Reproductive Biology of Plants

Editors:
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Preface

Reproduction is the biological process by which new individuals (offspring) are produced from their parents. Although plants reproduce both sexually and asexually, sexual reproduction generates recombination and enables the species to adapt to changing environment. In angiosperms, the flower is the unit of sexual reproduction. However, in a few species, seeds develop without fertilization; this phenomenon is termed apomixis. Reproductive biology has direct relevance in many fundamental and applied areas of plant sciences.

Reproduction is the basis of survival and sustenance of populations and species in natural habitats. Habitat degradation, overexploitation and climate change have threatened the sustainability of our biodiversity. The threat of species extinction is more pronounced in tropical countries such as Brazil, India, Malaysia, Indonesia and several others in South-East Asian where population pressure on forests and other natural resources has increased enormously in recent years. A large number of species have been pushed into the threatened status (rare, endangered and threatened category, RET species) as their populations have reduced markedly. Effective management of our biodiversity is going to be a major challenge in the coming decades. Unless suitable measures are taken, many of the threatened species would soon be extinct. According to many conservation biologists, an accelerating decline in world's biodiversity as a result of human activity is leading to the sixth major extinction event on our planet. Reproductive failure, as a result of constraints in one or several reproductive events, is the driving force for species extinction. Therefore, a comprehensive knowledge on their reproductive biology is required for effective management, conservation and sustainable utilization of our biodiversity.

In most of our crop plants, seeds and/or fruits are the economic products, and are the results of sexual reproduction. Reproductive events are susceptible to a range of environmental stresses that affect crop yields. Studies on reproductive ecology, therefore, have direct bearing not only for optimization of crop productivity but also on crop improvement through conventional and molecular approaches.

In recent years, reproductive biology has gained prominence in University education at different levels because of its importance in a range of fundamental and applied areas of plant Sciences. In the light of the prevailing specialization and super-specialization, it is difficult to get an overview of recent advances made on various aspects of reproductive biology. This book is aimed at providing an overview of reproductive biology essentially of flowering plants, although a few chapters on lower plants are also included. The chapters are written by recognized international specialists in their respective field of research. The book will be useful to students, teachers and researchers in wider disciplines of botany, molecular biology, agri-horticulture, forestry, environmental biology, conservation biology, entomology and biotechnology.

We would like to acknowledge ready cooperation of our contributors who have put in their efforts to ensure a high scientific quality to this book with up to date information. We wish to thank our publishers for their support and timely publication.

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Reproduction in Microalgae

Romain Gastineau,[1] *Nikolai A. Davidovich,*[2] *Gustaaf M. Hallegraeff,*[3] *Ian Probert*[4] and *Jean-Luc Mouget*[1,*]

ABSTRACT

Algae have traditionally been included in the group Cryptogamae ('hidden reproduction'), as opposed to Phanerogamae or Spermatophyta, the Seed Plants. Cryptogamae encompassed both macro- and microalgae, lichens, mosses, and ferns, and have also been referred to as 'Thallophyta', 'non-vascular plants', 'spore plants', seedless or flowerless plants, depending on the criteria used for classification. Microalgae have long been considered among the more primitive and less important organisms representing the plant-like characters of autotrophy and immobility. Reproduction in macroalgae was only observed in the 18th century, but the ability of microalgae to reproduce sexually was not demonstrated until the 19th century in microscopic green filamentous algae. In this chapter, we summarise up-to-date information on sexual reproduction in three major groups of

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microalgae, the diatoms, dinoflagellates and haptophytes, and discuss its relationship to biodiversity and ecological life cycle strategies. Microalgae obviously constitute the microscopic part of the 'plant-like' world, but they do not deserve being considered lower 'plants', as they exhibit a perplexing diversity of modalities of sexual reproduction (haplontic, haplo-diplontic, diplontic life cycles), compared to the 'higher plants' which appear strikingly uniform and rather limited in their life cycle options.

Keywords: auxospore, cyst, diatoms, dinoflagellates, frustule, haptophytes, microalgae, reproduction

Introduction

Algae have long been considered lower 'plants', and perceived by generations of students as among the more primitive, less attractive and less important organisms presenting the plant-like characters of autotrophy and immobility. In *Genera Plantarum* (1754) and *Species Plantarum* (1753a, b), Carl Linnaeus made prominent use of the study of sexual organs in his classification of 'Plantae', thus following traditions previously initiated by the Greek philosopher Theophrastus (a pupil of Aristotle)—the so-called 'father of botany'. 'Plantae' (embryophytes) are usually differentiated on the basis of the organization of their life cycle and sexual reproduction patterns. Since sexual organs were not visible in some of the 'Plantae', Linnaeus introduced the group Cryptogamae ('hidden reproduction'), as opposed to Phanerogamae or modern Spermatophyta, the seed plants. Cryptogamae are sometimes also referred to as 'thallophytes' (thallus = undifferentiated tissue without roots, leaves and stems, e.g., Endlicher 1836–1850), 'non-vascular plant', 'spore plants', seedless or flowerless plants, depending on the discriminating criteria used for classification. This latter group encompassed different organisms such as algae (both macro and microalgae), lichens, mosses, ferns, and even fungi. Fungi formerly treated as part of the plant kingdom are currently regarded as a separate kingdom, genetically more closely related to animals than plants. Having structures and organs which seemed more primitive than those of Phanerogamae, the 'thallophytes' were commonly named 'lower plants', a depreciative term implying some form of hierarchy compared to seed plants or 'higher plants'. From the 18th century onwards, 'lower plants' in the context of botanical nomenclature encountered the same qualitative terminology in many European languages (e.g., Payer 1850 in French; Strasburger 1906 in German). It is fair to state that human society has learned to better appreciate the importance of microalgae for the biosphere

and our planetary well-being in that they: (1) are responsible for more than 50% of photosynthesis and O_2 production on the planet; (2) are the basis of most aquatic food webs; (3) potentially represent an attractive fast growing alternative source for biofuels; (4) display a much greater genetic diversity than 'higher plants'; and finally (5) represent an invaluable heritage, as combined they experimented with a wide range of life cycle strategies over 3.5 billion years of evolution, from which the terrestrial land plants branched off only 400 million years ago.

Although qualified as 'hidden' in the absence of conceptual knowledge or because they were not easily visible, reproductive organs in macroalgae were observed as early as the beginning of the 18th century in the brown seaweed *Fucus* by Réaumur (1711; 1712), who misleadingly described them as flowers. Réaumur was contemporary to Van Leeuwenhoek, famous for the popularization of the light microscope. Later in the 19th century, several important and exhaustive botanical treatises were published, such as those of Thuret (1851) and Pringsheim (1855), who both described sexual organs in macroalgae and Cryptogamae.

The ability of microalgae to reproduce sexually may have been even more awkward for early naturalists, than macroalgae counterparts. First of all, the recognition of such microorganisms as being plant-like was not straightforward. They were sometimes associated with animal life forms as *Animalcules*, a word popularized by Van Leeuwenhoek (1702) or *Infusoria* (Marchand 1869). In light of broad morphological similarities, some microalgae (especially the green Chlorophyceae) were mistaken for gametes produced by macroalgae (Gay 1891; Kützing 1844). The motility of some microalgae invoked animalism, fuelling fierce controversy about the limits between plant and animal kingdoms (Agardh 1820; Kützing 1844), which encouraged some authors to propose a supplementary and intermediate kingdom, termed 'Psychodiaires' (Bory de Saint Vincent 1824) or Protista (Haeckel 1878). Despite these issues, sexual reproduction by conjugation in microscopic green filamentous algae (*Spirogyra*) has been known since the early 19th century (Vaucher 1803). Half a century later, Cohn dedicated an important part of his work to the study of sexual reproduction in green microalgae, such as *Sphaerococcus* and colonial *Volvox* species (for a recent review, see Kirk and Gruber 2005; and Chapter 2 of this book). Information on sexual reproduction in other phyla of microalgae was scarce, having been studied for a very limited number of species. In diatoms, sexual reproduction seems to have been described for the first time half a century later (Thwaites 1847), a few decades before the first observations on the dinoflagellates (Joseph 1879). In the haptophytes, sexual life cycles were first recognized in the 1960s only.

Reproduction in Diatoms

Despite being unicellular, diatoms are highly organized cells, presenting complex sexual reproduction processes, characterized by a common scheme well described in the literature (Geitler 1935, 1973; Drebes 1977; Round et al. 1990; Mann 1993; Edlund and Stoermer 1997; Chepurnov et al. 2004; Amato 2010).

Life Cycle

Diatoms are diplonts with meiosis in the final stage of gametogenesis (Fig. 1). The duration of the haploid phase is relatively short, not exceeding several hours, while the diploid state may last from months to years, depending on the species and the environmental conditions. The life cycle of diatoms is thus composed of two successive phases: a prolonged phase of vegetative multiplication and a very short period of sexual reproduction.

Depending upon the precise time and purpose of meiosis and gamete fertilization in the cycle, one can discriminate between **haplontic life cycles** (also called ***zygotic meiosis life cycles*** since meiosis occurs during germination of the zygote, the dominant part of the life cycle is haploid) or **diplontic life cycles** (also called ***gametic meiosis life cycles*** as meiosis is used to produce haploid gametes, the dominant part of the life cycle

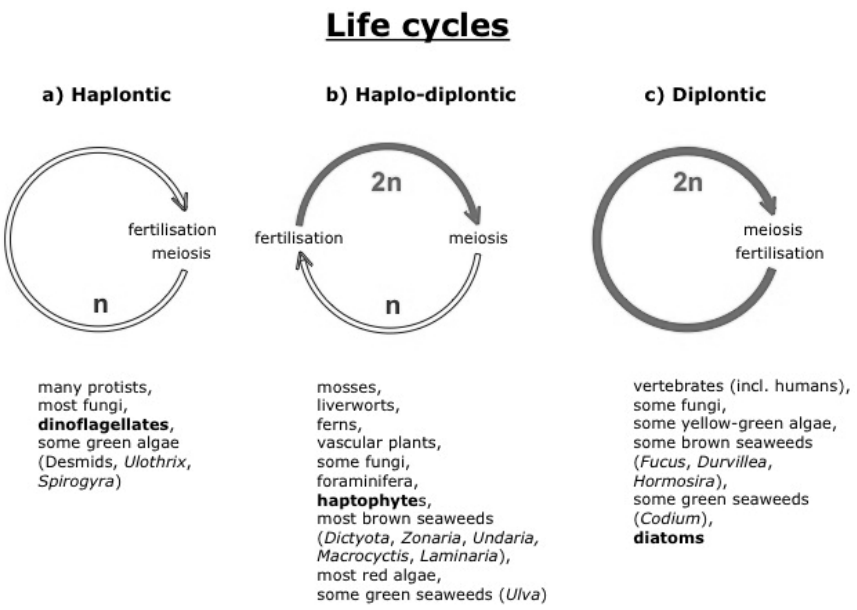

Figure 1. Different Types of Life Cycles Explored by Algal Groups and other Organisms.

is diploid)**. Haplo-diplontic** (= *diplobiontic*) **life cycles** (also called ***sporic meiosis life cycles*** as meiosis is used to produce haploid spores) represent a combination of both, with the gametophyte stage producing gametes and the sporophyte producing spores. Microscopic algae use zygotic (e.g., dinoflagellates), gametic meiosis (diatoms), or sporic meiosis (haptophytes). Sessile macroscopic seaweeds experiment (at least functionally) gametic or sporic meiosis, which ultimately is utilized by all land plants.

Vegetative multiplication is performed through common mitotic division. Under any circumstances, the biggest cells cannot reproduce sexually. However, most diatoms undergo significant size reduction over their life cycle, a phenomenon that puzzles many novice analysts of plankton samples. This happens because of the specific structure of the diatom cell wall also known as the frustule. The silicate frustule is rigid and consists of two parts, the epitheca and the hypotheca, which are joined to each other in a manner similar to the base and lid of a Petri dish. During mitotic division both daughter cells acquire new 'bases', i.e., hypothecae. As a result, after each act of vegetative multiplication via mitotic division one daughter cell keeps the parental size, while the other daughter cell becomes a little bit smaller in length (in pennate diatoms) or diameter (in centric diatoms). As cells divide, the mean cell size in a particular population gradually decreases, a phenomenon known as the MacDonald-Pfitzer rule (MacDonald 1869; Pfitzer 1869).The shift from the vegetative to the sexual phase of the life cycle in diatoms is size dependent. Upon reaching a critical size threshold, which is species specific, cells becomes sexually inducible and may enter sexual reproduction. It is not obligatory for cells below a critical size threshold to enter sexual reproduction, they are just allowed by the size factor to do this; many cells in the population continue vegetative multiplication. In some species there is also a second threshold below which cells could again not be induced to become sexual but rather divide mitotically until they die. When favourable conditions combine, such as the proper cell size, availability of the sexual partner (if needed), suitable environmental factors (light conditions, temperature, salinity, etc.), cells may shift from mitotic to meiotic division. The cells produce gametes, and can therefore be named gametangia. The process results in the reduction of the chromosome number to half that of parent cells (i.e., haploid). Gamete fusion leads to zygotes, restoring the diploid state. Zygotes commonly start to grow without any dormancy period. At this stage, the cells, which are called auxospores, are not surrounded by a siliceous frustule and are thus capable of expansion. The auxospore typically expands very quickly, reaching the maximum (or close to maximal) species specific size in a couple of hours. However, auxospore growth is not simple swelling of the cell and concomitant expansion of the cell wall; a more or less complex structure composed of silicate scales, rings and bands is formed in the growing cell

as a skeleton, named in pennate diatoms as the perizonium. The deposition of perizonial bands causes the rupture of the primary zygote wall (the membrane surrounding the zygote) and the remnants of such a membrane can be seen in many cases on both tips of the elongating auxospore in the form of caps. As soon as the auxospore has reached the maximum size, the diploid nucleus undergoes an acytokinetic division that precedes the deposition of the two initial valves (epitheca first, followed by hypotheca) inside the fully-grown auxospore. As a result, a new cell of the biggest size (an initial cell) arises. The initial cells may differ more or less from the vegetative cells in shape and structure of silica frustules. After being released from the auxospore envelope, the initial cell resumes vegetative multiplication and thereby creates a new clonal lineage of cells, which are renovated genetically and have the biggest sizes.

The close relationship between sexual reproduction and cell size restoration is an intriguing feature of the diatom life cycle. In a few species vegetative enlargement has also been observed (Gallagher 1983; Nagai 1995; Chepurnov et al. 2004). Usually, auxosporulation occurs in conditions that are favourable for vegetative growth. Certain centric and pennate diatoms can form resting stages in response to environmental stress (for details see Round et al. 1990). Nevertheless, in most cases the transition to dormant stages such as resting spores/cells or winter forms is not a typical characteristic of the diatom life cycle and is obligatory only in a few species (e.g., French and Hargraves 1985).

Generically, if compared with other groups of algae, 'lower plants', higher plants, and vertebrates (Mable and Otto 1998), the diatoms have a life cycle similar to those of the evolutionarily most advanced organisms (Fig. 1). In contrast to many other algae, where closely related taxa can exhibit widely variant life cycles with respect to the duration of haploid and diploid phases, the life cycle strategy in diatoms appears to be more permanent and uniform.

Patterns of sexual reproduction

A great diversity of copulation processes has been revealed in diatoms. Centric diatoms (Fig. 2) were shown to be oogamous (von Stosch and Drebes 1964; Schultz and Trainor 1968); they produce large non-motile female gametes (eggs) which are fertilized by small motile flagellated male gametes (sperms). Male cells usually undergo a series of successive differentiating mitoses giving rise to a variable number (2, 4, 8, 16, 32) of small diploid spermatogonia, which complete their further development by sperm formation (spermatogenesis). Two main types of spermatogenesis are known, depending on the formation of residual bodies, the merogenous and the hologenous types. The flagellum of the sperm cell is directed

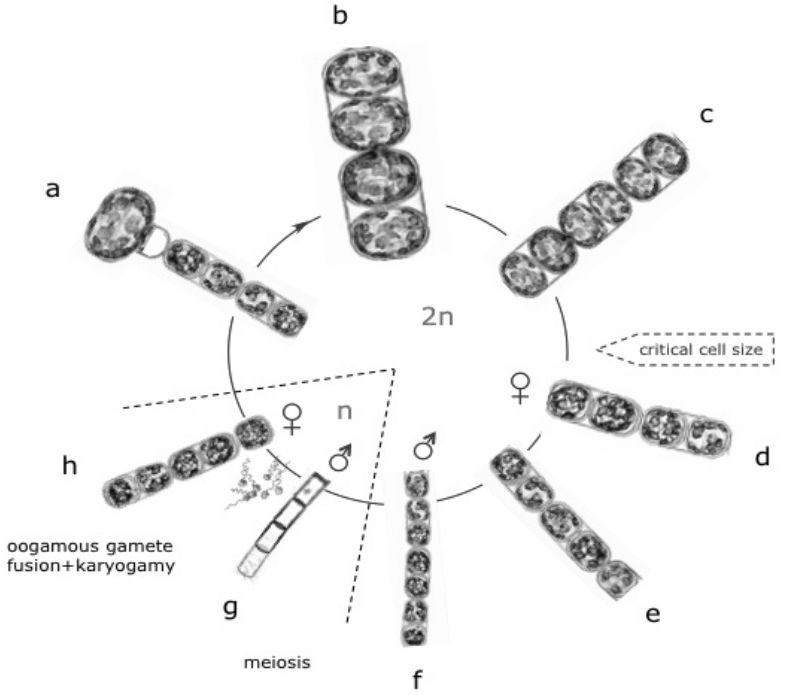

Figure 2. Diagrammatic Representation of the Life Cycle in a Centric Diatom *Melosira* sp. (a) an initial cell formed in a mature auxospore, (b-f), because of specific construction of the frustule the cell size decreases while cells pass through mitotic cycles, (g) male and (h) female gametogenesis; n and 2n, haplontic and diplontic phases (cell diameter range: ca. 20–80 μm).

Color image of this figure appears in the color plate section at the end of the book.

forwards during swimming and does not conform to the usual eukaryotic 9+2 arrangement, but shows a 9 + 0 pattern, i.e., central microtubules are lacking (Manton and von Stosch 1966; Heath and Darley 1972).

In contrast to the spermatogonia formed by depauperizing mitoses, the oogonia usually develop directly from vegetative cells. Three types of egg formation are recognized: (1) oogonia containing two eggs, (2) oogonia containing a single egg and a polar body, (3) oogonia containing a single egg. Independent from the meiotic nuclear stage of the oocyte, fertilization may happen as soon as the egg surface is mechanically (partly or totally) exposed, but in all cases nuclear fusion (karyogamy) does not follow before the female nucleus has reached the mature haploid stage (Drebes 1977). As a rule during fertilisation the flagellum is discarded.

Unlike centric, gametes released by pennate, diatoms are more or less equal in size (isogamy), however, they may differ morphologically and

behaviourally depending on the species (Fig. 3). Taken into account these and many other details, Geitler elaborated a comprehensive system of sexual reproduction patterns (Geitler 1973; Mann 1993). What is important, a flagellate stage has never been described in pennate diatoms (see also discussion in Subba Rao et al. 1991, 1992; Rosowski et al. 1992; Davidovich and Bates 1998). Automixis in the form of paedogamy or autogamy is also possible; and some diatoms are known to be apomictic (Geitler 1973; Vanormelingen et al. 2008).

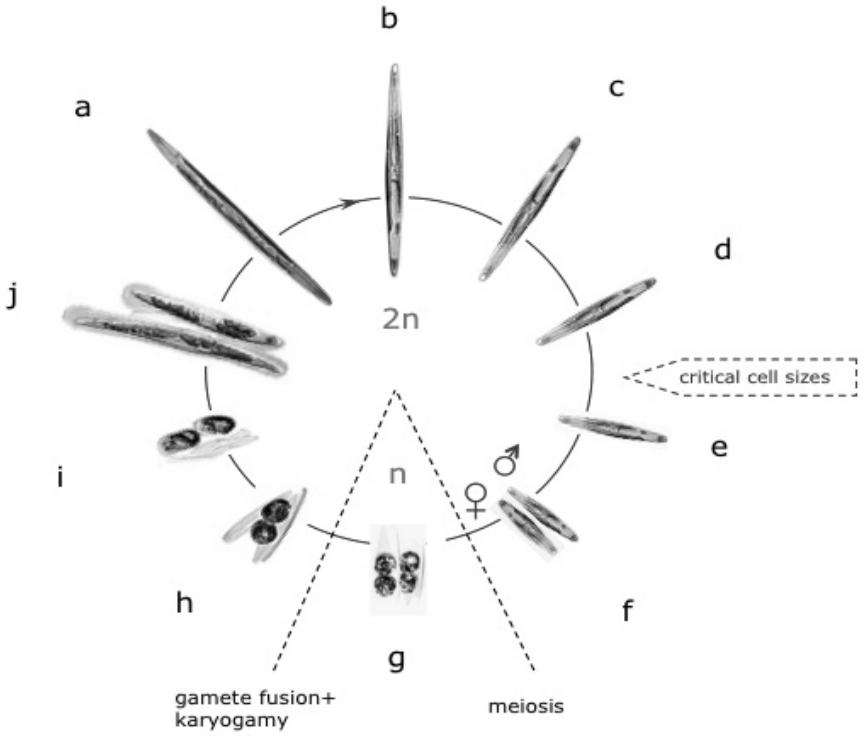

Figure 3. Diagrammatic Representation of the Life Cycle in the Pennate Diatom *Haslea karadagensis.* (a) an initial cell, (b-e) vegetative cells passing through mitotic cycles, (f) pairing of gametangia, (g) gametogenesis, (h) zygotes, (i-j) auxospore formation; n and 2n, haplontic and diplontic phases (cell length range: *ca.* 20–95 µm).

Color image of this figure appears in the color plate section at the end of the book.

Delivery of the gametes to the place of syngamy

The whole content of the gametangial cell transforms into only one or two gametes (more in centric males). Therefore, the cost of sex is very high in diatoms (Lewis 1984). Several mechanisms that promote the encounter of

gametes and thus their fusion have evolved. Oogamous fertilization in centric diatoms is effective because of motile male gametes; they swim by being dragged and not pushed by the anterior flagellum. Very often, the gametes of the raphe-bearing pennate diatoms are brought together by a prior pairing of their mother cells (gametangia) of the complementary sexes. For this reason the term 'gametangiogamy' is also in use (Wiese 1969). To secure fertilization, in several benthic pennates a mucilage capsule is secreted around the copulating partners. Once gametangia are physically close, another type of active movement, amoeboid movement may be employed. This movement allows gamete transfer to the place of syngamy and is sufficient for short distance translocation of the gamete from closely positioned gametangia. Vegetative cells of a very diverse group of araphid pennates, however, are sessile and only a few species are known for their slow motility (Kooistra et al. 2003; Sato and Medlin 2006). An unusual mechanism of male gamete motility has recently been described in araphid pennates, involving formation of thread-like cytoplasmic projections on the gamete cell surface (Sato et al. 2011; Davidovich et al. 2012).

Factors triggering sexual reproduction

If mating is heterothallic, interaction between sexual partners is required to initiate meiosis and gametogenesis. There is some evidence that the process of sexualisation is triggered by a sophisticated multi-stage exchange of several pheromones (Sato et al. 2011; Gillard et al. 2012). For now, it is unclear how species-specific these pheromones are. If pheromones or their combination are unique for each species, a minor change in their structure may lead to certain problems in the control of cell pairing that predetermine a very short and efficient way for speciation in diatoms, even in sympatric populations.

Comprehensive observations gained by previous investigators, mainly by Geitler (Geitler 1932), allowed Drebes to write in his review (Drebes 1977, p. 271): "In contrast to other protists, induction of sexualization in diatoms depends not only on the genotype and specific environmental conditions but also on a suitable cell size as an internal non-genetic factor". The concept of 'cardinal points' in the life cycle of diatoms developed by Geitler (Geitler 1932) declares that cells cannot be induced to become sexual until they have declined in size below the critical size. The upper threshold for sexual induction can range from 30% to 75% of the maximal size of the initial cells. However, for more than half of the species examined, the threshold was at 45% to 55% (Davidovich 2001).

Apart from suitable cell sizes, one of the necessary conditions for successful sexual reproduction is an excellent physiological state. Laboratory practice has shown that the best results can be achieved in exponentially

growing cultures. Stressed cultures never undergo sexual reproduction (Chepurnov et al. 2004). Beside internal cues, a number of external factors have been shown to influence sexual reproduction in diatoms (Drebes 1977). Appropriate light conditions are highly important, sometimes being a key factor in triggering sexual reproduction (e.g., Hiltz et al. 2000; Mouget et al. 2009).

Mating system

While centric diatoms are believed to be genetically monoecious and hence are capable of homothallic reproduction (Drebes 1977; however see comments in Chepurnov et al. 2004), pennate diatoms are for the most part heterothallic, or combine homo- and heterothallic modes of sexual reproduction (Roshchin and Chepurnov 1999; Chepurnov et al. 2004; Davidovich et al. 2009, 2010; Amato 2010). In the case of strict heterothally (genetic dioecy) clones are divided into two mating types, which are usually indistinguishable at the stage of vegetative growth, but in certain species may clearly differ by morphology and/or behaviour of their gametes (e.g., Stickle 1986; Davidovich et al. 2009). These diatoms (*cis*-anisogamous *sensu* Mann 1982) are most suitable as model species for studies of sex determination and sex inheritance. If differences are visible and correspond to two mating categories, these can be designated as 'male' and 'female'. Otherwise, mating types are conventionally termed plus and minus, '+' and '–'.

Sex determination

Mechanism of sex determination in diatoms is poorly understood. Sex chromosomes are unknown, at the same time obligate heterothallic reproduction and existence of two mating types in some pennates suggest genetic dioecy. In such a case sex factors must be located on different chromosomes. It has been shown that sibling clones, derived from the two initial cells formed during sexual reproduction of a single pair of gametangia (in those species where each gametangium produces two gametes) were of opposite sexes (Mann et al. 2003). This and other experiments (e.g., Chepurnov and Mann 2004) indicate that sex determination in heterothallic pennate species is not developmental or phenotypic.

Sex differentiation is impossible in the case of automictic reproduction, but automixis is not common among diatoms. A genetic base for sex determination can be reasonably elucidated in those species which are able to reproduce both intra- and interclonally. The mode of sex inheritance revealed in a homothallic progeny may give an answer to the question of how sex factors are distributed (Davidovich 2002; Chepurnov et al. 2004;

Davidovich et al. 2006). Data acquired suggests coupling of chromosomes bearing male (**M**) and female (**F**) genetic factors in combinations **MF** and **FF** for male and female sexes accordingly. Male sex is thus heterogametic. However, it cannot be ruled out that this simple model will prove to be more challenging as more data become available. For example, in several pennate diatoms some clones behaved as males when mated with female clones, but as females when mated with male clones (Chepurnov et al. 2004); this suggests mating system to be more complex than the bipolar and obscures the sex determination mechanism.

An example of phenotypic sex determination can be found in centric diatoms (Drebes 1977), where sex appearance generally depends on the stage of the life cycle. In one and the same clone, relatively big cells recently entered into the sexual size region produce eggs, while getting smaller they shift to spermatozoid production. At the same time, in some centric diatoms there were particular clones which acted as 'pure' males, never producing female gametes (Chepurnov et al. 2004).

Asexual auxosporulation

Auxospore formation is usually regarded as a process intrinsically connected with the phenomenon of sexual reproduction. However, sometimes auxospores may occur in the absence of any sexual process (e.g., Nagai et al. 1995; Sabbe et al. 2004; Chepurnov et al. 2004). Apomixis treated as diploid parthenogenesis is associated with asexual auxosporulation, and was reported both in centric and pennate diatoms. In other cases, unfused gametes can transform into auxospores (haploid parthenogenesis). The last is facultative in some *allogamous* pennate diatoms. During vegetative cell enlargement noted in some diatoms (e.g., Gallagher 1983; Nagai et al. 1995) the cells escape from the 'trap' of critical size diminution, but cells developed directly from vegetative cells do not produce typical perizonium and their size is approximately half the size of the normal auxospores produced by the same clone.

Reproduction in Dinoflagellates

Dinoflagellate sexual reproduction has long been disputed, and a 1973 textbook on protozoology contained no reference to this phenomenon (Grell 1973). The earliest documented report of sexual reproduction in dinoflagellates was Joseph's (1879) description of pairing and fusion of swimming cells of *Peridinium stygium*, but careful studies by von Stosch in the 1960s (1965; 1969; 1972; 1973) have since transformed our understanding of dinoflagellate sexuality. With researchers' increasing capacity to maintain laboratory cultures, sexuality has now been documented for some 100

species (Walker 1984; Wall and Dale 1968; Blackburn et al. 1989; Blackburn and Parker 2005). The reasons that sexual reproduction in dinoflagellates had so long been overlooked include: (1) gametes can look similar to vegetative cells; (2) gamete fusion is easily confused with cell division; (3) 'warty' zygotes have often been interpreted as aberrant cells (Pfiester and Andserson 1987). To date sexual life cycles are increasingly elucidated using nuclear staining and flow cytometric techniques, not only in culture but also field surveys.

Life Cycle

The life cycle of *Gymnodinium catenatum* is representative of those observed in many dinoflagellates (Fig. 4). Motile vegetative cells divide vegetatively (by mitosis) to form chains. With the onset of sexual reproduction, vegetative division results in single cell gametes. Usually two types of gametes from different clonal strains (heterothallism) are required for sexual reproduction, pairs of which fuse to give a planozygote. This cell loses motility to form a benthic resting cyst (hypnozygote). Excystment produces a planomeiocyte, similar to a planozygote, which divides (by meiosis) to re-establish the planktonic vegetative stage (after Blackburn et al. 1989). In Spanish cultures studied by Figueroa et al. (2006a, 2008) (dotted lines in Fig. 4), most planozygotes divided by binary fission to produce vegetative cells but without undergoing a cyst stage. Furthermore, some fusing gamete

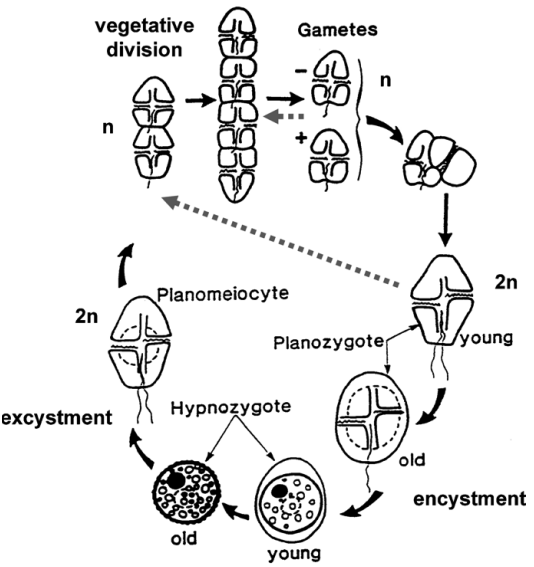

Figure 4. Diagrammatic Summary of the Sexual Life Cycle of *Gymnodinium catenatum* (from Hallegraeff et al. 2012).

pairs did not form planozygotes but went through a division process before completing cytoplasmic fusion.

Patterns of sexual reproduction

All dinoflagellates studied to date, but one, exhibit the *haplontic* type of life cycle, the dominant vegetative stage (the one undergoing vegetative growth) being haploid. The single exception is *Noctiluca* which is claimed to have a diplontic life cycle (Zingmark 1970). Asexual reproduction can happen much more quickly, and therefore is the predominant manner of reproduction during optimal environmental conditions, but sexual reproduction is essential for species adaptation and allows for genetic recombination. Under appropriate conditions, dinoflagellate gametes are produced and fuse to form a diploid planozygote. Often gametes swim faster, can be paler in colour and collect in 'dancing groups'. Fusing pairs of gametes can be distinguished from dividing vegetative cells because their cingula are perpendicular. Dinoflagellates often produce gametes that do not differ morphologically from vegetative cells (a condition called *hologamous*). Fusing gametes can be identical to each other (*isogamous*) or be different from each other (*anisogamous*, e.g., *Ceratium cornutum, C. horridum, Alexandrium tamarense*). As for diatoms, species are monoecious or *homothallic*, i.e., sexual reproduction can occur within a clone (e.g., *Alexandrium taylori*), or dioecious or *heterothallic*, i.e., two different mating types (designated plus or minus) must be combined. Sexual compatibility can comprise only two different mating types (simple heterothallism), such as in *Lingulodinium polyedrum* (Figueroa and Bravo 2005b) or more complex heterothallism, such as in *Alexandrium minutum* (Figueroa et al. 2007). Sexual mating compatibility has been used to elucidate species synonymy but also genetic affinities between geographic populations of the same species (Blackburn et al. 2001).

Three types of zygotes have been reported: (1) planozygote motile throughout and meiosis is completed without cyst formation (e.g., *Ceratium horridum*); (2) planozygote loses motility and forms a temporary cyst (e.g., *Helgolandinium subglobosum*); (3) planozygote forms a resting cyst or hypnozygote. The planozygote can be identified by two ('ski track') longitudinal flagella. This stage often develops into a resting cyst with a thick resistant cell wall and often requires a period of dormancy before germination is possible. Studies on freshwater dinoflagellates in particular have indicated that the timing of meiotic division in the sexual phase is variable (Pfiester 1975, 1976, 1977). Meiosis occurs (1) within the planozygote; or (2) after excystment to release an appropriate number (2–4) of daughter cells; or (3) by subsequent divisions of the single meiocyte released by excystment. In a growing number of dinoflagellate zygotes, the

nucleus has been observed to enlarge further and rotate rapidly within the cell (so called 'nuclear cyclosis' first described by Pouchet 1883) which von Stosch (1973) associated with the onset of meiosis.

Until recently the most common pathway observed was the transition of planozygote to resting cyst but it is now thought that the planozygote can also skip cyst formation (Figueroa and Bravo 2005a,b). Other possible pathways are: (1) gametes can revert to an asexual phase and undergo binary fission rather than fusion (e.g., *Gymnodinium nolleri*, *G. catenatum*, *Alexandrium taylori* or *Lingulodinium polyedrium*); and (2) planozygotes undergo meiosis and division without the production of a hypnozygote (Figueroa and Bravo 2005a,b; Figueroa et al. 2006a,b). In some species for which a sexual cycle has been reported no resting cyst is known (e.g., *Karlodinium veneficum, Karenia brevis;* Walker 1982). Asexual resting cysts are also known, e.g., in *Scrippsiella hangoei* (Kremp and Parrow 2006). Another type of quiescent stage is what is variably called temporary, pellicle or ecdysal cysts with a thin wall and limited capacity to withstand adverse environmental conditions, either produced sexually or asexually.

Cyst as survival strategies

Interest in dinoflagellate sexual reproduction was triggered in the early 1960s with the recognition that many fossil cysts (first described by Ehrenberg 1838 from Cretaceous flints as 'hystrichosphaeres') are in fact dinoflagellate hypnozygotes. Wall and Dale (1968) conducted the first experiments incubating living 'cysts' from Woods Hole bottom sediments. Excellent cyst preservation in the fossil record is due to the presence of sporopollenin in the wall of many (but not all) species (Fig. 5).

Currently more than 80 marine and 15 freshwater species of modern dinoflagellates are known to produce resting cysts. This number of cyst-producing species is small however compared with the total number of extant dinoflagellates (more than 2000). Resting cysts can survive harsh environmental conditions and thus play an important ecological role as the inoculum for recurrent blooms. Dinoflagellate cysts can remain viable in sediments for up to 100 yrs. They also facilitate expansion of the geographical distribution of a species through cyst dispersal via ocean currents and even ship ballast water discharge.

Factors triggering sexual reproduction

Sexuality has been traditionally achieved in culture through nutrient depletion, with temperature and light being important modulators of the cyst yield. However, gamete pairing and planozygote formation in nature may not be always linked to nutrient shortage, since sexuality has been

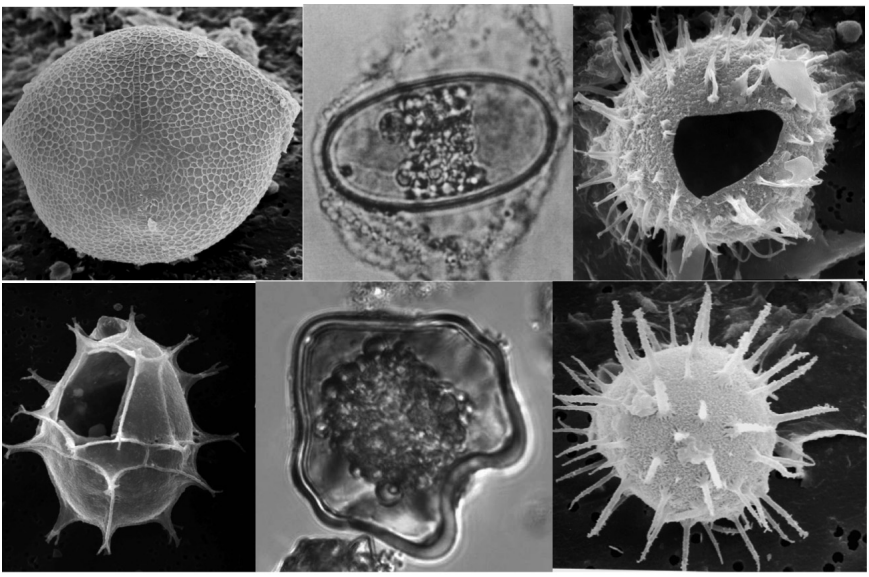

Figure 5. Sexually Produced Dinoflagellate Resting Cysts from Top Left to Bottom Right: *Gymnodinium catenatum* (36–62 μm diameter), *Alexandrium tamarense* (38–56 μm long, 23–32 μm wide), *Protoceratium reticulatum* (300 μm diameter; with archeopyle), *Gonyaulax elongata* (40–59 μm long, 26–42 μm wide; with archeopyle), *Protoperidinium oblongum* (55–65 μm long), *Lingulodinium polyedrum* (30–60 μm diameter).

observed either at termination of blooms or during active growth, or when reaching a cell abundance threshold (Garcés et al. 2002). The conditions that trigger cyst formation and cyst germination have become the key focus of predicting blooms of cyst forming toxic dinoflagellates such as *Alexandrium tamarense*, *Gymnodinium catenatum* and *Pyrodinium bahamense* (Anderson and Wall 1978; Dale 1983). The dormancy period is a maturation time during which biological activity is suspended; this can last from hours to days (*Kryptoperidinium foliaceum*), weeks (*Gymnodinium catenatum*) to months (*Alexandrium tamarense*). Germination cannot be induced during dormancy, but once completed the cyst enters quiescence, during which germination can occur if environmental conditions are suitable. A genetic (endogenous) control of dormancy has been documented in some species (Anderson and Kiefer 1987), but exogenous (abiotic and physiological) modulators and internal clocks (endogenous rhythms) also play a role. Germination patterns thus can drive bloom strategies and seasonal species succession. Once germination occurs, the size of the inoculum will be influenced not only by the number of germinating cysts but also by their viability.

Reproduction in Haptophytes

The division Haptophyta is a lineage of unicellular algae that are widespread and often very abundant in diverse marine settings. Most haptophytes occur as solitary motile or non-motile forms, but a few form colonies or short filaments. Haptophyte cells are usually covered with one or several layers of organic scales of varying degrees of complexity, these being formed intracellularly in Golgi-derived vesicles. Haptophytes are characterized by the presence of a unique organelle called a haptonema (from the Greek *hapsis*, touch, and *nema*, thread), which is superficially similar to a flagellum but differs in the arrangement of microtubules and in function, being implicated in attachment or capture of prey. The haptonema is present in most species, sometimes in a reduced or vestigial form, but may rarely be absent.

The Haptophyta includes 2 classes: the Pavlovophyceae with only 13 described species and the Prymnesiophyceae which contains the vast majority of the known diversity of haptophytes and which comprises 2 orders of non-calcifying taxa, the Phaeocystales and the Prymnesiales, together with the calcifying coccolithophores making up a monophyletic clade (the sub-class Calcihaptophycidae) containing 4 orders (Isochrysidales, Coccolithales, Syracosphaerales, Zygodiscales).

There are some well-known non-calcifying haptophyte taxa, such as *Phaeocystis*, *Prymnesium* and *Chrysochromulina*, that form periodic harmful or nuisance blooms in coastal environments. However, the most familiar haptophytes are the coccolithophores, members of the Prymnesiophyceae that, in addition to a proximal layer of organic body scales, are covered with a distal layer of calcified scales (coccoliths) that are also formed intracellularly and that often have complex ornamentation. Coccolithophores are responsible for a large part of modern oceanic carbonate production and are thus key actors in global carbon cycling (Rost and Riebesell 2004).

Heteromorphic life histories have been documented in many members of the haptophyte class Prymnesiophyceae. These include alternations between non-motile and flagellated stages, between colonial and single cell stages, and between benthic and planktonic stages. The earliest studies on haptophyte life cycles focused mainly on members of the coccolithophore families Pleurochrysidaceae and Hymenomonadaceae (order Coccolithales), these being relatively easy to maintain in laboratory culture. Alternation of a non-calcifying ('Apistonema') stage with a coccolith-bearing stage has been reported in *Ochrosphaera* (Schwarz 1932; Lefort 1975), *Hymenomonas* (Fresnel 1994) and *Pleurochrysis* (Leadbeater 1970, 1971; Gayral and Fresnel 1983). In *Pleurochrysis carterae* (Rayns 1962) and *Hymenomonas lacuna* (Fresnel 1994), chromosome counting confirmed that the non-calcifying stage in these

life cycles is haploid and the calcifying stage diploid, providing the first hard evidence of the existence of haplo-diplontic life cycles in haptophytes (Fig. 6).

These life cycles were relatively easy to discern due to the presence of coccoliths (that are visible in light microscopy) in one of the phases. Two main types of coccoliths exist: heterococcoliths (formed of a radial array of complex-shaped interlocking crystals units) and holococcoliths (constructed of numerous small, similar sized and simple-shaped calcite elements). A culture study by Parke and Adams (1960) on the non-motile heterococcolith-bearing stage of *Coccolithus braarudii* (Coccolithales) demonstrated an alternation with *Crystallolithus hyalinus*, a motile stage bearing holococcoliths. Prior to this observation, heterococcolithophores and holococcolithophores had been considered as taxonomically discrete groups of species.

Reviewing these and other studies, Billard (1994) suggested that haptophyte life cycles typically include haploid and diploid phases, each capable of independent asexual reproduction (haplo-diplonty), with distinct patterns of body scale ornamentation (and in some cases coccolith type) characteristic of each ploidy state. Prymnesiophycean body scales are composed of microfibrils and contain proteins and carbohydrates including cellulose (Leadbeater 1994 and references therein). The proximal ("body") scales are composed of two layers with the proximal face (facing the cell membrane) having a radial pattern of microfibrils often arranged

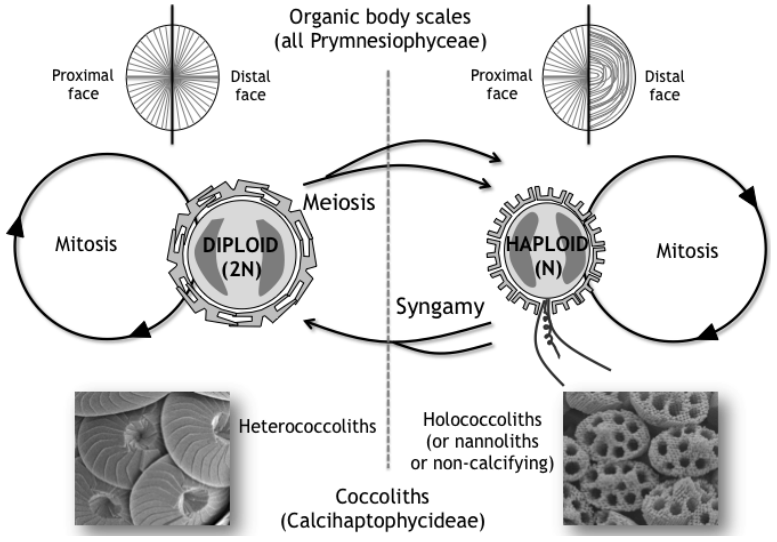

Figure 6. Schematic Representation of the Haplo-Diplontic Life Cycle of the Prymnesiophyceae and Scale Ornamentation in the Two Phases.

into quadrants, whereas the distal face either has a radial pattern or an interwoven spiral pattern of concentric rings. In this scheme, the body scales of the diploid cells have identical (radial) ornamentation on both sides, whereas those of the haploid stage have distinct patterns on the proximal and distal faces (radial and spiral, respectively). Billard (1994) predicted that the heterococcolith-bearing phase of *C. braarudii* is diploid and the holococcolith-bearing phase haploid, by likening the patterns of body scale ornamentation in the known haplo-diplontic life cycles of species in the Pleurochrysidaceae and Hymenomonadaceae with those in the life cycle of *C. braarudii* as illustrated by Manton and Leedale (1969). A number of other coccolithophores for which body scales have been illustrated in one phase only fit this pattern, including the heterococcolithophores *Syracosphaera pulchra* (Inouye and Pienaar 1988), *Umbilicosphaera hulburtiana* (Gaarder 1970) and *Jomonlithus littoralis* (Inouye and Chihara 1983), and the holococcolithophores *Calyptrosphaera sphaeroidea* (Klaveness 1973) and *Calyptrosphaera radiata* (Sym and Kawachi 2000).

DNA quantification by flow cytometry later confirmed the haplo-diplontic nature of the holococcolithophore-heterococcolithophore life cycle of *Coccolithus braarudii* as well as of two other species for which both phases were maintained in culture (Houdan et al. 2004). Further indirect evidence that haplo-diplontic life cycles are widespread in coccolithophores comes from observations in field samples from various locations of 'combination coccospheres' bearing both heterococcoliths and holococcoliths, interpreted as capturing the instant of a life cycle phase change (Thomsen et al. 1991; Kleijne 1992; Alcober and Jordan 1997; Young et al. 1998; Cros et al. 2000; Cortes and Bollmann 2002; Geisen et al. 2002; Cros and Fortuno 2002). These observations indicate that life cycles with alternating heterococcolith-bearing (diploid) and holococcolith-bearing (haploid) stages span a large part of the diversity of coccolithophores.

Over time, culture studies and observation of combination coccospheres have demonstrated that diploid generations in coccolithophore life cycles always bear heterococcoliths, whereas the haploid generations are covered by either holococcoliths (Coccolithaceae, Calcidiscaceae, Helicosphaeraceae, Syracosphaeraceae), aragonitic coccoliths (*Polycrater*), nannoliths (Ceratolithaceae), a non-calcifying benthic stage (Pleurochrysidaceae, Hymenomonadaceae) or a non-calcifying motile stage (Noëlhaerhabdaceae) (Billard and Inouye 2004 and references therein).

Alternation between generations of different ploidy levels are known to occur in each of the other two non-calcifying orders within the Prymnesiophyceae. In the Phaeocystales, the most complete information on the life cycle has been obtained for *Phaeocystis globosa* (reviewed by Rousseau et al. 2007). A haplo-diplontic life cycle has been described for this species, which includes diploid colonial cells (recorded either as free non-motile

cells or within colonies), diploid flagellate cells without organic scales, and two types of haploid flagellates surrounded by organic scales, differing in size (meso- and microflagellates). *P. antarctica* is thought to exhibit a similar life cycle (Zingone et al. 2011). In the Prymnesiales, *Prymnesium parvum* has been shown to be the diploid stage in a life cycle in which the haploid stage was originally described as a separate species, *P. patelliferum* (Larsen and Medlin 1997; Larsen and Edvardsen 1998) and *Prymnesium polylepi* (=*Chrysochromulina polylepis*) has also been demonstrated to have a haplo-diploid cycle (Edvardsen and Vaulot 1996; Edvardsen and Medlin 1998). In each of these cases, body scale ornamentation fits the scheme of Billard (1994) and in some cases morphological differences are also evident in the distal organic scales (e.g., Probert and Fresnel 2007). The details of these non-calcified scales can only be observed by electron microscopy, generally making life cycles in these non-calcifying haptophytes difficult to identify.

In summary, dimorphic haplo-diplontic life cycles appear to be widespread in the Prymnesiophyceae. To date, alternation of generations has not been demonstrated in members of the other haptophyte class, the Pavlovophyceae. The species within this distinctive clade do not possess the ornamented plate scales which have often proved indicative of ploidy state in the Prymnesiophyceae, and given the fact that different ploidy stages in many non-calcified members of the latter class can only be morphologically distinguished by this character, it is perhaps not surprising that the potential existence of haplo-diplontic life cycles in the Pavlovophyceae has not been recognized. Transitions from non-motile to motile cells are common in the Pavlovophyceae (Billard 1994; Bendif et al. 2011), but relative motility is not often a good indicator of ploidy state.

There are few reports of cysts in the Haptophyta. Cysts of *Prymnesium* were described by Carter (1937) and Conrad (1941) and have been investigated by Pienaar (1980) who has shown that the walls of *Prymnesium parvum* cysts are composed of layers of scales with siliceous material on the distal surfaces. It is not known whether formation of these cysts is related to ploidy level.

It should be noted that in haptophyte life cycles the existence and place of sexuality, if applicable, generally remains unknown (Billard 1994). In the coccolithophores, sexuality has been revealed by direct observation of syngamy in only three species, *Ochrosphaera neapolitana* (Schwarz 1932), *Pleurochrysis pseudoroscoffensis* (Gayral and Fresnel 1983) and *Coccolithus braarudii* (Houdan et al. 2004). In *O. neapolitana*, meiosis, isogamete formation and syngamy were reported by Schwarz (1932). From light microscope observations on *Coccolithus braarudii* (Houdan et al. 2004) and *Pleurochrysis pseudoroscoffensis* (Gayral and Fresnel 1983), certain general inferences can be made about the meiotic process in coccolithophore life cycles. In *Coccolithus*

braarudii, meiosis may occur within the heterococcosphere prior to production of the flagellar apparatus and subsequent liberation of the motile cell. Since only one viable cell emerges this would imply the redundancy of the other haploid nuclei formed by the meiotic divisions. Meiosis in the chlorophyte *Spirogyra* (Harada and Yamagishi 1984) is one of the best-known examples of this pattern. Alternatively, the motile cell that emerges from the heterococcosphere may still be diploid, with nuclear reduction occurring in subsequent divisions. The observation that holococcolith production does not commence immediately after formation of the flagellar apparatus may be interpreted as providing support for this second hypothesis (Houdan et al. 2004). In the life cycle of *P. pseudoroscoffensis*, four non-calcified motile haploid cells are formed within a heterococcosphere and following release, these cells remain motile for a short time before settling and dividing asexually to initiate the haploid non-calcified pseudofilamentous stage (Gayral and Fresnel 1983). Comparable 'meiospores' are formed in the life cycles of other members of the Pleurochrysidaceae (von Stosch 1967; Leadbeater 1971) and the Hymenomonadaceae (Fresnel 1994). In coccolithophores, the mode of initial gamete attraction and contact is not known, but once initiated, syngamy can clearly be completed within a very short period of time. The rapid initiation of heterococcolith production in the zygote observed in *C. braarudii* by Houdan et al. (2004) and reported for *P. pseudoroscoffensis* by Gayral and Fresnel (1983) indicates that the two haploid nuclei must fuse immediately following cytoplasmic fusion. In both species, a complete heterococcosphere was formed within 24 hours of the onset of fusion. From this limited evidence, it appears that in coccolithophores fusing gametes are isogamous and are morphologically indistinguishable from vegetative haploid cells, and that fusion can occur within a clone (homothallism). To our knowledge, crossing experiments between haploid coccolithophore strains have never been attempted.

In *Phaeocystis globosa*, micro- and mesoflagellates (haploid stages) are produced (presumably by meiosis) within the colony and are eventually released and multiply vegetatively. The life cycle is completed by syngamy between a micro- and mesoflagellate that develops into a diploid macroflagellate that is believed to develop into a new colony. However, the formation of colonial stages was never observed in cultures of *P. globosa* containing only haploid cells (Vaulot et al. 1994). This could be explained either by assuming that conjugation only occurs between the two heteromorphic types of haploid flagellates (anisogamy) or that different mating types exist amongst haploid flagellates (heterothallism). A non-motile zygote linking the haploid unicellular stages and the diploid colonial stages has been documented in *P. antarctica* (Gaebler-Schwarz et al. 2010). This zygote can divide vegetatively as a benthic palmelloid stage and not revert to the colonial stage at least in culture conditions.

Studies on the mechanisms of sexual reproduction in haptophytes are currently restricted by the limited number of cultures available and, moreover, by the lack of clear indications as to the factor(s) responsible for the induction of life cycle phase changes in this group. The transition between the life stages in haptophytes is presumably controlled by the interplay of endogenous and environmental factors, but the role and the relative importance of these factors are poorly known. A number of reports suggest that in the Pleurochrysidaceae factors such as temperature and light (Leadbeater 1970) and the addition of fresh medium (Inouye and Chihara 1979; Gayral and Fresnel 1983) may influence phase changes. In cultures of *Calyptrosphaera sphaeroidea*, Noel et al. (2004) demonstrated that exposure to selected vitamins and trace metals induced the transition to the heterococcolith-bearing phase, whereas a slightly higher concentration of components in the basic medium along with concomitant stresses of light and temperature induced formation of the holococcolith-bearing phase. Houdan et al. (2004) suggested that concentrations of inorganic or organic trace elements in the medium may have played a role in phase change induction in three coccolithophore species, and also hypothesized that a biological clock may be involved in this process.

Life cycle transitions with each phase adapted to distinct ecological niches may also be an integral part of the ecological strategy of haptophytes. In ecological terms, a haplo-diplontic life cycle is generally considered as an adaptation to an environment which is seasonally variable or that contains two different niches (see review by Valero et al. 1992). There is some evidence that heterococcolith-bearing and holococcolith-bearing or non-calcifying phases in the life cycle of certain coccolithophore species have differential in situ spatio-temporal distributions (Cros and Fortuno 2002; Frada et al. 2012). In general, holococcolith-bearing stages (which always possess flagella) may be adapted to warm stratified surface waters, whereas the more robust (and often non-motile) heterococcolith-bearing stages may be better suited to turbulent mixed-layer waters. Noel et al. (2004) extrapolated from growth medium preferences of the two stages of *Calyptrosphaera sphaeroidea* to propose a hypothetical ecological cycle in which the holococcolith-bearing stage occurs in offshore waters and the heterococcolith-bearing stage in coastal waters. Very few studies have experimentally compared the physiological characteristics of different ploidy stages in haptophytes, but in these studies differences in responses to physic-chemical and/or biotic parameters have consistently been found. For example, in the extremely abundant coccolithophore *Emiliania huxleyi*, which has a life cycle with diploid non-motile heterococcolith-bearing cells alternating with haploid flagellate non-calcifying cells, the haploid stage appears to be relatively sensitive to high light (Houdan et al. 2005), but not susceptible to *E. huxleyi* specific viruses (*Eh*Vs) that routinely infect

and kill diploid cells (Frada et al. 2008). Dramatic differentiation in gene expression between diploid and haploid phases of the coccolithophore *E. huxleyi* have been demonstrated, with greater transcriptome richness in diploid cells suggesting they may be more versatile for exploiting a diversity of rich environments whereas haploid cells appear to be intrinsically more streamlined (Von Dassow et al. 2009).

Concluding Remarks

Microalgae so far studied do represent a source of biodiversity much greater than 'higher plants', whatever the criteria considered (genetics, life cycles, strategies and modalities of sexual reproduction, plastid structures and pigment contents, impacts on biogeochemical cycles, etc.). For instance, compared to the perplexing diversity of life cycle strategies explored by microalgae, the 'higher plants' appear strikingly uniform and rather limited in their life cycle options. Due to their intrinsic characteristics, microalgae obviously constitute the microscopic part of the 'plant-like' world, but they do not deserve being considered lower 'plants', at the very least smaller 'plants' *lato sensu*, as green algae form the green lineage with Plantae.

References

Agardh, C.A. 1820. Dissertatio de metamorphosi algarum. Ex officina Berlingiana. Lundae (Lund.).

Amato, A. 2010. Diatom reproductive biology: living in a crystal cage. Int. J. Plant Reprod. Biol. 2: 1–10.

Alcober, J. and R.W. Jordan. 1997. An interesting association between *Neosphaera coccolithomorpha* and *Ceratolithus cristatus* (Haptophyta). Eur. J. Phycol. 32: 91–93.

Anderson, D.M. and D. Wall. 1978. Potential importance of benthic cysts of Gonyaulax tamarensis and G. excavata in initiating toxic dinoflagellate blooms. J. Phycol. 14: 224–234.

Anderson, D.M. and B.A. Keafer. 1987. An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*. Nature 325: 616–617.

Bendif, E.-M., I. Probert, A. Hervé, C. Billard, D. Goux , C. Lelong, J.-P. Cadoret and B. Véron. 2011. Integrative taxonomy of the Pavlovophyceae: a reassessment. Protist 162: 738–761.

Billard, C. 1994. Life Cycles. *In*: J.C. Green and B.S.C. Leadbeater (eds.). *The Haptophyte Algae*. The Systematics Association Special Volume 51. Oxford University Press, Oxford, pp. 167–186.

Billard, C. and I. Inouye. 2004. What's new in coccolithophore biology? *Coccolithophores: From Molecular Processes to Global Impact*. H.R. Thierstein and J.R. Young (eds.). Springer Verlag, pp. 1–29.

Blackburn, S.I., G.M. Hallegraeff and C.J.S. Bolch. 1989. Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. J. Phycol. 25: 577–90.

Blackburn, S.I. and N. Parker. 2005. Chapter 24. Microalgal Life cycles: encystment and excystment. *In*: R. Anderson (ed.). l. Algal culturing techniques. Elsevier, New York, pp. 399–417.

Blackburn, S.I., C.J.S. Bolch, K.A. Haskard and G.M. Hallegraeff. 2001. Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Bory de Saint-Vincent, J.B.G.M. 1824. « Psychodiaire ». In « Encyclopédie méthodique. Histoire naturelle des zoophytes ou animaux rayonnés faisant suite à l'histoire naturelle des vers de Bruguière. Imprimerie de la veuve Agasse. Paris.

Carter, N. 1937. New or interesting algae from brackish water. Arch. Protist. 90: 1–68.

Chepurnov, V.A., D.G. Mann, K. Sabbe and W. Vyverman. 2004. Experimental studies on sexual reproduction in diatoms. Int. Rev. Cytol. 237: 91–154.

Chepurnov, V.A. and D.G. Mann. 2004. Auxosporulation of Licmophora communis (Bacillariophyta) and a review of mating systems and sexual reproduction in araphid pennate diatoms. Phycol. Res. 52: 1–12.

Conrad, W. 1941. Sur les Chrysomonadines à trois fouets. Aperçu synoptique. Bull. Mus. Natl. Hist. (Belg.) 17: 1–16.

Cortés, M.Y. and J. Bollmann. 2002. A new combination coccosphere of the heterococcolith species *Coronosphaera mediterranea* and the holococcolith species *Calyptrolithina hasleana*. Eur. J. Phycol. 37: 145–146.

Cros, L., A. Kleijne, A. Zeltner, C. Billard and J.R. Young. 2000. New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorid biology. In Marine Micropaleontology. Nannoplankton Ecology and Palaeoecology. Proc. INA7 Conf., Puerto Rico 1998. 39: 1–34.

Cros, L. and J.-M. Fortuno. 2002. Atlas of northwestern Mediterranean coccolithophores. Sci. Mar. 66. pp. 186.

Dale, B. 1983. Dinoflagellate resting cysts. *In*: G.A. Fryxell (ed.). *Survival Strategies of the Algae*, Cambridge, Cambridge University Press, pp. 69–144.

Davidovich, N.A. 2001. Species specific sizes and size range of sexual reproduction in diatoms. *In:* Proceedings of the 16th International Diatom Symposium. Athens & Aegean Islands, 25 August–1 September 2000 (Ed. by A. Economou - Amilli), 601 pp. University of Athens, Faculty of Biology, Athens 2001. 191–196.

Davidovich, N.A. 2002. Sexual heterogeneity of the clones of *Nitzschia longissima* (Breb.) Ralfs (Bacillariophyta) Int. J. Algae. 4: 104–116.

Davidovich, N.A. and S.S. Bates. 1998. Sexual reproduction in the pennate diatoms *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima* (Bacillariophyceae). J. Phycol. 34: 126–137.

Davidovich, N.A., I. Kaczmarska and J.M. Ehrman. 2006. The sexual structure of a natural population of the diatom *Nitzschia longissima* (Bréb.) Ralfs *In*: Proceedings of the 18th International Diatom Symposium. Miedzyzdroje, Poland 2–7 September. 2004. (A. Witkowski ed.) Biopress Limited, Bristol 27–40.

Davidovich, N.A., I. Kaczmarska and J.M. Ehrman. 2010. Heterothallic and homothallic sexual reproduction in *Tabularia fasciculata* (Bacillariophyta). Fottea 10: 251–266.

Davidovich, N.A., I. Kaczmarska, S.A. Karpov, O.I. Davidovich, M.L. MacGillivary and L. Mather. 2012. Mechanism of male gamete motility in araphid pennate diatoms from the genus *Tabularia* (Bacillariophyta). Protist 163: 480–494.

Davidovich, N.A., J.-L. Mouget and P. Gaudin. 2009. Heterothallism in the pennate diatom *Haslea ostrearia* (Bacillariophyta). Eur. J. Phycol. 44: 251–261.

Drebes, G. 1977. Sexuality. *In*: The Biology of Diatoms. Botanical Monographs (D. Werner ed.). Blackwell Scientific Publications, Oxford 13: 250–283.

Edlund, M.B. and E.F. Stoermer. 1997. Ecological, evolutionary, and systematic significance of diatom life histories. J. Phycol. 33: 897–918.

Edvardsen, B. and D. Vaulot. 1996. Ploidy analysis of the two motile forms of *Chyrsochromulina polylepis* (Prymnesiophyceae). J. Phycol. 32: 94–102.

Edvardsen, B. and L. Medlin. 1998. Genetic analyses of authentic and alternate forms of *Chrysochromulina polylepis* (Haptophyta). Phycologia 37: 275–283.

Ehrenberg, C.G. 1838. Die Infusionsthierchen als vollkommene Organismen. Ein Blick in das Tiefere organische Leber der Natur. Leopold Voss, Leipzig.

Endlicher, S.L. 1836–1850. Genera Plantarum Secundum Ordines Naturales Disposita. Vindobonae (Vienna).

Figueroa, R.I. and I. Bravo. 2005a. A study of the sexual reproduction and determination of mating type of *Gymnodinium nolleri* (Dinophyceae) in culture. J. Phycol. 41: 74–83.
Figueroa, R.I. and I. Bravo. 2005b. Sexual reproduction and two different encystment strategies of *Lingulodinium polyedrum* (Dinophyceae) in culture. J. Phycol. 41: 370–379.
Figueroa, R.I., I. Bravo, E. Garcés and I. Ramilo. 2006a. Nuclear features and effect of nutrients on *Gymnodinium catenatum* (Dinophyceae) sexual stages. J. Phycol. 42: 67–77.
Figueroa, R.I., I. Bravo and E. Garcés. 2006b. The multiple routes of sexuality in *Alexandrium taylori* (Dinophyceae) in culture. J. Phycol. 42: 1028–1039.
Figueroa, R.I., E. Garcés and I. Bravo. 2007. Comparative study of the life cycles of *Alexandrium tamutum* and *Alexandrium minutum* (Gonyaulacales, Dinophyceae) in culture. J. Phycol. 43: 1039–1053.
Figueroa, R.I., I. Bravo, I. Ramilo, Y. Pazos and A. Moroño. 2008. New life cycle stages of *Gymnodinium catenatum* (Dinophyceae): Laboratory and field observations. Aq. Microbiol. Ecol. 52: 13–23.
Frada, M.J., I. Probert, M. Allen, W. Wilson and C. de Vargas. 2008. The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection. PNAS 105(41): 15944–15949.
Frada, M.J., K.D. Bidle, I. Probert and C. de Vargas. 2012. *In situ* survey of life cycle phases of the coccolithophore *Emiliania huxleyi*. Env. Microbiol. 14(6): 1558–1569.
French, F.W. III. and P.E. Hargraves. 1985. Spore formation in the life cycles of the diatoms *Chaetoceros diadema* and *Leptocylindrus danicus*. J. Phycol. 21: 477–483.
Fresnel, J. 1994. A heteromorphic life cycle in two coastal coccolithophorids, *Hymenomonas lacuna* and *Hymenomonas coronata* (Prymnesiophyceae). Can. J. Bot. 72: 1455–1462.
Gaarder, K.R. 1970. Three new taxa of Coccolithinae. Nytt Mag. Bot. 17: 113–126.
Gaebler-Schwarz, S., A. Davidson, P. Assmy, J. Chen, J. Henjes, E.M. Nothig, M. Lunau and L.K. Medlin. 2010. A new cell stage in the haploid–diploid life cycle of the colony-forming haptophyte *Phaeocystis antarctica* and its ecological implication. J. Phycol. 46: 1006–1016.
Gallagher, J.C. 1983. Cell enlargement in *Skeletonema costatum* (Bacillariophyceae). J. Phycol. 19: 539–542.
Garcés, E., A. Zingone, M. Montresor, B. Regera and B. Dale (eds.). 2002. LIFEHAB: Life history of microalgal species causing harmful blooms. European Commission. Research in Enclosed Seas series-12. Brussels.
Gay, F. 1891. Recherches sur le développement et la classification de quelques algues vertes. Librairie Des Sciences Naturelles Paul Klincksiek.
Gayral, P. and J. Fresnel. 1983. Description, sexualité et cycle de développement d'une nouvelle Coccolithophoracée (Prymnesiophyceae): *Pleurochrysis pseudoroscoffensis* sp. nov. Protistologica 19: 245–261.
Geisen, M., C. Billard, A.T.C. Broerse, L. Cros, I. Probert and J.R. Young. 2002. Life-cycle associations involving pairs of holococcolithophorid species: intraspecific variation or cryptic speciation? Eur. J. Phycol. 37: 531–550.
Geitler, L. 1932. Der Formwechsel der pennaten Diatomeen (Kieselalgen). Archiv Protistenkunde. 78: 1–226.
Geitler, L. 1935. Reproduction and life history in diatoms. Bot. Rev. 1: 149–161.
Geitler, L. 1973. Auxosporenbildung und Systematik bei pennaten Diatomeen und die Cytologie von Cocconeis-Sippen. Österreichische Botanische Zeitschrift. 122: 299–321.
Gillard, J., J. Frenkel, V. Devos, K. Sabbe, C. Paul, M. Rempt, D. Inzé, G. Pohnert, M. Vuylsteke and W. Vyverman. 2012. Metabolomics enables the structure elucidation of a diatom sex pheromone. Angew. Chem. Int. Ed. 51: 1–5.
Grell, K.G. 1973. Protozoology. Berlin, Heidelberg/New York: Springer. Haeckel E. 1878. Das Protistenreich. Eine populäre Übersicht über das Formengebiet der niedersten Lebewesen. Mit einem wissenschaftlichen Anhange: System der Protisten, E. Günther Verlag. Leipzig, 554 p.

Haeckel, E.H.P.A. 1878. Das Protistenreich [microform]: eine populäre Uebersicht über das Formengebiet der niedersten Lebewesen : mit wissenshaftlichen Anhange : System der Protisten. Leipzig. E. Günther.

Hallegraeff, G.M., S.I. Blackburn, C.J. Bolch and M.A. Doblin. 2012. Global toxicology, ecophysiology and population structure of the chainforming PST dinoflagellate *Gymnodinium catenatum*. Harmful Algae 14: 130–143.

Harada, A. and T. Yamagishi. 1984. Meiosis in *Spirogyra* (Chlorophyceae). Japanese J. Phycol. 32: 10–18.

Heath, I.B. and W.M. Darley. 1972. Observations on the ultrastructure of the male gametes of *Biddulphia laevis* Ehr. J. Phycol. 8: 51–59.

Hiltz, M., S.S. Bates and I. Kaczmarska. 2000. Effect of light: dark cycles and cell apical length on the sexual reproduction of the pennate diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae) in culture. Phycologia 39: 59–66.

Houdan, A., C. Billard, D. Marie, F. Not, A.G. Sáez, J.R. Young and I. Probert. 2004. Flow cytometric analysis of relative ploidy levels in holococcolithophore-heterococcolithophore (Haptophyta) life cycles. System. Biodiv. 1(4): 453–465.

Houdan, A., I. Probert, K. Van Lenning and S. Lefebvre. 2005. Comparison of photosynthetic responses in diploid and haploid life-cycle phases of *Emiliania huxleyi* (Prymnesiophyceae). Mar. Ecol. Progr. Ser. 292: 139–146.

Inouye, I. and M. Chihara. 1983. Ultrastructure and taxonomy of *Jomonlithus littoralis* gen. et sp. nov. (Class Prymnesiophyceae), a coccolithophorid from the Northwest Pacific. Bot. Mag. Tokyo 96: 365–376.

Inouye, I. and R.N. Pienaar. 1988. Light and electron microscope observations of the type species of Syracosphaera, *S. pulchra* (Prymnesiophyceae). Br. Phycol. J. 23: 205–17.

Joseph, G. 1879. Ueber Grotten-Infusorien. Zool. Anz. 2: 114–118.

Kirk, D.L. and H. Gruber. 2005. Ferdinand Cohn, multi-faceted microbiologist extraordinaire. Protist 156: 355–373.

Klaveness, D. 1973. The microanatomy of *Calyptrosphaera sphaeroidea*, with some supplementary observations on the motile stages of *Coccolithus pelagicus*. Norw. J. Bot. 20: 151–162.

Kleijne, A. 1992. Extant Rhabdosphaeraceae (coccolithophorids, class Prymnesiophyceae) from the Indian Ocean, Red Sea, Mediterranean Sea and North Atlantic Ocean. Scripta Geologica 100: 1–63.

Kooistra, W.H.C.F., M.D. Stefano, D.G. Mann, N. Salma and L.K. Medlin. 2003. Phylogenetic position of *Toxarium*, a pennate-like lineage within centric diatoms (Bacillariophyceae). J. Phycol. 39: 185–197.

Kremp, A. and M.W. Parrow. 2006. Evidence for asexual resting cysts in the life cycle of the marine peridinoid dinoflagellate, *Scrippsiella hangoei*. J. Phycol. 42: 400–409.

Kützing, F.T. 1844. Über die Verwandlung der Infusorien in niedere Algenformen. W. Köhne Verlag. Nordhausen.

Larsen, A. and L.K. Medlin. 1997. Inter- and intraspecific genetic variation in twelve *Prymnesium* (Haptophyceae) clones. J. Phycol. 33: 1007–1015.

Larsen, A. and B. Edvardsen. 1998. Relative ploidy levels in *Prymnesium parvum* and *P. patelliferum* (Haptophyta) analyzed by flow cytometry. Phycologia 37: 412–424.

Leadbeater, B.S.C. 1970. Preliminary observations on differences of scale morphology at various stages in the life-cycle of '*Apistonema-Syracosphaera*' *sensu* von Stosch. Br. Phycol. J. 5: 57–69.

Leadbeater, B.S.C. 1971. Observations on the life-history of the haptophycean alga *Pleurochrysis scherffelii* with special reference to the microanatomy of the different types of motile cell. Ann. Bot. 35: 429–439.

Leadbeater, B.S.C. 1994. Cell coverings. *In*: J.C. Green and B.S.C. Leadbeater (eds.). *The Haptophyte Algae*. The Systematics Association Special Volume 51. Oxford University Press, Oxford, pp. 23–46.

Lefort, F. 1975. Étude de quelques Coccolithophoracées marines rapportées aux genres *Hymenomonas* et *Ochrosphaera.* Cah. Biol. Mar. 16: 213–229.

Linnaeus, C. 1753a. Species plantarum: exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Tomus I. Stockholm: Impensis Laurentii Salvii. First edition.
Linnaeus, C. 1753b. Species plantarum: exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Tomus II. Stockholm: Impensis Laurentii Salvii. First edition.
Linnaeus, C. 1754. Genera plantarum: eorumque characteres naturales secundum numerum, figuram, situm, et proportionem omnium fructificationis partium. Stockholm: Impensis Laurentii Salvii. Fifth edition.
Lewis, W.M. Jr. 1984. The diatom sex clock and its evolutionary significance. The American Naturalist 123: 73–80.
Mable, B.K. and S.P. Otto. 1998. The evolution of life cycles with haploid and diploid phases. BioEssays 20: 453–462.
MacDonald, J.D. 1869. On the structure of the diatomaceous frustule, and its genetic cycle. Annals and Magazine of Natural History 3: 1–8.
Mann, D.G. 1982. Auxospore formation in *Licmophora* (Bacillariophyta). Plant Systematics and Evolution. 139: 289–294.
Mann, D.G. 1993. Patterns of sexual reproduction in diatoms. (H. van Dam (ed.), 12th International Diatom Symposium, Renesse (Netherlands), 30 Aug–5 Sep 1992. Kluwer Academic Publishers. Printed in Belgium) Hydrobiologia. 269/270: 11–20.
Mann, D.G., V.A. Chepurnov and M. Idei. 2003. Mating system, sexual reproduction, and auxosporulation in the anomalous raphid diatom *Eunotia* (Bacillariophyta). J. Phycol. 39: 1067–1084.
Manton, I. and G.F. Leedale. 1969. Observations on the micro-anatomy of *Crystallolithus hyalinus* Gaarder and Markali. Arch. Mikrobiol. 47: 115–136.
Manton, I. and H.A. von Stosch. 1966. Observations on the fine structure of the male gamete of the marine centric diatom *Lithodesmium undulatum*. J. Roy. Microsc. Soc. 85: 119–134.
Marchand, L. 1869. De la reproduction des animaux infusoires. F. Savy éditeurs. Paris.
Mouget, J.-L., R. Gastineau, O. Davidovich, P. Gaudin and N.A. Davidovich. 2009. Light is a key factor in triggering sexual reproduction in the pennate diatom Haslea ostrearia. FEMS Microbiol. Ecol. 69: 194–201.
Nagai, S., Y. Hori, T. Manabe and I. Imai. 1995. Restoration of cell size by vegetative cell enlargement in *Coscinodiscus wailesii* (Bacillariophyceae). Phycologia 34: 533–535.
Noël, M.H., M. Kawachi and I. Inouye. 2004. Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (*Prymnesiophyceae, Haptophyta*). J. Phycol. 40: 112–129.
Parke, M. and I. Adams. 1960. The motile (*Crystallolithus hyalinus* Gaarder and Markali) and nonmotile phases in the life history of *Coccolithus pelagicus* (Wallich) Schiller. J. Mar. Biol. Ass. UK 39: 263–274.
Payer, J.B. 1850. Botanique cryptogamique ou histoire des familles naturelles des plantes inférieures. Victor Masson. Paris.
Pfiester, L.A. 1975. Sexual reproduction of *Peridinium cinctum* f. *ovoplanum* (Dinophyceae). J. Phycol. 11: 259–265.
Pfiester, L.A. 1976. Sexual reproduction of *Peridinium willei* (Dinophyceae). J. Phycol. 12: 34–8.
Pfiester, L.A. 1977. Sexual reproduction of *Peridinium gatunense* (Dinophyceae). J. Phycol. 13: 92–95.
Pfiester, L.A. and D.M. Anderson. 1987. Dinoflagellate reproduction. *In*: The biology of dinoflagellates. Botanical monographs 21 (ed. by F.J.R. Taylor), Blackwell Scientific Publications, pp. 611–648.
Pfitzer, E. 1869. Über Bau und Zellteilung der Diatomeen. Botanische Zeitung 27: 774–776.
Pienaar, R.N. 1980. Observations on the structure and composition of the cyst of *Prymnesium* (Prymnesiophyceae). Ann. Conf. Proc. Electron Microscopy Society of South Africa, pp. 73–74.
Pouchet, G. 1883. Contribution à l'histoire des Cilio-flagelles. J. Anat. Physiol. 19: 399–455.

Pringsheim, N. 1855. Über die Befruchtung und Keimung der Algen und das Wesen des Zeugungsactes. August Kirschwald. Berlin.

Probert, I. and J. Fresnel. 2007. *Prymnesium lepailleurii* sp. nov., a new toxic flagellate from the Mediterranean Sea. Eur. J. Phycol. 42: 289–294.

Rayns, D.G. 1962. Alternation of generations in a coccolithophorid *Cricosphaera carterae* (Braarud and Fagerland) Braarud. J. Mar. Biol. Ass. UK 42: 481–484.

Réaumur, R.A.F. 1711. Description des fleurs et des graines de divers fucus et quelques autres observations physiques sur ces mêmes plantes. Mémoires de l'Académie Royale des Sciences.

Réaumur, R.A.F. 1712. Suite d'un mémoire imprimé en 1711 page 282 sur les fleurs et les graines de diverses plantes marines. Mémoires de l'Académie Royale des Sciences.

Roshchin, A.M. and V.A. Chepurnov. 1999. Dioecy and monoecy in the pennate diatoms (with reference to the centric taxa). *In*: Proceedings of the 14th International Diatom Symposium. (ed. by M. Mayama, M. Idei and I. Koizumi). Koeltz Scientific Books, Koenigstein 241–261.

Rosowski, J.R., L.M. Johnson and D.G. Mann. 1992. Comment on the report of gametogenesis, ogamy, and uniflagellated sperm in the pennate diatom *Nitzschia pungens* (1991. J. Phycol. 27: 21–26). J. Phycol. 28: 570–574.

Rost, B. and U. Riebesell. 2004. Coccolithophore calcification and the biological pump: reponse to environmental changes. In *Coccolithophores: From the Molecular Processes to Global Impact*. H.R. Thierstein and J.R. Young (eds.). New York, USA: Springer Verlag, pp. 99–125.

Round, F.E., R.M. Crawford and D.G. Mann. 1990. The Diatoms. Biology and Morphology of the Genera. Cambridge University Press, Cambridge 747 p.

Rousseau, V., M.J. Chretionnot-Dinet, A. Jacobsen, P. Verity and S. Whipple. 2007. The life cycle of *Phaeocystis*: state of knowledge and presumptive role in ecology. Biogeochem. 83: 29–47.

Sabbe, K., V.A. Chepurnov, W. Vyverman and D.G. Mann. 2004. Apomixis in *Achnanthes* (Bacillariophyceae); development of a model system for diatom reproductive biology. Eur. J. Phycol. 39: 327–341.

Sato, S., G. Beakes, M. Idei, T. Nagumo and D.G. Mann. 2011. Novel sex cells and evidence for sex pheromones in diatoms. PLoS ONE 6(10): e26923. doi:10.1371/journal.pone.0026923.

Sato, S. and L.K. Medlin. 2006. Motility of non-araphid diatoms. Diatom Research 21: 473–477.

Schultz, M.E. and F.R. Trainor. 1968. Production of male gametes and auxospores in the centric diatoms *Cyclotella meneghiniana* and *C. cryptica*. J. Phycol. 4: 85–88.

Schwarz, E. 1932. Beiträge zur Entwicklungsgeschichte der Protophyten. IX. Der Formwechsel von *Ochrosphaera neapolitana.* Arch. Protist 77: 434–462.

Stickle, A.J. 1986. Mastogloia smithii has a method of sexual reproduction hitherto unknown in raphid diatoms. Diatom Research 1: 271–282.

Strasburger, A.E. 1906. Lehrbuch der Botanik für Hochschulen: mit 779 Abb. 8. Auflage Fischer. Jena.

Subba Rao, D.V., F. Partensky, G. Wohlgeschaffen and W.K.W. Li. 1991. Flow cytometry and microscopy of gametogenesis in *Nitzschia pungens*, a toxic, bloom-forming, marine diatom. J. Phycol. 27: 21–26.

Subba Rao, D.V., F. Partensky, G. Wohlgeschaffen and W.K.W. Li. 1992. Comment. Gametogenesis in *Nitzschia pungens f.multiseries* J. Phycol. 28: 574–576.

Sym, S. and M. Kawachi. 2000. Ultrastructure of *Calyptrosphaera radiata*, sp. nov. (Prymnesiophyceae, Haptophyta). Eur. J. Phycol. 35: 283–293.

Thomsen, H.A., J.B. Østergaard and L.E. Hansen. 1991. Heteromorphic life histories in Arctic coccolithophorids (Prymnesiophyceae). J. Phycol. 27: 634–642.

Thuret, G.A. 1851. Recherches sur les zoospores des algues et les anthéridies des cryptogames. Imprimerie L. Martinet. Paris.

Thwaites, G.K. 1847. On conjugation in the Diatomaceae. Report of the 17th meeting of the British Association for the Advancement of Science held at Oxford, transactions of the sections.

Valero, M., S. Richerd and V. Perrot. 1992. Evolution of alternation of haploid and diploid phases in life cycles. Trends Ecol. Evol. 7: 25–29.

van Leeuwenhoek, A. 1702. Letter from Mr Antony van Leeuwenhoek, F. R. S. concerning Green Weeds Growing in Water, and Some Animalcula Found about Them Phil. Trans. 1702 23 1304-1311; doi:10.1098/rstl.1702.0042.

Vanormelingen, P., V.A. Chepurnov, D.G. Mann, K. Sabbe and W. Vyverman. 2008. Genetic divergence and reproductive barriers among morphologically heterogeneous sympatric clones of *Eunotia bilunaris Sensu Lato* (Bacillariophyta). Protist 159: 73–90.

Vaucher, J.P. 1803. Histoire des Conferves d'eau douce contenant leurs différents modes de reproduction, et la description de leurs principales espèces suivie de l'histoire des Trémelles et des Ulves d'eau douce. J.J. Paschoud Editeur. Genève.

Vaulot, D., J.L. Birrien, D. Marie, R. Casotti, M.J.W. Veldhuis, G.W. Kraay and M.J. Chretionnot-Dinet. 1994. Morphology, ploidy, pigment composition, and genome size of cultured strains of *Phaeocystis* (Prymnesiophyceae). J. Phycol. 30: 1022–1035.

von Dassow, P., H. Ogata, I. Probert, P. Wincker, C. Da Silva, S. Audic, J.M. Claverie and C. de Vargas. 2009. Transcriptome analysis of functional differentiation between haploid and diploid cells of *Emiliania huxleyi*, a globally significant photosynthetic calcifying cell. Genome Biol. 10: R114.

von Stosch, H.A. 1967. Haptophyceae. *In*: W. Rhuland (ed.). Vegetative Fortpflanzung, Parthogenese und Apogamie bei Algen. Encylopedia of Plant Physiology. Springer-Verlag, Berlin, pp. 646–656.

von Stosch, H.A. 1969. Dinoflagellaten aus der Nordsee II. *Helgolandinium subglobosum* gen. et. spec. nov. Helgolander wiss. Meeresunt. 19: 569–577.

von Stosch, H.A. 1972. La signification cytologique de la 'cyclo nucléaire' dans le cycle de vie des dinoflagelles. Soc. Bot. Fr., Mémoires, 201–202.

von Stosch, H.A. 1973. Observation on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. Br. Phycol. J. 8: 105–134.

von Stosch, H.A. and G. Drebes. 1964. Entwicklungsgeschichtliche Untersuchungen an zentrischen Diatomeen. IV. Die Planktohdiatomee *Stephanopyxis turris*-ihre Behandlung und Entwicklungsgeschichte. Hegoländer wiss. Meeresunt. 11: 209–257.

von Stosch, H.A. 1965. Sexualitat bei *Ceratium cornutum* (Dinophyta). Naturwissenschaften, 52: 112–113.

Walker, L.M. 1982. Evidence for a sexual cycle in the Florida red tide dinoflagellate *Ptychodiscus brevis (=Gymnodinium breve*). Bioscience 32: 809–810.

Walker, L.M. 1984. Life histories, dispersal and survival in marine, planktonic dinoflagellates. *In*: K.A. Steidinger and L.M. Walker (eds.). Marine Plankton Life Cycle Strategies. CRC press, Florida, USA, pp. 19–34.

Wall, D. and B. Dale. 1968. Modern dinoflagellate cysts and evolution of the Peridiniales. Micropaleontology 14: 265–304.

Wiese, L. 1969 Algae. *In:* Fertilisation. Comparative morphology, biochemistry, and immunology. C.B. Metz and A.Monroy (eds.). Vol. 2. Academic Press, London and New York 169–188.

Young, J.R., R.W. Jordan and L. Cros. 1998. Notes on nannoplankton systematics and life-cycles- *Ceratolithus cristatus*, *Neosphaera coccolithomorpha* and *Umbilicosphaera sibogae*. J. Nannoplankton Res. 20: 89–99.

Zingmark, R. 1970. Sexual reproduction in the dinoflagellate *Noctiluca miliaris* Suriray. J. Phycol. 6: 122–126.

Zingone, A., G. Forlani, I. Percopo and M. Montresor. 2011. Morphological characterization of *Phaeocystis antarctica* (Prymnesiophyceae). Phycologia 50(6): 650–660.

2

Mechanism of Sexual Reproduction in Fresh Water Microalgae

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ABSTRACT

The sexual reproductive processes of three representative freshwater green algae are reviewed. The *Chlamydomonas reinhardtii* is a heterothallic species having two mating types: mating type plus and mating type minus, which are controlled by a single complex mating type locus (MT^+ and MT^-). The differentiation of respective gametes is triggered by nitrogen starvation. The sexual adhesion between the gametes is mediated by sex-specific agglutinin molecules on their flagellar membranes. Then, the intracellular cAMP level is elevated, triggering dramatic alterations in the cell. Cell fusion is initiated by an adhesive interaction between the mt^+ and mt^- mating structures, followed by localized membrane fusion. Two proteins (FUS1 and GCS1/HAP2) are known to be an essential for the membrane fusion reaction. The *Volvox carteri* is a dioecious species and is composed of only two cell types, 2000–4000 biflagellate *Chlamydomonas*-like somatic cells and 16 reproductive cells (gonidia). Sexual reproduction is initiated by a mutation-like switch, which leads to the formation of the first sexual male colony. The sex-inducing pheromone is produced and released

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by this sexual male colony and acts on the asexual gonidia of both sexes. It alters their developmental pathway such that sexual forms (egg- or sperm-bearing forms) are produced in the next generation. Sexual reproduction of heterothallic *Closterium peracerosum-strigosum-littorale* complex is controlled by two multifunctional sex pheromones, PR-IP and PR-IP Inducer that independently promote multiple steps in conjugation at the appropriate times through different induction mechanisms. Conjugation processes of the homothallic strain are also controlled by pheromone(s) orthologous to the heterothallic sex pheromone. In addition, it is suggested that the division of one vegetative cell into two sister gametangial cells during conjugation processes is a segregative process capable of producing complementary mating types in homothallic strains.

Keywords: Closterium, Chlamydomonas, Volvox, sexual reproduction

Introduction

Sexual reproduction is of undisputed importance for both the prosperity of a species and the production of new progeny that can overcome environmental changes. The sexual reproductive process of living organisms consists of several steps, including meiosis, sex determination or differentiation, induction and differentiation of sexual structures, mutual recognition by individuals of the opposite sex, release and fusion of gametes, and the formation of zygotes, although the sequence of these events differs between species. For successful fertilization to occur, concurrent expression of both types of gametes or sexes is required. Synchronization of this step depends on various mechanisms, including genetic programs, environmental factors, endogenous rhythms, physical contact, and exposure to chemical substances.

In some algae, dormant zygotes are formed as a result of sexual reproduction and show resistance to severe environmental conditions, such as drought. In the case of brown algae, sex pheromones are always involved in successful fertilization (Maier 1993, 1995; Sekimoto 2005). The sex pheromones secreted by immotile female gametes or freshly released eggs induce the chemoattraction or kinetic orientation of the motile male gametes toward female gametes. In some species, pheromones are also responsible for the release of male gametes from the male reproductive organs (antheridia). All of the currently identified pheromones are volatile unsaturated C_{11} or C_8 hydrocarbons. Although several possible mechanisms of action have been proposed, to date, none have been confirmed (Maier and Müller 1986; Maier and Calenberg 1994). Recently, the 214 million base pair (Mbp) genome sequence for *Ectocarpus siliculosus* was reported, and the presence of a family of receptor kinases was indicated (Cock et al. 2010). This information has the potential to be useful for identifying a putative pheromone receptor.

The green algae are monophyletic with embryophyte green plants (land plants), although the sister of the embryophytes includes only a few green algae. Their methods of sexual reproduction are quite varied. The green algae have evolved in two major lineages: the chlorophyte clade and the charophyte clade. The chlorophycean algae include the majority of species traditionally called green algae. The charophycean algae form a relevant monophyly with land plants. Charophyceans and land plants share many distinctive characteristics with respect to cellular structures and metabolism, and are evolutionarily distant from the chlorophycean algae (Graham and Wilcox 2000; Karol et al. 2001; Graham et al. 2009). In this review, the sexual reproductive processes of freshwater green algae, especially chlorophycean (*Chlamydomonas reinhardtii* and *Volvox carteri*) and charophycean (the *Closterium peracerosum–strigosum–littorale* complex; *Cl. psl.* complex) are presented.

Sexual Reproduction in *Chlamydomonas reinhardtii*

Overview of the Life Cycle of *Chlamydomonas reinhardtii*

Chlamydomonas (*Ch.*) *reinhardtii* was first isolated as a green soil alga and is used as a model organism in plants. It retains two flagella subsequently lost by most plants and a chloroplast being functionally equivalent to the chloroplasts of green plants. Its complete genome sequence is available (Merchant et al. 2007) and its life cycle is well characterized. *Ch. reinhardtii* has two mating types: mating type plus (mt^+) and mating type minus (mt^-), which are controlled by a single complex mating type locus (MT^+ or MT^-) (Ferris et al. 2002). They proliferate asexually when an adequate source of nitrogen exists in the environment. After mitosis in the mitotic cell cycle, the newly formed daughter cells are liberated by the effect of sporangin (a subtilisin-like serine protease) acting on the breakdown of the sporangial cell walls (Matsuda et al. 1995; Kubo et al. 2009). When nitrogen levels fall below a certain threshold, vegetative cells having the MT^+ locus differentiate into mt^+ gametes and cells having the MT^- locus differentiate into mt^- gametes. Three hierarchically regulated gene expression programs are generally recognized as a response to nitrogen depletion: a program to adapt to nitrogen starvation, a gamete differentiation program, and a zygote formation program (Abe et al. 2004, 2005; Kubo et al. 2008). Within minutes of being mixed, gametes start to agglutinate. Gametes of opposite mating types pair with each other and fuse to form binucleate quadriflagellated cells (QFCs). The two nuclei in the cell fuse, and a novel set of zygote-specific genes is expressed to form a dormant zygote that is resistant to both freezing and desiccation. When conditions improve, the dormant zygote initiates meiosis and the four recombinant haploid products resume vegetative growth (Fig. 1).

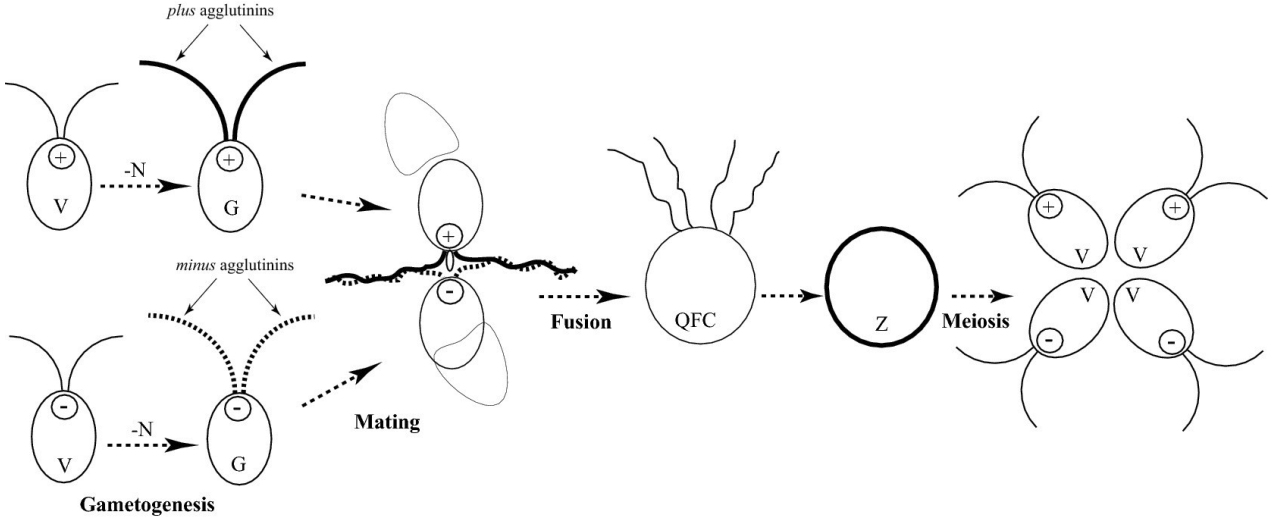

Figure 1. The life cycle of *Chlamydomonas reinhardtii*. Vegetative cells (V) differentiate into mt^+ and mt^- gametes (G) during nitrogen starvation (–N). Mating types are restricted by mating-type loci (+ and –). When gametes are mixed, the *plus* and *minus* agglutinin molecules on their flagellar surfaces adhere to each other and adhesion generates an elevation of intracellular cAMP. The signal triggers gamete cell wall release and mating-structure activation. Cells then fuse to form binucleate quadriflagellated cells (QFCs). Zygotes having thick cell walls (Z) germinate in response to light and nitrogen supplementation, and undergo meiosis to release four haploid vegetative cells.

Sexual Adhesion

The sexual adhesion between the gametes is mediated by agglutinin molecules on their flagellar membranes. The plus and minus agglutinins are sex-specifically displayed by nitrogen-starved mt^+ and mt^- gametes, respectively. These molecules are encoded by different genes and possess complementary adhesive properties. They are both huge monomeric glycoproteins (>1000 kDa; Adair et al. 1983), possessing a large globular head and a fibrous shaft (Goodenough et al. 1985), and are members of the hydroxyproline-rich glycoprotein (HRGP) family (Cooper et al. 1983). Ferris et al. (2005) isolated the *SEXUAL AGGLUTINATION1* (*Sag1*) and *SEXUAL ADHESION1* (*Sad1*) genes encoding plus and minus agglutinins, respectively. Gene expression is restricted to gametes of one mating type. The presence of the *Minus-dominance* (*Mid*) gene localized on the MT^- locus suppresses the expression of *Sag1* but induces the expression of *Sad1*. Both deduced proteins are organized into three distinct domains: a head (C-terminal), a shaft, and an N-terminal domain. The plus and minus heads are quite large domains (2006 and 2404 amino acids, respectively) having 12 and 14 putative *N*-glycosylation sites, respectively. Six of the putative *N*-glycosylation sites are in similar locations. They are poorly conserved in the amino acid sequence except for two regions of the predicted hydrophobic α-helix. The shafts contain numerous repeats of the PPSPX motif. Head–head interactions, heads–shafts interactions, and antiparallel shaft–shaft interactions may be involved in the sexual adhesion between two specific agglutinins (Ferris et al. 2005).

Signal Transduction after the Interaction of Agglutinins

After the interactions between plus and minus agglutinin molecules on the flagellar membranes, a gamete-specific flagellar adenylyl cyclase is activated via a protein kinase- and kinesin-II-dependent pathway (Saito et al. 1993; Zhang and Snell 1994; Pan and Snell 2002) and the intracellular cAMP level is elevated nearly tenfold, triggering dramatic alterations in the cell (Pasquale and Goodenough 1987; Saito et al. 1993; Zhang and Snell 1994). The addition of a cell-permeable analog of cAMP, dibutyryl cAMP, can induce most of the cellular changes (Pasquale and Goodenough 1987). The mating-related effects of cAMP elevation include the following. First, flagellar motility is altered and the adhesiveness of the flagellar surface is increased by the translocation of inactive agglutinin molecules from the plasma membrane of the cell body onto the contiguous flagella membrane where the agglutinins become active (Saito et al. 1985; Goodenough 1989; Hunnicutt et al. 1990). The process is mediated by the kinesin/dynein-mediated intraflagellar transport system (Snell et al. 2004; Wang et al. 2006; Piao et al. 2009). Second,

activated gametes secrete a serine protease (*p*-lysinase) that converts an extracellularly stored prometalloprotease into an active matrix-degrading enzyme (Buchanan et al. 1989; Snell et al. 1989; Kinoshita et al. 1992), and the cell wall (multilayered glycoproteinaceous extracellular matrix) surrounding each cell is degraded so that the gametes are able to fuse. Third, mt^+ gametes erect an actin-filled microvillus ("fertilization tube") as a mating structure at the apical ends near the bases of the flagella and the mt^- gametes also erect a small, dome-like, actin-free mating structure. The mating structures of both types of gametes display an extracellular coat of material referred to as fringe (Goodenough et al. 1982).

Molecules Required for the Fusion

Cell fusion is initiated by an adhesive interaction between the mt^+ and mt^- mating structures, followed by localized membrane fusion. Two proteins are known to be an essential for the membrane fusion reaction. The first is FUS1, which is a single transmembrane protein on the mating structure of the mt^+ gamete (Ferris et al. 1996; Misamore et al. 2003). The *FUS1* gene is sex-specifically expressed and is located in the *MT*$^+$ locus (Ferris et al. 1996). FUS1 is an about 95-kDa protein and has domains related to the Ig-like domains of prokaryotic invasins and adhesins. It is essential for the adhesion of the mt^+ mating structure to an unidentified receptor on the mating structure of mt^- gametes (Misamore et al. 2002, 2003). The *fus1-1* mutant undergoes normal flagellar adhesion and gamete activation, and produces an actin-filled fertilization tube in response to cAMP; however, the *fus1-1* fertilization tube fails to fuse with the activated *minus* mating structure and the cells continue to agglutinate for several days. The mt^+ fringe is encoded by the *FUS1* gene (Misamore et al. 2003). Fertilization tubes on *fus1-1* gametes do not contain the fringe (Goodenough et al. 1982). When mt^+ gametes are incubated with the anti-FUS1 antibody, fusions with mt^- gametes are blocked.

The second protein required for the membrane fusion reaction is GCS1/HAP2, which is expressed on the surface of the mt^- mating structure as a single transmembrane protein (Liu et al. 2008). The gene has also been identified in other algae, protists, and higher plants (Mori et al. 2006; von Besser et al. 2006; Steele and Dana 2009; Wong and Johnson 2010). In *Arabidopsis thaliana*, the *GCS1/HAP2* gene is specifically expressed in sperm cells, and the mutant fails to fuse with both egg and central cells (Mori et al. 2006). In the case of the malaria organism *Plasmodium berghei*, GCS1/HAP2 is required at a particular step in the membrane fusion reaction between gamete membranes (Liu et al. 2008). The knockout mutant shows male sterility (Hirai et al. 2008). In *Ch. reinhardtii*, expression of the *GCS1/HAP2* gene is confirmed in both mt^+ and mt^- gametes but is far stronger in

mt^- gametes (Mori et al. 2006). GCS1/HAP2 protein localizes at the fusion site of mt^- gametes and mt^- *gcs1/hap2* mutant gametes can form tight pre-fusion membrane attachments with mt^+ gametes, but they fail to fuse (Liu et al. 2008). Both FUS1 and GCS1/HAP2 proteins are degraded rapidly upon fusion, as would be expected to block polygamy (Liu et al. 2010).

Development of the Zygote

The zygote developmental program is triggered by the heterodimerization of two homeoproteins, Gamete specific *plus*1 (Gsp1) and Gamete specific *minus*1 (Gsm1), which are contributed by the mt^+ gamete and mt^- gamete, respectively (Lee et al. 2008). Gsp1 is distantly related to the BELL class homeoproteins and Gsm1 is an ortholog of the KNOTTED1-like homeobox (KNOX) class (Hake et al. 2004; Scofield and Murray 2006). The expression of the *GSM1* gene is dependent on the expression of the *MINUS DOMINANCE* (*MID*) gene on the MT^- locus, while *GSP1* expression is inhibited by MID. *GSP1* was identified as a gene expressed specifically in mt^+ gametes (Kurvari et al. 1998). Ectopic expression of *GSP1* in mt^- gametes is responsible for the formation of the zygotic cell walls and the expression of several zygote-specific genes (Zhao et al. 2001). When both the *GSP1* and the *GSM1* genes were ectopically expressed in vegetative cells, a zygote developmental program was activated: they formed zygote-specific cell walls and expressed zygote specific genes, despite being in a nitrogen-supplemented medium. With ectopic expression of both *GSP1* and *GSM1* in a generated mt^+ diploid background, the resulting zygotes undergo normal meiosis (Lee et al. 2008). GSP1 is also important for the uniparental inheritance of chloroplast and mitochondrial DNA. A mutant, *biparental31* (*bp31*), having a deletion of about 60 kb on chromosome 2, including the *GSP1* gene, impairs the uniparental inheritance of chloroplast and mitochondrial DNA. The mutant phenotype can be rescued by a co-transformation with both the *GSP1* and *INOSITOL MONOPHOSPHATASE-LIKE1* (*INM1*) genes (Nishimura et al. 2012).

Sex Determination in *Chlamydomonas reinhardtii*

As explained previously, mating types of *Ch. reinhardtii* are controlled by a single complex mating type locus (MT^+ or MT^-) on linkage group VI (Ferris et al. 2002). Heterozygous mt^+/mt^- diploids, which are occasionally formed after mating, always mate as mt^- gametes, indicating that MT^- is dominant to MT^+ (Harris 1989). The core of the two *MT* loci encompasses 200–300 kb (Ferris and Goodenough 1994; Ferris et al. 2002, 2010). The *MT* loci contain highly rearranged DNA sequences, characterized by several large inversions and translocations, which act to suppress the recombination. Some genes

are specifically linked to either *MT* locus. The *FUS1* gene on the MT^+ locus, and the *MT locus region d* (*MTD1*) and *MID* genes on the MT^- locus have been assigned mating type-specific functions in gametogenesis and mating. Mt^+ cells transformed with the *MID* gene differentiate as mt^- gametes and the functional mutant in an mt^- background differentiates into an mt^+ gamete having all of the molecules required of an mt^+ gamete, except for the FUS1 protein (Ferris and Goodenough 1997; Ferris et al. 2002). These results indicate that *MID* is necessary both to activate mt^- gene expression and to prevent mt^+ gene expression, allowing the conversion of wild-type mt^+ gametes to mt^- gametes. *MID* encodes a RWP-RK family putative transcription factor. Vegetative mt^- cells express basal levels of *MID*. A pulse of upregulated expression (level 1, threefold increase to basal level) occurs at 30 min after nitrogen removal, followed by a return to the basal level at 1 h. The expression is strongly upregulated (level 2, eightfold) at 4–6 h together with the acquisition of mating competency (Lin and Goodenough 2007). Knockdown of *MTD1* in mt^- cells results in a failure to differentiate into gametes of either mating type after nitrogen removal. From the results, Lin and Goodenough (2007) proposed that the first increase of Mid (level 1) is sufficient to activate *MTD1* transcription and to repress mt^+ gamete-specific genes, and that *MTD1* expression in turn allows the second increase (level 2) that is necessary to turn on mt^- gamete-specific genes.

Sexual Reproduction in *Volvox carteri*

Overview of the *Volvox*

The spheroidal chlorophycean *Volvox* and its close relatives are suitable model organisms for addressing fundamental issues in the evolution of multicellularity and the development of a germ-soma dichotomy (Kirk 1998; Nozaki et al. 2000; Kirk and Nishii 2001; Nozaki 2003; Hallmann 2011). They form a group of genera closely related to the genus *Volvox* within the order Volvocales. This group ranges in complexity from unicellular organisms, such as *Ch. reinhardtii*, to homocytic colonial organisms, such as *Gonium pectorale*, to heterocytic multicellular organisms with different cell types, as is found in *Volvox*, which is the most highly developed genus. Several patterns of sexual reproduction are exhibited by the different species of the genus. Some species are monoclonic, while others are diclonic. Monoclonic species are either monoecious or dioecious. All diclonic species are dioecious and produce male and female spheroids in separate clones. *Volvox* mostly reproduces asexually, although it is able to switch to the sexual pathway. The involvement of sex pheromones in switching was first described by Darden who showed that asexual *V. aureus* (a monoclonic, monoecious species) cultures could be induced to change to the sexual pathway by

supplementing the cell-free cultured medium of mature male spheroids (Darden 1966). The same phenomena were subsequently reported for other species of *Volvox* (Kochert 1981).

Life cycles of *Volvox carteri*

V. carteri, a dioecious species, is the most intensively studied *Volvox* species (Kirk and Nishii 2001). It is composed of only two cell types, 2000–4000 biflagellate *Chlamydomonas*-like somatic cells, which form a monolayer at the surface of a hollow sphere and 16 reproductive cells (gonidia) that lie just below the sheet of somatic cells (Starr 1969). During embryogenesis, the embryos first cleave symmetrically five times to form a 32-cell embryo with identical cells, and then 16 cells divide asymmetrically to each produce one large gonidial cell precursor and one small somatic cell precursor (Kirk and Kirk 2004). These gonidial precursors divide asymmetrically two more times and produce additional somatic precursors at each division. The gonidial precursors then temporarily stop cell division, while the somatic precursors divide symmetrically about three more times. At the end of embryogenesis, the volume of the gonidial precursors expands to about 30-times that of their somatic precursors.

Sexual reproduction is initiated by a mutation-like switch with a probability of 2×10^{-4}, which leads to the formation of the first sexual male colony (Weisshaar et al. 1984). A pheromone, named "sex inducer" or "sex-inducing pheromone", has been the focus of considerable attention (Starr 1970). The sex-inducing pheromone is produced and released by this sexual male colony and acts on the asexual gonidia of both sexes. It alters their developmental pathway such that sexual forms (egg- or sperm-bearing forms) are produced in the next generation. In sexually induced male embryos, asymmetric cell division is postponed from the sixth to the eighth division cycle (Starr 1969, 1970; Hallmann et al. 1998). At this point, somatic cell precursors no longer divide and the large gonidial precursors each symmetrically divide seven times to form sperm bundles containing up to 128 sperm cells. Thus, the male ends up with 128 sperm bundles and 128 somatic cells. In female embryos, the first asymmetrical cell division is also postponed from the sixth to the seventh division cycle. Asymmetrical cell division then occurs and the somatic cell precursors continue to further cleave, with the large gonidial precursors developing as about 32 eggs and about 2000 somatic cells. Sperm bundles generated on the male colony contact the female colony by chance rather than by directed swimming (Coggin et al. 1979; Kirk 1998), after which a specific transient binding to somatic cells occurs. The sperm bundles break up into individual sperm cells and the sperm penetrate the extracellular matrix (ECM) of the female to reach the eggs inside the spheroids. The fusion of gametes results in the

formation of a dormant diploid zygote that survives the drought. Under favorable environmental conditions, the germination of the zygote occurs with meiosis to form only a single viable germling and three nonviable polar bodies. The germling will produce a haploid female or male and then reproduce asexually (Starr 1975; Fig. 2).

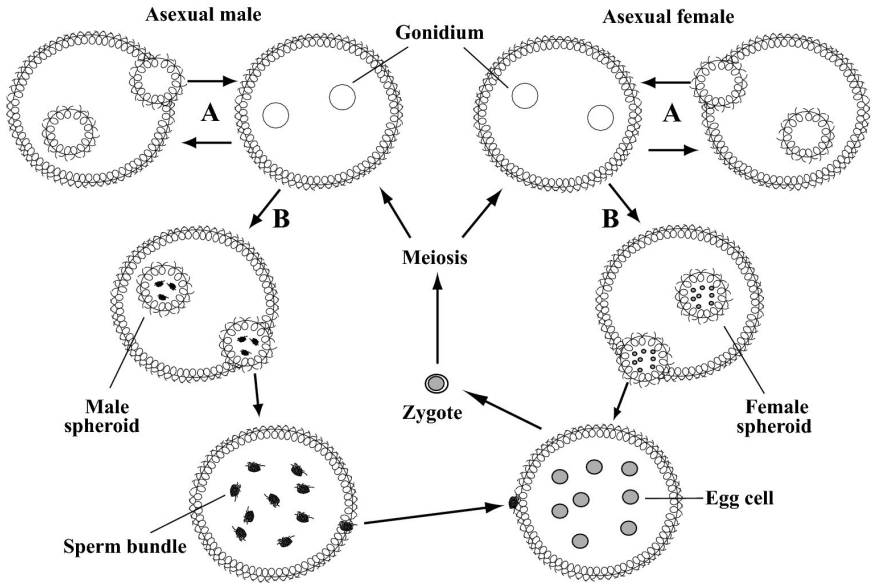

Figure 2. A life cycle of *Volvox carteri*. A: Asexual reproduction, B: Sexual differentiation induced by sex-inducing pheromone.

The *Volvox carteri* Sex-inducing Pheromone

Because the production of pheromone could not be detected in sexual females or the somatic cells of sexual males, it was believed that expression of the gene encoding the pheromone was tightly linked to sperm development (Starr 1970; Gilles et al. 1981). However, both asexual females and asexual males were able to produce the pheromone following exposure to a heat shock (Kirk and Kirk 1986). The participation of reactive oxygen species in both the production of the pheromone and its activity in triggering sexual development in gonidia has been suggested (Nedelcu and Michod 2003).

Sex-inducing pheromones were independently purified from two isolates of *V. carteri*: one from Japan (*V. carteri* f. *nagarensis*) and the other from the United States (*V. carteri* f. *weismannia*) (Kochert and Yates 1974; Starr and Jaenicke 1974). Both were glycoproteins of about 30-kDa. The inducer of the *V. carteri* f. *nagarensis* was strictly competent for its own gonidia whereas that of *V. carteri* f. *weismannia* induced sexuality of both

of them (Al-Hasani and Jaenicke 1992). The pheromone of *V. carteri* f. *nagarensis* is one of the most potent biological effector molecules known, exhibiting its full effectiveness below 10^{-16} M. As a result of successful large-scale production, partial amino acid sequences were obtained, allowing the cloning of a genomic clone encoding the pheromone (Tschochner et al. 1987) and the cDNA (Mages et al. 1988).

Possible Mode of Action of the Pheromone

The pheromone seems to have at least two modes of action. One is that the pheromone molecules act directly on the receptors of the gonidial cells. For this, the pheromone molecules must pass through the ECM. The other is that the pheromone molecules exert the effect by binding to the receptors on somatic cells, surrounding the spheroid. The binding triggers the synthesis of extracellular proteins, generating signal amplification. Because the very first experimentally detectable cellular responses to the sex pheromone come from the somatic cells at the surface, but not from the gonidial cells, the latter mode of action seems to be more plausible (Hallmann 2003).

The pheromone induces the synthesis of the deep zone hydroxyproline-rich glycoprotein (DZ-HRGP; Ender et al. 1999), chitinase/lysozyme, chitin-binding protein (Amon et al. 1998), and metalloproteinases (VMPs; Hallmann et al. 2001). Some of the ECM glycoproteins that are inducible by the sex pheromone are also inducible by mechanical wounding (Amon et al. 1998; Ender et al. 1999) but wounding does not cause the production of the sex pheromone itself. The majority of proteins synthesized shortly after the pheromone treatment in the ECM are part of a single family of glycoproteins: the pherophorins (Sumper et al. 1993; Godl et al. 1995, 1997). At least 34 different pherophorins occur in *Volvox* (Hallmann 2003, 2006; Prochnik et al. 2010). The carboxy-terminal domains of all pherophorins are similar to the pheromone. Pherophorin-II is considered to be responsible for the signal amplification mechanism of the pheromone (Sumper et al. 1993; Godl et al. 1995). Pherophorin-II is a glycoprotein that consists of three domains: the N-terminal domain, whose sequence is related to a motif of another ECM protein, SSG185; the polyproline spacer; and the carboxy-terminal domain, which is 30% identical to the sex-inducing pheromone. The carboxy-terminal domain is proteolytically liberated from the parent glycoprotein, after its pheromone induces synthesis. Because the inhibition of processing by protease inhibitors coincides with a suppression of sexual induction, and the induction of the gene expression of pherophorin-II by the pheromone could not be observed in all three independently isolated sterile mutants, the liberated domain may potentially act as an analog of the sex-inducing pheromone (Sumper et al. 1993; Godl et al. 1995). Transformed *V. carteri* expressing recombinant pherophorin-II, in which the carboxy-terminus

had been fused with green fluorescent protein (GFP), indicated that the carboxy-terminal domain including GFP was cleaved proteolytically, as in the native protein (Ishida 2007). The GFP signal of the transformant was located at the ECM directly surrounding the gonidium, the final target of the sexual-induction signal. However, no sex-inducing activity of the domain has been experimentally demonstrated.

Genes showing similarity with pherophorins have also been identified in other Volvocales (*Ch. reinhardtii*, *G. pectorale* and *Pandorina morum*), although information regarding their expression conditions is not available (Hallmann 2006). These pherophorins contain a (hydroxyl-) proline-rich (HR) rodlike domain and are abundant within the extracellular compartment, in a similar manner to the extensins of higher plants. In addition, pherophorins show a striking general structural similarity with a special class of extensin: the solanaceous lectins. Pherophorins have been suggested to be used as the versatile building blocks for the ECM architecture. In view of the large number of pherophorins, the pheromone is considered to be a pherophorin paralog and might have evolved a new function over time (Hallmann 2006).

A small cysteine-rich extracellular protein, named VCRP, which was quickly synthesized by somatic cells in response to the pheromone, has been identified (Hallmann 2007). In addition, a VCRP-related protein, VCRP2, has also been found using genome information from *V. carteri* (Hallmann 2008). Both VCRPs are speculated to be candidates for the extracellular second messenger from somatic cells to gonidial cells.

Mating-type Loci of *Volvox carteri*

As indicated previously, sexual differentiation in *Ch. reinhardtii* is largely controlled by the *MID* gene, encoding the RWP-RK family putative transcription factor. Nozaki et al. (2006) isolated an orthologous *MID* gene from the oogaomous volvocacean *Pleodorina starrii.* The gene, named *PlestMID*, is only present in the male genome and the protein is abundantly present in sperm nuclei. This finding strongly suggests that maleness was probably established from the minus mating type of its isogamous unicellular ancestor during the evolution of oogamy. The *MID* homolog has been identified in other volvocaceans, e.g., *G. pectorale* (*GpMID* in mt^- genome; Hamaji et al. 2008). Recently, both alleles of the *Volvox MT* were sequenced. Only two sex-limited genes, *MID* and *MTD1*, located on the *MT* loci of *Ch. reinhardtii* have recognizable homologs in the *Volvox MT*, and both are in the male *MT*. However, both *Volvox MID* (*VcMID*) and *Volvox MTD1* (*VcMTD1*) are expressed constitutively (Ferris et al. 2010). The *retinoblastoma-related protein1* (*RBR1*) gene, a homolog of *Ch. reinhardtii MATING-TYPE LINKED3* (*MAT3*), is located on the *Volvox* female *MT* and has a very

different structure from the male *MAT3* homolog (Kianianmomeni et al. 2008; Ferris et al. 2010). Both *MAT3* homologs display sexually regulated alternative splicing and sex-specific selection. The predominant *MAT3* splicing variant in sexual males includes an early termination codon. The downregulation of *MAT3* in *Volvox* males may be linked to the production of small-celled sperm because *mat3* mutants in *Ch. reinhardtii* are known to produce tiny gametes (Umen and Goodenough 2001). However, *MAT3* homologs from the five colonial species examined (isogamous *G. pectorale* and *Yamagishiella unicocca*, anisogamous *Eudorina* sp. and *P. starrii*, and oogamous *Volvox africanus*) had almost identical nucleotide sequences between the two sexes. The extreme gender-based *MAT3* divergence observed in *V. carteri* species may not be directly related to the evolution of male and female dimorphism within the colonial Volvocales as a whole (Hiraide et al. 2013).

Sexual Reproduction in the *Closterium peracerosum–strigosum–littorale* Complex

Overview of sexual reproduction in *Closterium*

The desmid *Closterium* belongs to the Zygnematophyceae and is the most successfully characterized unicellular charophycean in terms of the maintenance of strains and sexual reproduction (Ichimura 1971). Recently, studies have suggested that either the Zygnematophyceae or a clade consisting of Zygnematophyceae and Coleochaetophyceae might be a likely sister group of land plants (Turmel et al. 2006; Wodniok et al. 2011). Additional data are required to confirm this and biological studies of the *Closterium* are likely to generate great interest in the near future. The sexual reproduction of species in the genus *Closterium* has been of interest to many investigators for more than 100 years, and the morphological details and modes of sexual reproduction are well documented (Cook 1963; Lippert 1967; Pickett-Heaps and Fowke 1971; Ichimura 1973; Noguchi and Ueda 1985; Noguchi 1988). *Closterium* has no flagellum-like machinery for active movement and has been considered to use diffusible substances for the intercellular communication essential for sexual reproduction. Ichimura (1971) reported a technique for promoting the sexual reproduction of *Closterium* in an axenic culture using a synthetic culture medium and many studies using this system have subsequently been published (Hamada et al. 1982; Watanabe and Ichimura 1982; Ichimura 1983; Kato et al. 1983; Ichimura and Kasai 1987; Kasai and Ichimura 1987, 1990; Ichimura and Kasai 1995).

In *Closterium*, two types of conjugation produce zygotes (Tsuchikane et al. 2010b; Sekimoto et al. 2012). One is a conjugation between two

complementary mating-type cells (mt^+ and mt^-) and the other is a conjugation between clonal cells. The former is referred to as heterothallism and the latter as homothallism (Graham and Wilcox 2000). The conjugation process can be divided into several steps: sexual cell division (SCD), which produces sexually competent gametangial cells, pairing, formation of conjugation papillae, condensing of their cytoplasms, release and fusion of gametic protoplasts (gametes), and the formation of zygotes.

After the formation of zygotes, they become dormant and acquire resistance against dryness. Once they are exposed to dry conditions followed by a water supply, they start meiosis. Two non-sister nuclei of the second meiotic division survive and the other two degenerate. As a result, the two surviving nuclei carry opposite mating type genes in the absence of crossing over, and a pair of mt^+ and mt^- cells would arise from one zygote in the case of heterothallic strains (Brandham and Godward 1965; Lippert 1967; Hamada et al. 1982; Watanabe and Ichimura 1982).

Sex Pheromones in the Heterothallic *Closterium peracerosum–strigosum–littorale* Complex

When mt^+ and mt^- cells of the heterothallic *Cl. psl.* complex are mixed together in a nitrogen-depleted mating medium under light conditions, cells of both types differentiate to gametangial cells as a result of SCD and become paired. These paired cells then release their protoplasts to form zygotes (Fig. 3).

A pheromone, named protoplast-release-inducing protein (PR-IP), was isolated from the *Cl. psl.* complex (Sekimoto et al. 1990). This pheromone is a glycoprotein that consists of subunits of 42- and 19-kDa. It is released by mt^+ cells (NIES-67, obtained from the National Institute for Environmental Studies, Ibaraki, Japan) and is responsible for inducing the release of protoplasts from mt^- cells (NIES-68). The latter process proceeds only after appropriate preculture under continuous light conditions, during which the mt^- cells differentiate from vegetative cells into sexually competent cells (Sekimoto and Fujii 1992) and PR-IP receptors appear on the plasma membranes of mt^- cells. Specific binding of the biotinylated 19-kDa subunit of PR-IP to the cells has been clearly demonstrated (Sekimoto et al. 1993b).

Another pheromone, which induces the synthesis and release of PR-IP, has been detected in a medium in which only mt^- cells had been cultured (Sekimoto et al. 1993a). The pheromone, named PR-IP Inducer, was subsequently purified and found to be a glycoprotein with a molecular mass of 18.7 kDa (Nojiri et al. 1995). PR-IP Inducer is released constitutively from mt^- cells in the presence of light and directly induces the production and release of PR-IP from mt^+ cells. Furthermore, cDNAs encoding the subunits of PR-IP (Sekimoto et al. 1994a, b) and PR-IP Inducer (Sekimoto et al. 1998)

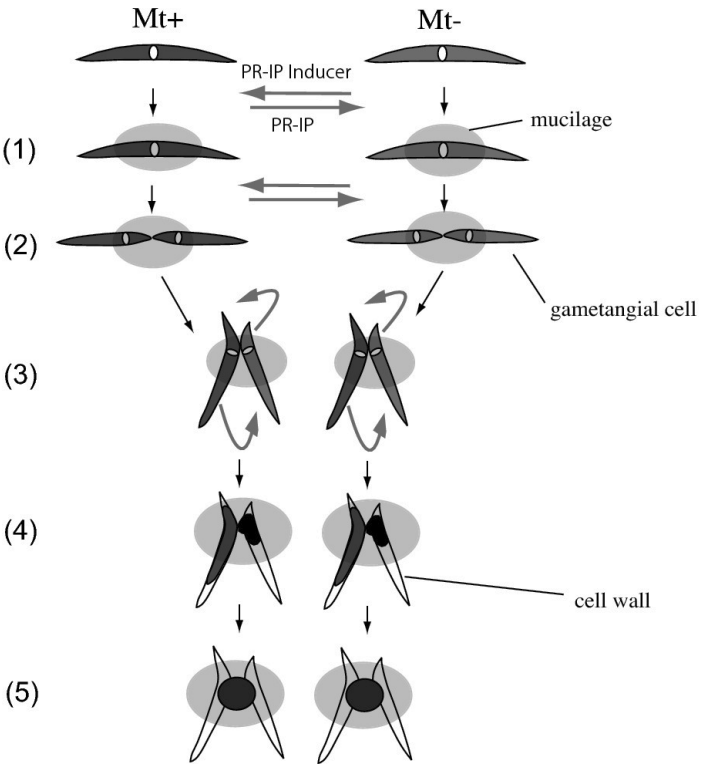

Figure 3. Schematic illustrations of the sexual reproduction of heterothallic *Closterium peracerosum-strigosum-littorale* complex. (1) mucilage secretion, (2) sexual cell division, (3) sexual pair formation induced by unknown chemoattractant pheromone(s), (4) protoplast release, (5) zygote. Most of processes are induced by the PR-IP and the PR-IP Inducer. Gray arrows indicate pheromonal communication.

have been isolated. A computer search using the nucleotide sequences and the deduced amino-acid sequences failed to reveal any homologies to known proteins. Genes for these pheromones can be detected in cells of both mating types by genomic Southern hybridization analysis, but are only expressed in cells of the respective mating types, suggesting the sex-specific regulation of gene expression (Sekimoto et al. 1994c, 1998). The sequences of 500 bp immediately upstream of the transcriptional initiation sites from mt^- and mt^+ cells are almost identical, indicating the existence of the putative mt^+ cells specific *trans*-acting factor(s) (Endo et al. 1997). From the recent whole genome analysis, both 19-kDa and 42-kDa subunits are encoded by a single gene locus each, but PR-IP Inducer is encoded by a multigene family (unpublished data). Also, many paralogous genes may be encoding PR-IP Inducer-like proteins.

In the sexual reproductive processes of *Closterium* species, gametangial cells are produced from haploid vegetative cells. Ichimura (1971) reported that vegetative cells of the *Cl. psl.* complex divided before the formation of sexual pairs when both mating type cells were mixed (Ichimura 1971). This SCD of each mating-type cell could be induced in a medium in which both mating type cells had been co-cultured (Tsuchikane et al. 2003). The mt^- cells release an SCD-inducing pheromone specific for the mt^+ cells and are designated SCD-IP-minus (sexual-cell division-inducing pheromone-minus), whereas a pheromone specific to mt^- cells released from mt^+ cells is designated SCD-IP-plus. Time-lapse video analyses have revealed that SCD was not always required for successful pairing because some of the non-divided vegetative cells can form pairs (unpublished data).

Closterium exhibits a gliding locomotory behavior, mediated by the forceful extrusion of mucilage from one pole of the cell that causes the cell to glide in the opposite direction (Domozych et al. 1993). Substances with the ability to stimulate the secretion of uronic-acid-containing mucilage from mt^+ and mt^- cells were detected in media in which mt^- and mt^+ cells had been cultured separately, and were designated as mucilage-secretion-stimulating pheromone (MS-SP)-minus and MS-SP-plus, respectively (Akatsuka et al. 2003).

Both MS-SP-minus and SCD-IP-minus displayed similar characteristics to the PR-IP Inducer, whereas both MS-SP-plus and SCD-IP-plus displayed similar characteristics to the PR-IP, with respect to molecular mass, heat stability, and their dependency on light for secretion and function, indicating the presence of close relationships among these pheromones. Recombinant PR-IP Inducer produced in yeast cells generated the induction of both PR-IP and SCD by mt^+ cells, although SCD could be induced by exposure to lower concentrations of recombinant PR-IP Inducer (Sekimoto 2002; Tsuchikane et al. 2005). Moreover, the SCD could be induced by a shorter period of treatment with the pheromone than the production of PR-IP (Tsuchikane et al. 2005). In addition, PR-IP Inducer also displayed mucilage-secretion-stimulating activity against mt^+ cells (Akatsuka et al. 2003).

However, purified PR-IP also exhibited mucilage-secretion-stimulating, SCD-inducing, and protoplast-releasing activities against mt^- cells, although the effective concentrations were different (Akatsuka et al. 2006). These results strongly suggest that both PR-IP and PR-IP Inducer are multifunctional pheromones that independently promote multiple steps in conjugation at the appropriate times through different induction mechanisms.

Mode of Sexual Reproduction in the *Closterium peracerosum–strigosum–littorale* Complex

Based on the results described here, postulated sexual reproductive events can be summarized. The PR-IP Inducer is released from mt^- cells when cells are exposed to nitrogen-depleted conditions in a light environment. The mt^+ cells then receive a signal and begin to release PR-IP into the medium. During this communication, mucilage is secreted into the surrounding medium. Concentrations of these pheromones are gradually elevated and SCD is then induced with respective gametangial cells being formed as a result. The mt^+ and mt^- cells then move together and become paired due to the effects of unknown chemotactic pheromones. After the final communication by PR-IP and PR-IP Inducer, the mt^- cells begin to release their protoplasts. The release of protoplasts from mt^+ cells is eventually induced by the direct adhesion of cells, and these protoplasts fuse to form a zygote (Fig. 3).

EST and Microarray Analyses to Elucidate Sexual Reproduction

To elucidate the molecular mechanism of intercellular communication during sexual reproduction, a normalized cDNA library was established from a mixture of cDNA libraries prepared from cells at various stages of sexual reproduction and from a mixture of vegetative mt^+ and mt^- cells. The aim was to reduce redundancy, and 3236 ESTs were generated, which were classified into 1615 nonredundant groups (Sekimoto et al. 2003, 2006). The EST sequences were compared with nonredundant protein sequence databases in the public domain using the BLASTX program, and 1045 nonredundant sequences displaying similarity to previously registered genes in the public databases were confirmed. The source group with the highest similarity was land plants, including *Arabidopsis thaliana*.

A cDNA microarray was then constructed and expression profiles were obtained using mRNA isolated from cells in various stages of the life cycle. Finally, 88 pheromone-inducible, conjugation-related, and/or sex-specific genes were identified (Sekimoto et al. 2006), although their functions during sexual reproduction have not been characterized.

Of the 88 genes identified, a gene encoding receptor-like protein kinase (RLK) was the most notable and named *CpRLK1*. The gene is expressed during sexual reproduction and treatment of mt^+ cells with the PR-IP Inducer also induces the expression, indicating that the CpRLK1 protein probably functions during sexual reproduction (Sekimoto et al. 2006). The full-length cDNA has been isolated and an amino acid sequence containing

an extracellular domain (ECD) was obtained (unpublished data). In *A. thaliana*, the RLK family is the largest gene family with more than 600 family members (Shiu and Bleecker 2001, 2003; Shiu et al. 2004), although the functions of most of these genes are still unknown. Only two RLK genes have been found in the genome of *Ch. reinhardtii*; however, the predicted proteins do not have recognizable ECDs. No RLK gene was found in the genome of *Ostreococcus tauri* (Lehti-Shiu et al. 2009). In contrast, RLKs having transmembrane domains and/or ECDs have been isolated from two charophyceans (*Nitella axillaris* and *Closterium ehrenbergii*) (Sasaki et al. 2007), indicating that the receptor configuration was likely established before the divergence of land plants from charophyceans but after the divergence of charophyceans from chlorophytes (Graham and Wilcox 2000; Karol et al. 2001). The receptor configuration is likely to function for intercellular communication, especially during sexual reproduction; however, the confirmation of genomic information from early diversified nonsexual charophyceans such as Klebsormidiophyceae and Chlorokybophyceae is necessary to confirm this assumption.

Recently, a nuclear transformation system for *Cl. psl.* complex was developed (Abe et al. 2008a, 2008b, 2011). It should provide not only a basis for molecular investigation of *Closterium* but also an insight into important processes regarding the mechanism and evolution of intercellular communication between the egg and sperm cells of land plants.

Conjugation Processes of the Homothallic *Closterium peracerosum–strigosum–littorale* Complex

In isogamous organisms, if gametes from the same individual are able to conjugate to each other and produce viable progeny, the organism is termed homothallic (self-fertile). If gametes from two individuals of different genetic makeup are required for successful mating, the organism is termed heterothallic (self-sterile; Graham and Wilcox 2000). These two types of zygote formation exist in natural populations of *Closterium*.

The detailed conjugation processes of the homothallic strain in the *Cl. psl.* complex (kodama20; NIES-2666) were revealed by a time-lapse analysis (Tsuchikane et al. 2010b). The first step in the conjugation process is cell division resulting in the formation of two sister gametangial cells from one vegetative cell. Two gametangial cells form a pair and then form a zygote. In contrast to the heterothallic cells, the formation of gametangial cells by cell division is absolutely indispensable for the next pairing step. Approximately 90% of homothallic zygotes originate as a result of conjugation of two sister gametangial cells derived from one vegetative cell (sister conjugation; Fig. 4B left). Hence, sister gametangial cells of the homothallic strain can recognize each other. The resultant zygotes

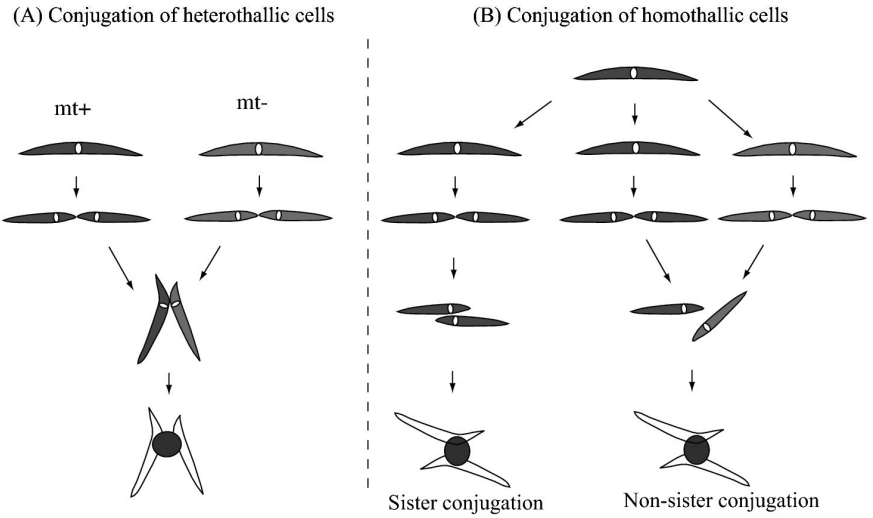

Figure 4. Schematic illustrations of the processes involved in zygote formation in the *Cl. psl.* complex. (A) Zygote formation of heterothallic strain. (B) Sister and non-sister conjugation of homothallic strain. Sister conjugation proceeds between two sister gametangial cells derived from one vegetative cell (left). Non-sister conjugation was observed between gametangial cells of separately adjoined individuals (right).

are referred to as sister zygotes. The remaining 10% of zygotes originate from the gametangial cells of separately adjoined individuals (non-sister conjugation; Fig. 4B right) and are referred to as non-sister zygotes.

Conjugation-regulating Sex Pheromones in Homothallic Strains

For conjugation to occur in the homothallic cells, cell density in the culture is critical. Cells likely discern and regulate their density to achieve conjugation through a mechanism similar to the quorum sensing observed in some types of bacteria (Camilli and Bassler 2006). Two conjugation-related activities were successfully detected in a cell-free cultured medium (Tsuchikane et al. 2010a). One of the activities stimulated the formation of gametangial cells by cell division and promoted the formation of zygotes (conjugation-promoting activity). The other suppressed the progress of the steps in conjugation (conjugation-suppressing activity). Both active substances displayed similar characteristics to those of the heterothallic sex-pheromone, PR-IP Inducer. The cDNAs encoding orthologous PR-IP Inducer were cloned from homothallic cells using a combination of degenerate and rapid amplification of cDNA ends (RACE)-polymerase chain reaction (PCR). Three representative recombinant PR-IP Inducers produced by yeast cells were shown to display conjugation-promoting activity, but not -suppressing activity (Tsuchikane et al. 2010a).

As explained previously, PR-IP Inducer from the heterothallic strain is released from mt^- cells in a nitrogen-depleted medium under light conditions. In homothallic cells, conjugation is also regulated by a pheromone, which is an ortholog of heterothallic PR-IP Inducer; however, both the homothallic cells and the resultant gametangial cells are theoretically clones and do not appear to be differentiated in either mating type. In addition, most homothallic zygotes originated by sister conjugation, apparently recognizing each other (Fig. 4B). To confirm this, the relationship between homothallic cells and heterothallic cells has been further characterized as discussed below.

Relationships between Heterothallism and Homothallism

Heterothallic mating group II-B and homothallic strains (kodama20) are phylogenetically closely related (Tsuchikane et al. 2010b; Tsuchikane et al. 2012). One can assume that the type of conjugation (heterothallic vs. homothallic) has been shifted by the mutation of a few important genes.

Because approximately 90% of the homothallic zygotes are sister zygotes, originating as a result of the conjugation of two sister gametangial cells, one can hypothesize that these sister gametangial cells are sexually differentiated to their respective mating-type cells, as with hetorothallic strains. In laboratory studies, homothallic cells have been mixed with heterothallic group II-B cells, which had been surface labeled with calcofluor white, permitting fusions with homothallic cells to be identified. The formation of hybrid zygotes between the homothallic cells and heterothallic mt^+ cells was confirmed (Tsuchikane et al. 2012). These results suggest that at least some of the homothallic gametangial cells possess the same characteristics as heterothallic mt^- cells. In heterothallic strains, mt^+ and mt^- cells recognize each other through the mating-type-specific sex pheromones PR-IP Inducer and PR-IP. Thus, homothallic cells and heterothallic mt^+ cells may recognize each other through sex pheromones. These findings support the idea that the division of one vegetative cell into two sister gametangial cells is a segregative process capable of producing complementary mating types.

The sister conjugation has also been observed in other unicellular isogamous charophycean alga (*Penium margaritaceum*; Tsuchikane et al. 2011), as well as the *Cl. psl.* complex. Whether homothallism or heterothallism represents the ancestral reproductive strategy has not yet been determined. To clarify the evolution of sex within algal species in detail, the phylogenetic relationship of homothallic and heterothallic strains in various taxonomic groups must be studied in the near future.

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References

Abe, J., Y. Hiwatashi, M. Ito, M. Hasebe and H. Sekimoto. 2008a. Expression of exogenous genes under the control of endogenous *HSP70* and *CAB* promoters in the *Closterium peracerosum-strigosum-littorale* complex. Plant Cell Physiol. 49: 625–632.

Abe, J., S. Hori, Y. Tsuchikane, N. Kitao, M. Kato and H. Sekimoto. 2011. Stable nuclear transformation of the *Closterium peracerosum-strigosum-littorale* complex. Plant Cell Physiol. 52: 1676–1685.

Abe, J., T. Kubo, T. Saito and Y. Matsuda. 2005. The regulatory networks of gene expression during the sexual differentiation of *Chlamydomonas reinhardtii*, as analyzed by mutants for gametogenesis. Plant Cell Physiol. 46: 312–316.

Abe, J., T. Kubo, Y. Takagi, T. Saito, K. Miura, H. Fukuzawa and Y. Matsuda. 2004. The transcriptional program of synchronous gametogenesis in *Chlamydomonas reinhardtii*. Curr. Genet. 46: 304–315.

Abe, J., K. Sakayori and H. Sekimoto. 2008b. Effect of antibiotics on cell proliferation in the *Closterium peracerosum-strigosum-littorale* complex (Charophyceae, Chlorophyta). Biologia 63: 932–936.

Adair, W.S., C. Hwang and U.W. Goodenough. 1983. Identification and visualization of the sexual agglutinin from the mating-type plus flagellar membrane of *Chlamydomonas*. Cell 33: 183–193.

Akatsuka, S., H. Sekimoto, H. Iwai, R. Fukumoto and T. Fujii. 2003. Mucilage secretion regulated by sex pheromones in *Closterium peracerosum-strigosum-littorale* complex. Plant Cell Physiol. 44: 1081–1087.

Akatsuka, S., Y. Tsuchikane, R. Fukumoto, T. Fujii and H. Sekimoto. 2006. Physiological characterization of the sex pheromone protoplast-release-inducing protein from the *Closterium peracerosum-strigosum-littorale* complex (Charophyta). Phycol. Res. 54: 116–121.

Al-Hasani, H. and L. Jaenicke. 1992. Characterization of the sex-inducer glycoprotein of *Volvox carteri* f. *weismannia*. Sex. Plant Reprod. 5: 8–12.

Amon, P., E. Haas and M. Sumper. 1998. The sex-inducing pheromone and wounding trigger the same set of genes in the multicellular green alga *Volvox*. Plant Cell 10: 781–789.

Brandham, P.E. and M.B.E. Godward. 1965. The inheritance of mating type in desmids. New Phytol. 64: 428–435.

Buchanan, M.J., S.H. Imam, W.A. Eskue and W.J. Snell. 1989. Activation of the cell wall degrading protease, lysin, during sexual signalling in *Chlamydomonas*: the enzyme is stored as an inactive, higher relative molecular mass precursor in the periplasm. J. Cell Biol. 108: 199–207.

Camilli, A. and B.L. Bassler. 2006. Bacterial small-molecule signaling pathways. Science 311: 1113–1116.

Cock, J.M., L. Sterck, P. Rouze, D. Scornet, A.E. Allen, G. Amoutzias, V. Anthouard, F. Artiguenave, J.M. Aury, J.H. Badger, B. Beszteri, K. Billiau, E. Bonnet, J.H. Bothwell, C. Bowler, C. Boyen, C. Brownlee, C.J. Carrano, B. Charrier, G.Y. Cho, S.M. Coelho, J. Collen, E. Corre, C. Da Silva, L. Delage, N. Delaroque, S.M. Dittami, S. Doulbeau, M. Elias, G. Farnham, C.M. Gachon, B. Gschloessl, S. Heesch, K. Jabbari, C. Jubin, H. Kawai, K. Kimura, B. Kloareg, F.C. Kupper, D. Lang, A. Le Bail, C. Leblanc, P. Lerouge, M. Lohr, P.J. Lopez, C. Martens, F. Maumus, G. Michel, D. Miranda-Saavedra, J. Morales, H. Moreau, T. Motomura, C. Nagasato, C.A. Napoli, D.R. Nelson, P. Nyvall-Collen, A.F. Peters, C. Pommier, P. Potin, J. Poulain, H. Quesneville, B. Read, S.A. Rensing, A. Ritter, S. Rousvoal, M. Samanta, G. Samson, D.C. Schroeder, B. Segurens, M. Strittmatter, T. Tonon, J.W. Tregear, K. Valentin, P. von Dassow, T. Yamagishi, Y. Van de Peer and P. Wincker. 2010. The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. Nature 465: 617–621.

Coggin, S.J., W. Hutt and G. Kochert. 1979. Sperm bundle-female somatic cell interaction in the fertilization process of *Volvox carteri* f. *weismannia* (Chlorophyta). J. Phycol. 15: 247–251.

Cook, P.A. 1963. Variation in vegetative and sexual morphology among the small curved species of *Closterium*. Phycologia 3: 1–18.

Cooper, J.B., W.S. Adair, R.P. Mecham and J.E. Heuser. 1983. *Chlamydomonas* agglutinin is a hydroxyproline-rich glycoprotein. Proc. Natl. Acad. Sci. USA 80: 5898–5901.

Darden, W.H.J. 1966. Sexual differentiation in *Volvox aureus*. J. Protozool. 13: 239–255.

Domozych, C.R., K. Plante and P. Blais. 1993. Mucilage processing and secretion in the green alga *Closterium*. 1. Cytology and biochemistry. J. Phycol. 29: 650–659.

Ender, F., A. Hallmann, P. Amon and M. Sumper. 1999. Response to the sexual pheromone and wounding in the green alga *Volvox*: induction of an extracellular glycoprotein consisting almost exclusively of hydroxyproline. J. Biol. Chem. 274: 35023–35028.

Endo, B., T. Fujii, Y. Kamiya and H. Sekimoto. 1997. Analysis of genomic sequences encoding a sex pheromone from the *Closterium peracerosum-strigosum-littorale* complex. J. Plant Res. 110: 463–467.

Ferris, P., E.V. Armbrust and U.W. Goodenough. 2002. Genetic structure of the mating-type locus of *Chlamydomonas reinhardtii*. Genetics 160: 181–200.

Ferris, P., B.J. Olson, P.L. De Hoff, S. Douglass, D. Casero, S. Prochnik, S. Geng, R. Rai, J. Grimwood, J. Schmutz, I. Nishii, T. Hamaji, H. Nozaki, M. Pellegrini and J.G. Umen. 2010. Evolution of an expanded sex-determining locus in *Volvox*. Science 328: 351–354.

Ferris, P., S. Waffenschmidt, J.G. Umen, H. Lin, J.-H. Lee, K. Ishida, T. Kubo, J. Lau and U.W. Goodenough. 2005. Plus and minus sexual agglutinins from *Chlamydomonas reinhardtii*. Plant Cell 17: 597–615.

Ferris, P.J. and U.W. Goodenough. 1994. The Mating-type locus of *Chlamydomonas reinhardtii* contains highly rearranged DNA sequences. Cell 76: 1135–1145.

Ferris, P.J. and U.W. Goodenough. 1997. Mating type in *Chlamydomonas* is specified by *mid*, the minus-dominance gene. Genetics 146: 859–869.

Ferris, P.J., J.P. Woessner and U.W. Goodenough. 1996. A sex recognition glycoprotein is encoded by the *plus* mating-type gene *fus1* of *Chlamydomonas reinhardtii*. Mol. Biol. Cell 7: 1235–1248.

Gilles, R., C. Bittner and L. Jaenicke. 1981. Site and time of formation of the sex-inducing glycoprotein in *Volvox carteri*. FEBS Lett. 124: 57–61.

Godl, K., A. Hallmann, A. Rappel and M. Sumper. 1995. Pherophorins: a family of extracellular matrix glycoproteins from *Volvox* structurally related to the sex-inducing pheromone. Planta 196: 781–787.

Godl, K., A. Hallmann, S. Wenzl and M. Sumper. 1997. Differential targeting of closely related ECM glycoproteins: the pherophorin family from *Volvox*. EMBO J. 16: 25–34.

Goodenough, U.W. 1989. Cyclic AMP enhances the sexual agglutinability of *Chlamydomonas* flagella. J. Cell Biol. 109: 247–252.

Goodenough, U.W., W.S. Adair, P. Collin-Osdoby and J.E. Heuser. 1985. Structure of the *Chlamydomonas* agglutinin and related flagellar surface proteins *in vitro* and *in situ*. J. Cell Biol. 101: 924–941.

Goodenough, U.W., P.A. Detmers and C. Hwang. 1982. Activation for cell fusion in *Chlamydomonas*: analysis of wild-type gametes and nonfusing mutants. J. Cell Biol. 92: 378–386.

Graham, L.E., J.E. Graham and L.W. Wilcox. 2009. Algae 2nd ed. Peason Education, San Francisco, CA.

Graham, L.E. and L.W. Wilcox. 2000. Algae. Prentice-Hall, Upper Saddle River, New Jersey.

Hake, S., H.M. Smith, H. Holtan, E. Magnani, G. Mele and J. Ramirez. 2004. The role of *knox* genes in plant development. Annu. Rev. Cell Dev. Biol. 20: 125–151.

Hallmann, A. 2003. Extracellular matrix and sex-inducing pheromone in Volvox. Int. Rev. Cytol. 227: 131–182.

Hallmann, A. 2006. The pherophorins: common, versatile building blocks in the evolution of extracellular matrix architecture in Volvocales. Plant J. 45: 292–307.

Hallmann, A. 2007. A small cysteine-rich extracellular protein, VCRP, is inducible by the sex-inducer of *Volvox carteri* and by wounding. Planta 226: 719–727.

Hallmann, A. 2008. VCRPs, small cysteine-rich proteins, might be involved in extracellular signaling in the green alga *Volvox*. Plant Signal. Behav. 3: 124–127.

Hallmann, A. 2011. Evolution of reproductive development in the volvocine algae. Sex. Plant Reprod. 24: 97–112.

Hallmann, A., P. Amon, K. Godl, M. Heitzer and M. Sumper. 2001. Transcriptional activation by the sexual pheromone and wounding: a new gene family from *Volvox* encoding modular proteins with (hydroxy)proline-rich and metalloproteinase homology domains. Plant J. 26: 583–593.

Hallmann, A., K. Godl, S. Wenzl and M. Sumper. 1998. The highly efficient sex-inducing pheromone system of *Volvox*. Trends Microbiol. 6: 185–189.

Hamada, J., T. Yoshizawa-Katoh and K. Tsunewaki. 1982. Genetic study on mating type genes by a new type of tetrad analysis in *Closterium ehrenbergii*. Bot. Mag. Tokyo 95: 101–108.

Hamaji, T., P.J. Ferris, A.W. Coleman, S. Waffenschmidt, F. Takahashi, I. Nishii and H. Nozaki. 2008. Identification of the minus-dominance gene ortholog in the mating-type locus of *Gonium pectorale*. Genetics 178: 283–294.

Harris, E.H. 1989. The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego, CA.

Hirai, M., M. Arai, T. Mori, S.Y. Miyagishima, S. Kawai, K. Kita, T. Kuroiwa, O. Terenius and H. Matsuoka. 2008. Male fertility of malaria parasites is determined by GCS1, a plant-type reproduction factor. Curr. Biol. 18: 607–613.

Hiraide, R., H. Kawai-Toyooka, T. Hamaji, R. Matsuzaki, K. Kawafune, J. Abe, H. Sekimoto, J. Umen and H. Nozaki. 2013. The evolution of male-female sexual dimorphism predates the gender-based divergence of the mating locus gene *MAT3/RB*. Mol. Biol. Evol. doi: 10.1093/molbev/mst018.

Hunnicutt, G.R., M.G. Kosfiszer and W.J. Snell. 1990. Cell body and flagellar agglutinins in *Chlamydomonas reinhardtii*: the cell body plasma membrane is a reservoir for agglutinins whose migration to the flagella is regulated by a functional barrier. J. Cell Biol. 111: 1605–1616.

Ichimura, T. 1971. Sexual cell division and conjugation-papilla formation in sexual reproduction of *Closterium strigosum*. *In*: K. Nishizawa (ed.). Proceedings of the 7th International Seaweed Symposium. University of Tokyo Press, Tokyo, pp. 208–214.

Ichimura, T. 1973. The life cycle and its control in some species of *Closterium*, with special reference to the biological species problem. *In*: D. Sci. thesis, University of Tokyo, 69 pp.

Ichimura, T. 1983. Hybrid inviability and predominant survival of mating type minus progeny in laboratory crosses between two closely related mating groups of *Closterium ehrenbergii*. Evolution 37: 252–260.

Ichimura, T. and F. Kasai. 1987. Time-lapse analyses of sexual isolation between two closely related mating groups of the *Closterium ehrenbergii* species complex (Chlorophyta). J. Phycol. 23: 523–534.

Ichimura, T. and F. Kasai. 1995. Dissection of conjugants and mating type plus and minus cells in selfing clones of the isogamous green alga *Closterium ehrenbergii*. Sex. Plant Reprod. 8: 44–48.

Ishida, K. 2007. Sexual pheromone induces diffusion of the pheromone-homologous polypeptide in the extracellular matrix of *Volvox carteri*. Eukaryot. Cell 6: 2157–2162.

Karol, K.G., R.M. McCourt, M.T. Cimino and C.F. Delwiche. 2001. The closest living relatives of land plants. Science 294: 2351–2353.

Kasai, F. and T. Ichimura. 1987. Stable diploids from intragroup zygospores of *Closterium ehrenbergii* Menegh. (Conjugatophyceae). J. Phycol. 23: 344–351.

Kasai, F. and T. Ichimura. 1990. A sex determining mechanism in the *Closterium ehrenbergii* (Chlorophyta) species complex. J. Phycol. 26: 195–201.

Kato, A., T. Takagi and K. Sasaki. 1983. Light conditions for sexual reproduction in heterothallic strains of *Closterium*. Plant Cell Physiol. 24: 93–100.

Kianianmomeni, A., G. Nematollahi and A. Hallmann. 2008. A gender-specific retinoblastoma-related protein in *Volvox carteri* implies a role for the retinoblastoma protein family in sexual development. Plant Cell 20: 2399–2419.

Kinoshita, T., H. Fukuzawa, T. Shimada, T. Saito and Y. Matsuda. 1992. Primary structure and expression of a gamete lytic enzyme in *Chlamydomonas reinhardtii*: similarity of functional domains to matrix metalloproteases. Proc. Natl. Acad. Sci. USA 89: 4693–4697.

Kirk, D.L. 1998. *Volvox*: Molecular-genetic origins of multicellularity and cellular differentiation. Cambridge University Press, Cambridge.

Kirk, D.L. and M.M. Kirk. 1986. Heat shock elicits production of sexual inducer in *Volvox*. Science 231: 51–54.

Kirk, D.L. and I. Nishii. 2001. *Volvox carteri* as a model for studying the genetic and cytological control of morphogenesis. Dev. Growth Diff. 43: 621–631.

Kirk, M.M. and D.L. Kirk. 2004. Exploring germ-soma differentiation in *Volvox*. J. Biosci. 29: 143–152.

Kochert, G. 1981. Sexual pheromones in *Volvox* development. *In*: P.A. Horgen (ed.). Sexual interactions in eukaryotic microbes. Academic Press, New York, pp. 73–93.

Kochert, G. and I. Yates. 1974. Purification and partial characterization of a glycoprotein sexual inducer from *Volvox carteri*. Proc. Natl. Acad. Sci. USA 71: 1211–1214.

Kubo, T., J. Abe, T. Oyamada, M. Ohnishi, H. Fukuzawa, Y. Matsuda and T. Saito. 2008. Characterization of novel genes induced by sexual adhesion and gamete fusion and of their transcriptional regulation in *Chlamydomonas reinhardtii*. Plant Cell Physiol. 49: 981–993.

Kubo, T., S. Kaida, J. Abe, T. Saito, H. Fukuzawa and Y. Matsuda. 2009. The *Chlamydomonas* hatching enzyme, sporangin, is expressed in specific phases of the cell cycle and is localized to the flagella of daughter cells within the sporangial cell wall. Plant Cell Physiol. 50: 572–583.

Kurvari, V., N.V. Grishin and W.J. Snell. 1998. A gamete-specific, sex-limited homeodomain protein in *Chlamydomonas*. J. Cell Biol. 143: 1971–1980.

Lee, J.H., L. Huawen, S. Joo and U.W. Goodenough. 2008. Early sexual origins of homeoprotein heterodimerization and evolution of the plant KNOX/BELL family. Cell 133: 829–840.

Lehti-Shiu, M.D., C. Zou, K. Hanada and S.H. Shiu. 2009. Evolutionary history and stress regulation of plant receptor-like kinase/pelle genes. Plant Physiol. 150: 12–26.

Lin, H. and U.W. Goodenough. 2007. Gametogenesis in the *Chlamydomonas reinhardtii* minus mating type is controlled by two genes, *MID* and *MTD1*. Genetics 176: 913–925.

Lippert, B.E. 1967. Sexual reproduction in *Closterium moniliferum* and *Closterium ehrenbergii*. J. Phycol. 3: 182–198.

Liu, Y., M.J. Misamore and W.J. Snell. 2010. Membrane fusion triggers rapid degradation of two gamete-specific, fusion-essential proteins in a membrane block to polygamy in *Chlamydomonas*. Development 137: 1473–1481.

Liu, Y., R. Tewari, J. Ning, A.M. Blagborough, S. Garbom, J. Pei, N.V. Grishin, R.E. Steele, R.E. Sinden, W.J. Snell and O. Billker. 2008. The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in *Chlamydomonas* and *Plasmodium* gametes. Genes Dev. 22: 1051–1068.

Mages, H.W., H. Tschochner and M. Sumper. 1988. The sexual inducer of *Volvox carteri*. Primary structure deduced from cDNA sequence. FEBS Lett. 234: 407–410.

Maier, I. 1993. Gamete orientation and induction of gametogenesis by pheromones in algae and plants. Plant Cell Env. 16: 891–907.

Maier, I. 1995. Brown algal pheromones. *In*: D.J. Chapman (ed.). Progress in Phycological Research. Biopress Ltd., Bristol, pp. 51–102.

Maier, I. and M. Calenberg. 1994. Effect of extracellular Ca^{2+} and Ca^{2+}-antagonists on the movement and chemoorientation of male gametes of *Ectocarpus siliculosus* (Phaeophyceae). Bot. Acta 107: 451–460.

Maier, I. and D.G. Müller. 1986. Sexual pheromones in algae. Biol. Bull. 170: 145–175.

Matsuda, Y., M. Koseki, T. Shimada and T. Saito. 1995. Purification and characterization of a vegetative lytic enzyme responsible for liberation of daughter cells during the proliferation of *Chlamydomonas reinhardtii*. Plant Cell Physiol. 36: 681–689.

Merchant, S.S., S.E. Prochnik, O. Vallon, E.H. Harris, S.J. Karpowicz, G.B. Witman, A. Terry, A. Salamov, L.K. Fritz-Laylin, L. Marechal-Drouard, W.F. Marshall, L.H. Qu, D.R. Nelson, A.A. Sanderfoot, M.H. Spalding, V.V. Kapitonov, Q. Ren, P. Ferris, E. Lindquist, H. Shapiro, S.M. Lucas, J. Grimwood, J. Schmutz, P. Cardol, H. Cerutti, G. Chanfreau, C.L. Chen, V. Cognat, M.T. Croft, R. Dent, S. Dutcher, E. Fernandez, H. Fukuzawa, D. Gonzalez-Ballester, D. Gonzalez-Halphen, A. Hallmann, M. Hanikenne, M. Hippler, W. Inwood, K. Jabbari, M. Kalanon, R. Kuras, P.A. Lefebvre, S.D. Lemaire, A.V. Lobanov, M. Lohr, A. Manuell, I. Meier, L. Mets, M. Mittag, T. Mittelmeier, J.V. Moroney, J. Moseley, C. Napoli, A.M. Nedelcu, K. Niyogi, S.V. Novoselov, I.T. Paulsen, G. Pazour, S. Purton, J.P. Ral, D.M. Riano-Pachon, W. Riekhof, L. Rymarquis, M. Schroda, D. Stern, J. Umen, R. Willows, N. Wilson, S.L. Zimmer, J. Allmer, J. Balk, K. Bisova, C.J. Chen, M. Elias, K. Gendler, C. Hauser, M.R. Lamb, H. Ledford, J.C. Long, J. Minagawa, M.D. Page, J. Pan, W. Pootakham, S. Roje, A. Rose, E. Stahlberg, A.M. Terauchi, P. Yang, S. Ball, C. Bowler, C.L. Dieckmann, V.N. Gladyshev, P. Green, R. Jorgensen, S. Mayfield, B. Mueller-Roeber, S. Rajamani, R.T. Sayre, P. Brokstein, I. Dubchak, D. Goodstein, L. Hornick, Y.W. Huang, J. Jhaveri, Y. Luo, D. Martinez, W.C. Ngau, B. Otillar, A. Poliakov, A. Porter, L. Szajkowski, G. Werner, K. Zhou, I.V. Grigoriev, D.S. Rokhsar and A.R. Grossman. 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318: 245–250.

Misamore, M.J., S. Gupta and W.J. Snell. 2002. FUS1, the cell surface protein required for *Chlamydomonas* fertilization, contains invasin-like internal repeats and requires actin for its localization. Mol. Biol. Cell 13: 625.

Misamore, M.J., S. Gupta and W.J. Snell. 2003. The *Chlamydomonas* Fus1 protein is present on the mating type plus fusion organelle and required for a critical membrane adhesion event during fusion with minus gametes. Mol. Biol. Cell 14: 2530–2542.

Mori, T., H. Kuroiwa, T. Higashiyama and T. Kuroiwa. 2006. Generative Cell Specific 1 is essential for angiosperm fertilization. Nat. Cell Biol. 8: 64–71.

Nedelcu, A.M. and R.E. Michod. 2003. Sex as a response to oxidative stress: the effect of antioxidants on sexual induction in a facultatively sexual lineage. Proc. R Soc. Lond. B 270: S136–S139.

Nishimura, Y., T. Shikanai, S. Nakamura, M. Kawai-Yamada and H. Uchimiya. 2012. Gsp1 triggers the sexual developmental program including inheritance of chloroplast DNA and mitochondrial DNA in *Chlamydomonas reinhardtii*. Plant Cell 24: 2401–2414.

Noguchi, T. 1988. Numerical and structural changes in dictyosomes during zygospore germination of *Closterium ehrenbergii*. Protoplasma 147: 135–142.

Noguchi, T. and K. Ueda. 1985. Cell walls, plasma membranes, and dictyosomes during zygote maturation of *Closterium ehrenbergii*. Protoplasma 128: 64–71.

Nojiri, T., T. Fujii and H. Sekimoto. 1995. Purification and characterization of a novel sex pheromone that induces the release of another sex pheromone during sexual reproduction of the heterothallic *Closterium peracerosum-strigosum-littorale* complex. Plant Cell Physiol. 36: 79–84.

Nozaki, H. 2003. Origin and evolution of the genera *Pleodorina* and *Volvox* (Volvocales). Biologia 58/4: 425–431.

Nozaki, H., K. Misawa, T. Kajita, M. Kato, S. Nohara and M.M. Watanabe. 2000. Origin and evolution of the colonial Volvocales (Chlorophyceae) as inferred from multiple, chloroplast gene sequences. Mol. Phylogenet. Evol. 17: 256–268.

Nozaki, H., T. Mori, O. Misumi, S. Matsunaga and T. Kuroiwa. 2006. Males evolved from the dominant isogametic mating type. Curr. Biol. 16: R1018–R1020.

Pan, J. and W.J. Snell. 2002. Kinesin-II is required for flagellar sensory transduction during fertilization in *Chlamydomonas*. Mol. Biol. Cell 13: 1417–1426.

Pasquale, S.M. and U.W. Goodenough. 1987. Cyclic AMP functions as a primary sexual signal in gametes of *Chlamydomonas reinhardtii*. J. Cell Biol. 105: 2279–2292.

Piao, T., M. Luo, L. Wang, Y. Guo, D. Li, P. Li, W.J. Snell and J. Pan. 2009. A microtubule depolymerizing kinesin functions during both flagellar disassembly and flagellar assembly in *Chlamydomonas*. Proc. Natl. Acad. Sci. USA 106: 4713–4718.

Pickett-Heaps, J.D. and L.C. Fowke. 1971. Conjugation in the desmid *Closterium littorale*. J. Phycol. 7: 37–50.

Prochnik, S.E., J. Umen, A.M. Nedelcu, A. Hallmann, S.M. Miller, I. Nishii, P. Ferris, A. Kuo, T. Mitros, L.K. Fritz-Laylin, U. Hellsten, J. Chapman, O. Simakov, S.A. Rensing, A. Terry, J. Pangilinan, V. Kapitonov, J. Jurka, A. Salamov, H. Shapiro, J. Schmutz, J. Grimwood, E. Lindquist, S. Lucas, I.V. Grigoriev, R. Schmitt, D. Kirk and D.S. Rokhsar. 2010. Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*. Science 329: 223–226.

Saito, T., L. Small and U.W. Goodenough. 1993. Activation of adenylyl cyclase in *Chlamydomonas reinhardtii* by adhesion and by heat. J. Cell Biol. 122: 137–147.

Saito, T., Y. Tsubo and Y. Matsuda. 1985. Synthesis and turnover of cell body agglutinin as a pool of flagellar surface agglutinin in *Chlamydomonas reinhardtii* gamete. Arch. Microbiol. 142: 207–210.

Sasaki, G., K. Katoh, N. Hirose, H. Suga, K. Kuma, T. Miyata and Z.H. Su. 2007. Multiple receptor-like kinase cDNAs from liverwort Marchantia polymorpha and two charophycean green algae, *Closterium ehrenbergii* and *Nitella axillaris*: Extensive gene duplications and gene shufflings in the early evolution of streptophytes. Gene 401: 135–144.

Scofield, S. and A.H. Murray. 2006. *KNOX* gene function in plant stem cell niches. Plant Mol. Biol. 60: 929–946.

Sekimoto, H. 2002. Production and secretion of a biologically active *Closterium* sex pheromone by *Saccharomyces cerevisiae*. Plant Physiol. Biochem. 40: 789–794.

Sekimoto, H. 2005. Plant sex pheromones. Vitam. Horm. 72: 457–478.

Sekimoto, H., J. Abe and Y. Tsuchikane. 2012. New insights into the regulation of sexual reproduction in *Closterium*. Int. Rev. Cell Mol. Biol. 297: 309–338.

Sekimoto, H., R. Fukumoto, N. Dohmae, K. Takio, T. Fujii and Y. Kamiya. 1998. Molecular cloning of a novel sex pheromone responsible for the release of a different sex pheromone in *Closterium peracerosum-strigosum-littorale* complex. Plant Cell Physiol. 39: 1169–1175.

Sekimoto, H., S. Satoh and T. Fujii. 1990. Biochemical and physiological properties of a protein inducing protoplast release during conjugation in the *Closterium peracerosum-strigosum-littorale* complex. Planta 182: 348–354.

Sekimoto, H. and T. Fujii. 1992. Analysis of gametic protoplast release in the *Closterium peracerosum-strigosum-littorale* complex (Chlorophyta). J. Phycol. 28: 615–619.

Sekimoto, H., Y. Inoki and T. Fujii. 1993a. Detection and evaluation of an inducer of diffusible mating pheromone of heterothallic *Closterium peracerosum-strigosum-littorale* complex. Plant Cell Physiol. 37: 991–996.

Sekimoto, H., S. Satoh and T. Fujii. 1993b. Analysis of binding of biotinylated protoplast-release-inducing protein that induces release of gametic protoplasts in the *Closterium peracerosum-strigosum-littorale* complex. Planta 189: 468–474.

Sekimoto, H., Y. Sone and T. Fujii. 1994a. cDNA cloning of a 42-kilodalton subunit of protoplast-release-inducing protein from *Closterium*. Plant Physiol. 104: 1095–1096.

Sekimoto, H., Y. Sone and T. Fujii. 1994b. A cDNA encoding a 19-kilodalton subunit of protoplast-release-inducing protein from *Closterium*. Plant Physiol. 105: 447.

Sekimoto, H., Y. Sone and T. Fujii. 1994c. Regulation of expression of the genes for a sex pheromone by an inducer of the sex pheromone in the *Closterium peracerosum-strigosum-littorale* complex. Planta 193: 137–144.

Sekimoto, H., Y. Tanabe, M. Takizawa, N. Ito, R. Fukumoto and M. Ito. 2003. Expressed sequence tags from the *Closterium peracerosum-strigosum-littorale* complex, a unicellular charophycean alga, in the sexual reproduction process. DNA Res. 10: 147–153.

Sekimoto, H., Y. Tanabe, Y. Tsuchikane, H. Shirosaki, H. Fukuda, T. Demura and M. Ito. 2006. Gene expression profiling using cDNA microarray analysis of the sexual reproduction stage of the unicellular charophycean alga *Closterium peracerosum-strigosum-littorale* complex. Plant Physiol. 141: 271–279.

Shiu, S.H. and A.B. Bleecker. 2001. Plant receptor-like kinase gene family: diversity, function, and signaling. Science's STKE: signal transduction knowledge environment 2001: re22.

Shiu, S.H. and A.B. Bleecker. 2003. Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. Plant Physiol. 132: 530–543.

Shiu, S.H., W.M. Karlowski, R. Pan, Y.H. Tzeng, K.F. Mayer and W.H. Li. 2004. Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. Plant Cell 16: 1220–1234.

Snell, W.J., W.A. Eskue and M.J. Buchanan. 1989. Regulated secretion of a serine protease that activates an extracellular matrix-degrading metalloprotease during fertilization in *Chlamydomonas*. J. Cell Biol. 109: 1689–1694.

Snell, W.J., J. Pan and Q. Wang. 2004. Cilia and flagella revealed: from flagellar assembly in *Chlamydomonas* to human obesity disorders. Cell 117: 693–697.

Starr, R.C. 1969. Structure, reproduction, and differentiation in *Volvox carteri* f. *nagariensis* Iyengar, strains HK9 & 10. Arch. Protistenk. 111: 204–222.

Starr, R.C. 1970. Control of differentiation in *Volvox*. Dev. Biol. Suppl. 4: 59–100.

Starr, R.C. 1975. Meiosis in *Volvox carteri* f. *nagariensis*. Arch. Protistenkd. 117: 187–191.

Starr, R.C. and L. Jaenicke. 1974. Purification and characterization of the hormone initiating sexual morphogenesis in *Volvox carteri* f. *nagariensis* Iyengar. Proc. Natl. Acad. Sci. USA 71: 1050–1054.

Steele, R.E. and C.E. Dana. 2009. Evolutionary history of the *HAP2/GCS1* gene and sexual reproduction in metazoans. PloS one 4: e7680.

Sumper, M., E. Berg, S. Wenzl and K. Godl. 1993. How a sex pheromone might act at a concentration below 10^{-16} M. EMBO J. 12: 831–836.

Tschochner, H., F. Lottspeich and M. Sumper. 1987. The sexual inducer of *Volvox carteri*: purification, chemical characterization and identification of its gene. EMBO J. 6: 2203–2207.

Tsuchikane, Y., R. Fukumoto, S. Akatsuka, T. Fujii and H. Sekimoto. 2003. Sex pheromones that induce sexual cell division in the *Closterium peracerosum-strigosum-littorale* complex (Charophyta). J. Phycol. 39: 303–309.

Tsuchikane, Y., M. Ito, T. Fujii and H. Sekimoto. 2005. A sex pheromone, protoplast-release-inducing protein (PR-IP) Inducer, induces sexual cell division and production of PR-IP in *Closterium*. Plant Cell Physiol. 46: 1472–1476.

Tsuchikane, Y., Y. Kokubun and H. Sekimoto. 2010a. Characterization and molecular cloning of conjugation-regulating sex pheromones in homothallic *Closterium*. Plant Cell Physiol. 51: 1515–1523.

Tsuchikane, Y., M. Sato, T. Ootaki, Y. Kokubun, H. Nozaki, M. Ito and H. Sekimoto. 2010b. Sexual processes and phylogenetic relationships of a homothallic strain in the *Closterium peracerosum-strigosum-littorale* complex (Zygnematales, Charophyceae). J. Phycol. 46: 278–284.

Tsuchikane, Y., M. Tsuchiya, F. Hindak, H. Nozaki and H. Sekimoto. 2012. Zygospore formation between homothallic and heterothallic strains of *Closterium*. Sex. Plant Reprod. 25: 1–9.

Tsuchikane, Y., M. Tsuchiya, Y. Kokubun, J. Abe and H. Sekimoto. 2011. Conjugation processes of *Penium margaritaceum* (Zygnemophyceae, Charophyta). Phycol. Res. 59: 74–82.

Turmel, M., C. Otis and C. Lemieux. 2006. The chloroplast genome sequence of *Chara vulgaris* sheds new light into the closest green algal relatives of land plants. Mol. Biol. Evol. 23: 1324–1338.

Umen, J.G. and U.W. Goodenough. 2001. Control of cell division by a retinoblastoma protein homolog in *Chlamydomonas*. Genes Dev. 15: 1652–1661.

von Besser, K., A.C. Frank, M.A. Johnson and D. Preuss. 2006. *Arabidopsis HAP2* (*GCS1*) is a sperm-specific gene required for pollen tube guidance and fertilization. Development 133: 4761–4769.

Wang, Q., J. Pan and W.J. Snell. 2006. Intraflagellar transport particles participate directly in cilium-generated signaling in *Chlamydomonas*. Cell 125: 549–562.

Watanabe, M.M. and T. Ichimura. 1982. Biosystematic studies of the *Closterium peracerosum-strigosum-littorale* complex. IV. Hybrid breakdown between two closely related groups, Group II-A and Group II-B. Bot. Mag. Tokyo 95: 241–247.

Weisshaar, B., R. Gilles, R. Moka and L. Jaenicke. 1984. A high frequency mutation starts sexual reproduction in *Volvox carteri*. Z. Naturforsch. 39c: 1159–1162.

Wodniok, S., H. Brinkmann, G. Glockner, A.J. Heidel, H. Philippe, M. Melkonian and B. Becker. 2011. Origin of land plants: do conjugating green algae hold the key? BMC Evol. Biol. 11: 104.

Wong, J.L. and M.A. Johnson. 2010. Is HAP2-GCS1 an ancestral gamete fusogen? Trends Cell Biol. 20: 134–141.

Zhang, Y.H. and W.J. Snell. 1994. Flagellar adhesion-dependent regulation of *Chlamydomonas* adenylyl cyclase *in vitro*—a possible role for protein kinases in sexual signaling. J. Cell Biol. 125: 617–624.

Zhao, H., M. Lu, R. Singh and W.J. Snell. 2001. Ectopic expression of a *Chlamydomonas* mt+-specific homeodomain protein in mt^- gametes initiates zygote development without gamete fusion. Genes Dev. 15: 2767–2777.

3

Reproduction in Bryophytes

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ABSTRACT

Liverworts, mosses and hornworts are together called bryophytes, terrestrial plants with perennial/photosyntetically dominant gametophytes and ephemerous/dependent sporophytes. These plants are classified, according to their sexual system, as monoicous and dioicous, with a few lesser categories recognized by bryologists. Sexual systems in bryophytes are apparently related to breeding systems, with out-crossing occurring in dioicous species and self-fertilization in monoicous ones. Dioicous species are associated with low frequency of fertilization and rarity of sporophytes, which can be caused by biased sex ratios, spatial separation of sexes, and absence of males or failure of males to express sex among populations. After fertilization and sporophyte development, bryophytes produce spores that give rise to new plants. However, other forms of reproduction are present among bryophytes, with asexual structures as gemmae, propagules and regeneration of fragments that are able to form new plants. In the three lineages of bryophytes evolutionary processes resulted in reduction of gametophytic and sporophytic traits, reverse transitions of sexual systems (i.e., monoicy to dioicy), "abnormal" ploidy shifts (through

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apospory and apogamy), precocious development and maintenance of juvenile traits in the adulthood (e.g., intracapsular germination and neoteny). Despite the impressive diversity of reproductive modes in bryophytes, these plants are still poorly known, and only recently have attracted more attention from botanists. Studies related to reproduction in bryophytes increased progressively from 1987, quickly reaching around 20 papers per year in 2001 and a maximum of 25 papers in 2011 in the "Web of Science" database. Many species and ecosystems remain unexplored regarding the bryophytes and their reproductive biology, especially in the tropics, claiming for natural history studies that will identify and characterize interesting systems for research. Moreover, future studies with focus on evolutionary biology, biogeography and functional ecology will promote a comparative framework of the reproductive patterns and processes among all plants and not only bryophytes.

Keywords: Asexual reproduction, cross-fertilization, dioecy, dioicy, gemmae, hornworts, liverworts, monoecy, monoicy, mosses, self-fertilization, sex ratios, sexual reproduction, spores, sporophyte production

Introduction

Liverworts (Marchantiophyta), mosses (Bryophyta) and hornworts (Anthocerotophyta), commonly known as bryophytes, are represented by approximately 18,700 species in the world. Bryophytes are terrestrial atracheophyte plants, whose life cycle has alternation of generations (Fig. 1A–F), with a green and perennial gametophyte and an ephemerous dependent sporophyte (Schofield 1985; Vanderpoorten and Goffinet 2009; Goffinet and Buck 2013).

Gametophytes are leafy (in all mosses—Fig. 2A–F, and the majority of liverworts—Fig. 2G–I) or thallose (in some liverworts—Fig. 2J–L, and all hornworts—Fig. 2M–O). Gametangia are composed by one or more sterile cell layers forming the wall, and inside contain gametes. Antheridia (male gametangia—Fig. 1B) have numerous antherozoids or sperm (motile male gametes), and archegonia (female gametangia—Fig. 1B) contain one single oosphere or egg (not motile female gamete). Fertilization (Fig. 1C) can occur when an antherozoid reaches, mediated by water, an oosphere. After that, a zygote develops into an embryo inside the archegonium. Embryo gives rise to a sporophyte with a foot, seta or stalk (except in hornworts) and capsule (sporangium, Fig. 1D).

Distinct from all terrestrial plants, bryophytes produces a single sporangium in each sporophyte (Singer 2010). Liverwort capsules generally open through four longitudinal slits or valves; hornworts by one or two

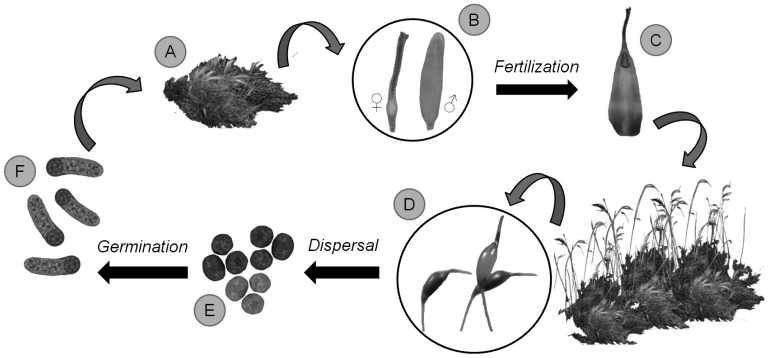

Figure 1A–F. Life cycle of a monoicous moss (*Pyrrhobryum spiniforme*). A. Leafy gametophytes; B. Female (archegonium) and male (antheridium) gametangia; C. Fertilized archegonium; D. Sporophytes attached to gametophytes; E. Spores; F. Protonemata (chloronema phase).

Color image of this figure appears in the color plate section at the end of the book.

longitudinal slits; and the majority of mosses have capsules opening through a transversal line resulting from the operculum detachment (Crandall-Stotler et al. 2009; Goffinet et al. 2009; Renzaglia et al. 2009; Goffinet and Buck 2013).

Inside a capsule, mother cells of spores (sporocytes) split out meiotically, generally originating tetrads of haploid spores. After maturation, spores are released from the capsule and dispersed usually by wind (Fig. 1E). In mosses, after the spore germination a filamentous phase called protonema is produced. The moss protonema differentiates into chloronema (cells with numerous chloroplasts and right transverse walls (Fig. 1F)), caulonema (cells with needle-shaped chloroplasts and oblique transverse walls) and rhizoids (brownish cells with no chloroplasts and oblique transverse walls). Leafy buds are generally formed on the caulonema and give rise to many genetically identical leafy gametophytes (Nishida 1978; Nehira 1983; Duckett et al. 1998). Among liverworts and hornworts, the protonema is much more ephemerous, being restricted to a short-filamentous, globose or cylindrical phase, which commonly develop into a single plant (Nehira 1983; Goffinet and Buck 2013).

In leafy species the gametophyte has rhizoids, caulid (stem), and phyllids (leaves). Rhizoids attach the gametophyte to the substrate; are unicellular and filamentous structures, generally hyaline in liverworts and hornworts; and multicellular, branched and brown-colored in mosses. Caulid is a vertically or horizontally growing axis, with an undifferentiated, very simple or complex anatomy. Respective of species, caulid may contain epidermis, cortex and central cylinder. In the central cylinder of some mosses, especially in the family Polytrichaceae, there are cells specialized

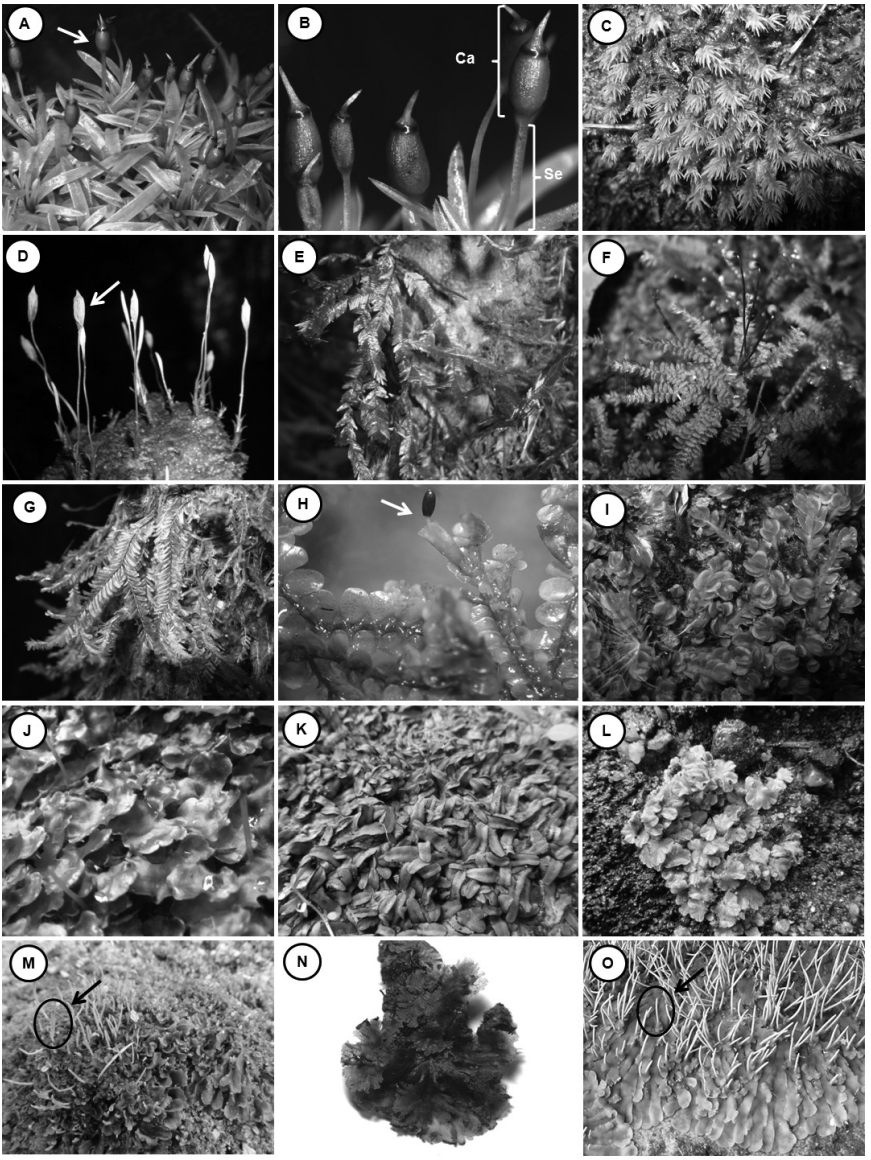

Figure 2 A–N. Diversity of bryophytes. A-D. Acrocarpous mosses; B. Detail of the sporophytes. E-F. Pleurocarpous mosses; G-I. Leafy liverworts; J-L. Thallose liverworts. M-O. Hornworts. Ca. Capsule; Se. Seta; Arrows indicate sporophytes. Pictures K-M by Nivea D. Santos and pictures N-O by Juan Vilarreal.

Color image of this figure appears in the color plate section at the end of the book.

in water and mineral conduction, the hydroids (tissue hydrom), and in photosynthates conduction, the leptoids (tissue leptom). In thalloid species, gametophyte has rhizoids attached to a flattened thallus with one or multiple cell layers, dichotomically branched or with a rosette-shape (Crandall-Stotler et al. 2009; Goffinet etal. 2009; Renzaglia et al. 2009; Goffinet and Buck 2013).

Phyllids are attached to the caulid, and are generally green, small and one-cell layered. In mosses, phyllids are commonly helicoidally arranged, and have a thick central area (with more than one cell layer) similar to the central vein in leaves of other plants, called costa. Conversely, in liverworts, phyllids are arranged in three lines, or rarely in two or four, but do not have a true costa (Crandall-Stotler et al. 2009; Goffinet et al. 2009; Goffinet and Buck 2013).

Leafy bryophytes have gametangia at tips, axils or intercalary in branches or caulid. It is common to find modified phyllids covering gametangia and forming sexual branches. Terms used to classify the sexual branches among bryophytes can vary in the literature, and it is common to find perigonium (♂) and perichaetium (♀) for mosses, and androecium (♂) and gynoecium (♀) for leafy liverworts (Gradstein et al. 2001). Moreover, sterile structures, known as paraphyses, probably protect mosses' gametangia against mechanical damages and dehydration, and also have a role in the dehiscence of the antheridia and secretion of substances to attract microarthropods that disperse sperm cells (Cronberg et al. 2006; Crandall-Stotler et al. 2009; Goffinet et al. 2009; Goffinet and Buck 2013). In hornworts gametangia are located inside the thallus; and complex thalloid liverworts have special receptacles or specialized branches, antheridiophores and archegoniophores, which contain the gametangia. In the thallose liverworts Ricciaceae, gametangia and sporophytes are formed and are totally embedded inside the thallus, capsules are cleistocarpous and release spores only after tissue decaying (Crandall-Stotler et al. 2009; Renzaglia et al. 2009).

In acrocarpous mosses, which comprise nearly half the species in the phylum, perichaetia are produced at the apex of the stem (terminal perichaetia), whereas in pleurocarpous species the perichaetia are located on lateral buds or short side branches, without vegetative leaves. There is a third condition where the perichaetia are terminal, although formed on lateral branches with vegetative leaves (cladocarpous mosses) (La Farge-England 1996). Traditionally, acrocarpy, pleurocarpy and cladocarpy were recognized as growth forms among the mosses. Nowadays, these terms are used to indicate perichaetium, and consequently, the sporophyte position in the gametophyte (La Farge-England 1996). Although the direction of growth in a moss is generally a cue for the growth form classification, it does not

correspond to the perichaetial position, and some acrocarpous mosses grow horizontally and some pleurocarpous grow vertically (Glime 2007).

Retention and development of an embryo that is attached to mother plant is shared by bryophytes and all land plants, a reason for the clade name Embryophytes. A multicellular matrotrophic embryo depends on maternal tissues nutritionally and developmentally for at least some time during its early development (Graham 1996). Embryo nutrition occurs through transfer cells at the intersection between gametophyte and sporophyte, forming a tissue known as placenta. The embryo, and posteriorly the sporophyte, receive water, minerals and organic substances necessary for development from the gametophyte through the placenta (Goffinet et al. 2009; Vanderpoorten and Goffinet 2009). Moreover, in mosses a maternal calyptra (a remnant of the archegonial neck), with a multilayered cuticle, has a role of dehydration protection of the immature sporophyte and is able to increase the offspring fitness. The moss calyptra is the most ancient form of maternal protection by a cuticle in green plants (Budke et al. 2013).

Sexual Systems in Bryophytes and their Consequences

The sexual systems in bryophytes are classified into two main groups, monoicy and dioicy, each one with a few categories that are usually recognized by bryologists (Anderson 1980; Wyatt 1985; Mishler 1988; Table 1 and Fig. 3).

In bryophytes, unlike ferns and angiosperms, species having unisexual plants (the photossynthetically dominant phase) are overrepresented compared to bisexual ones. Among liverworts, about 70% of species are dioicous; in mosses dioicy reaches 55–60%; and in hornworts monoicy predominates among species (Wyatt 1982, 1994; Vanderpoorten and Goffinet 2009). On the other hand, ca. 6% of angiosperm species are dioecious (Renner and Ricklefs 1995).

Wyatt (1985) highlighted the importance for a unified terminology of sexual systems in plants, attributing to the lack of concordant terms in bryophytes and other embryophytes. However, there are good reasons to prefer terms like monoicous/dioicous over monoecious/dioecious among bryophytes. The sexual system in bryophytes describes the sexuality of the gametophyte while in other embryophytes (e.g., seed plants) it describes where and how unisexual gametophytes are borne on the sporophyte. All seed plants have dioicous gametophytes. The terms monoecious and dioecious (sporophyte having one or two unisexual (dioicous) gametophytes) are meaningless for bryophytes since the sporophytes among these plants do not bear gametophytes (Allen and Magill 1987). Moreover, bryophytes are homosporous, whereas seed plants are heterosporous. In homosporous plants products of meiotic divisions (spores) give rise to

Table 1. Sexual Systems in Bryophytes with Examples of Some Species.

Sexual systems	Definition	Examples	References
Monoicous Plants	**♀ and ♂ gametangia on a same plant**		
1. Synoicous plants	♀ and ♂ gametangia mixed on a same sexual branch	*Neckeropsis disticha*	Merced-Alejandro and Sastre-de-Jesús 2009
		N. undulata	Maciel-Silva and Válio 2011
2. Paroicous plants	♂ gametangia in the axils of leaves of ♀ branches	*Cheilolejeunea compacta*	Reiner-Drehwald 2006
		Trichostomum perligulatum	Stark and Castteter 1995
		Pyhhrobryum spniniforme	Maciel-Silva and Válio 2011
		Physcomitrella patens subsp. *californica*	Une and Tateishi 1996
3. Autoicous plants	♀ and ♂ gametangia on separate sexual branches		
a. cladautoicous	♀ and ♂ branches completely separate	*Octoblepharum albidum*	Pôrto and Oliveira 2002
b. gonioautoicous	♀ closer to ♂ branches, being ♂ branches bud-like and axillary on the same stem or branch of the ♀ branch	*Forsstroemia trichomitria*	Stark 1986
		Pyhhrobryum spniniforme	Maciel-Silva and Válio 2011
		Trichostomum perligulatum	Stark and Castteter 1995
		Entodon cladorrhizans	Stark 1983
c. rhizautoicous	♂ branch on a short branch attached to ♀ by rhizoids.	*Aloina bifrons*	Stark and Delgadillo 2001 Stark and Brinda 2013
		Fissidens hornschuchi	Pursell 1989
4. Heteroicous plants (= polyoicous)	♀ and ♂ branches completely mixed or separate on a same plant	*Frullania kunzei*	Grastein and Uribe 2011 Manyanga et al. 2011
		Anastrophyllum sphenoloboides	
Dioicous Plants	**♀ and ♂ gametangia on different plants (plants in both sexes with normal sizes)**	*Bryum dunense*	Herrnstadt and Kidron 2005
		Syntrichia caninervis	Bowker et al. 2000
		Phyllogonium viride	Maciel-Silva and Válio 2011
		Plagiochila disticha	
		Bazzania heterostipa	
		Marchantia inflexa	Fuselier and McLetchie 2004
1. pseudautoicous plants	dwarf ♂ plants on caulid of a ♀ plant	*Dicranum majus*	Solli et al. 2000
		Dicranum polisetum	Bisang and Ehrlén 2002
		Leucobryum crispum	Maciel-Silva and Válio 2011
		L. clavatum	
2. phyllodioicous plants	dwarf ♂ plants on leaves of a ♀ plant	*Macromitrium holomitrioides*	Une 1984
		M. comatum	Une 1985
		M. ferrieri	
		M. prolongatum	

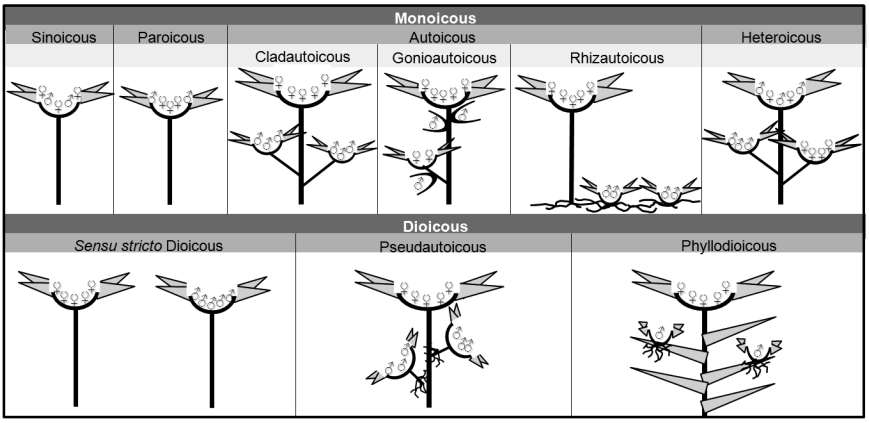

Figure 3. Sexual systems with sub-divisions in bryophytes.

egg-bearing and sperm-bearing gametophytes (dioicous) or gametophytes with both sex cells (monoicous). In heterosporous plants, after meiosis, gametophytes give rise to only one type of sexual cell (Zander 1984; Allen and Magill 1987). Therefore, the breeding systems in bryophytes and seed plants are differently influenced by the sexual systems, i.e., self-fertilization in bryophytes and in seed plants have different evolutionary consequences (Crawford et al. 2009). In the monoicous system, egg and sperm are originated from mitotic divisions in the same gametophyte, being genetically identical cells. Conversely, in the monoecious system egg and sperm cells are formed on different gametophytes (independent meiotic events in the sporophyte) and are genetically variable (Allen and Magill 1987).

Sexual and breeding systems in bryophytes are probably related, with out-crossing in dioicous species and self-fertilization in monoicous ones (Gemmell 1950). Several records of self-compatibility are currently known among monoicous bryophytes, but recent studies suggest that self-incompatibility (intra-gametophytic level) also occurs in this group (Stark and Brinda 2013). According to Longton and Schuster (1983) the sub-divisions within the monoicy in bryophytes may reflect selection for out-crossing, from sinoicy (high selfing likelihood) to rhizautoicy (high crossing likelihood).

There are other mechanisms to prevent the self-fertilization among monoicous bryophytes, such as protandry (maturation of antheridia before archegonia on the same plant) and protogyny (maturation of archegonia before antheridia). Apparently, in most species of mosses, antheridia have earlier and longer maturation than the archegonia (Gemmell 1952; Longton and Schuster 1983; Stark 2002).

Sporophyte Production, Sexual Systems and Sex Ratios

Although the dioicy promotes out-crossing, this is balanced by a decrease in the sporophyte production (Mishler 1988; Crawford et al. 2009). Therefore, sporophyte production in bryophytes seems to be directly linked to the sexual system, since the rarity or absence of sporophytes has been mostly detected among dioicous species compared to monoicous ones (Longton 1976, 1992; Longton and Schuster 1983; Reese 1984; Laaka-Lindberg et al. 2000; Oliveira and Pôrto 1998, 2002; Pôrto and Oliveira 2002; Maciel-Silva et al. 2012a).

For instance, a study on British mossflora (Gemmell 1950) showed a significant low frequency of sporophyte-bearing dioicous species, mostly explained by separation of the sexes and consequent mechanical difficulties in the transport of sperm to archegonia. The same study reported a higher frequency of successful sporophyte production among monoicous species, possibly due to self-fertilization, even though dioicous species were more widely distributed than monoicous ones. Still among British mosses, 87% of species with unknown sporophytes were dioicous and 83% of the monoicous species had sporophytes (Longton 1997). The other two studies on British moss (Longton 1992) and liverwort (Laaka-Lindberg et al. 2000) flora compared the life history traits (e.g., monoicy *vs.* dioicy; presence *vs* absence of sporophytes) of rare and common species and detected that 1) many rare species had no sporophytes compared to common species (consequences of sexual reproduction), 2) many monoicous species were rare compared to dioicous ones (probably adverse consequences of self-fertilization).

Among bryophytes in the Brazilian Atlantic rainforest, dioicous species that produced high numbers of sexual branches and gametangia per sexual branch (i.e., high reproductive performance) failed to produce sporophytes, probably due to the spatial separation of sexes (Maciel-Silva et al. 2012a). Spatial separation of colonies having different sexes is commonly verified among dioicous species of bryophytes, and occurs when a diaspore (spore, gemma or propagule) reaches a substrate, germinates and plants grow clonally forming a colony (Stark et al. 2005).

Dioicous bryophytes have obligatory cross-fertilization that results in high genetic variability compared to monoicous ones. However, this advantage is counter balanced with a low sporophyte production commonly recorded among dioicous species (Eppley et al. 2007). Main reasons for a low sporophyte production among dioicous species are related to spatial separation of female and male colonies (and populations), lower number of males than females, and absence of male plants (or failure to express sex among male plants) (Gemmell 1950; Longton and Schuster 1983; Bowker et al. 2000; Oliveira and Pôrto 2002; Stark et al. 2005, 2010).

Low number of male plants compared to female ones is commonly recorded in populations of dioicous bryophytes and results in a pattern recognized as female biased sex-ratios (Bowker et al. 2000; Bisang and Hedenäs 2005; Bisang et al. 2006; Stark et al. 2010). The real causes for this phenomenon remain unclear, but some hypotheses have been suggested to explain why the initial sex-ratio 1:1 verified among the spores (Stark et al. 2010) usually change during the gametophyte growth and maturation. Explanations are possibly related to sex-differential spore abortion, germination, protonema growth, and gametophore development and mortality in response to competition and/or abiotic effects (for a more detailed discussion see Stark et al. 2010, and Bisang and Hedenäs 2013).

Some studies on female-biased sex-ratio in bryophytes have begun to elucidate this biological puzzle. Constraints on one of the two sexes may begin during spore development, with spore abortion resulting in female and male biased sex-ratios. For instance sex-ratios of germinating spores of 4.1♀:1♂ in the moss *Mnium undulatum* and 0.89♀:1♂ in *M. hornum* (Newton 1972), and 1.5♀:1♂ in *Ceratodon purpureus* (Shaw and Gaughan 1993). In some species, biased sex-ratios begin after spore germination. Liverwort *Sphaerocarpos texanus* had high abundance of females in the field and growth chamber (cultures from 1:1 male to female spores), suggesting lower survival rate in males than in females (McLetchie 1992). In the laboratory, moss *Bryum argenteum* had unbiased (or slightly biased) sporeling sex-ratios, whilst a female-biased gametophyte sex-ratio predominated in the field (Stark et al. 2010).

Failure to express sex among male plants has been suggested to explain the female skewed ratio verified in bryophyte populations (Longton 1990). A hypothesis called "the shy male hypothesis" presumes that males and females have similar frequencies, but males produce sexual structures less often than females (Mishler and Oliver 1991; Stark et al. 1998, 2001, 2005), having a "*reservoir of male plants in the nonexpressing state*" (Stark et al 2010). Male plants are generally rare (or do not express sex) in high-stress habitats like deserts (Bowker et al. 2000; Stark et al. 2010). However, recently the moss *Drepanocladus lycopodioides* was recorded having both genetically determined and phenotipically expressed sex-ratios being female-biased, which indicate that male plants compared to female ones do not necessarily fail to express sex. Sex-ratios were, indeed, female biased among sex-expressing and nonexpressing plants (Bisang and Hedenäs 2013).

The spatial separation of males and females among dioicous bryophytes creates a potential obstacle for fertilization. Bryophyte sperm moves to the female archegonium through a water-film, implying in a maximum distance for fertilization of no more than a few centimeters (Longton and Schuster 1983; Crum 2001). However, some species have developed strategies to increase the chances of fertilization over larger distances. Mosses in the

genus *Plagiomnium* and in the family Polytrichaceae have splash cups with the antheridia exposed to rain action (Rohrer 1982; Andersson 2002); the liverwort *Conocephalum conicum*, after contact with water, produce airborne sperm that can travel long distances (Shimamura et al. 2008; Springer Videos 2010). Furthermore, microarthropods have been reported to mediate fertilization through sperm dispersal in the moss *Bryum argenteum* (Cronberg et al. 2006). Recently, Rosenstiel et al. (2012) demonstrated that sperm-dispersing microarthropods are guided by scents (volatile cue substances) emitted from fertile shoots of the moss *Ceratodon purpureus*, suggesting a fertilization syndrome somewhat comparable to insect pollination of flowering plants (Cronberg 2012; Rosenstiel et al. 2012).

Dioicous species seem to compensate their frequent failure to produce sporophytes through the asexual propagation for the species maintenance (Longton and Schuster 1983; Longton 1992, 2006). For instance, Une (1986) observed that 77.5% of Japanese dioicous species had asexual structures, which are found in only 9.9% of the monoicous species. Recently, Pôrto and Silva (2012) found 54% dioicous *vs*. 46% monoicous species in northeastern Brazilian Atlantic rainforest, where monoicous mosses produced sporophytes more frequently than dioicous ones (ca. 70% and 30%, respectively), and dioicous mosses and liverworts had large frequency of asexual structures compared to monoicous ones (ca. 35% and 15% for liverworts and 20% and 0.5% for mosses, respectively). Conversely, Crawford et al. (2009) analyzed life history data for 367 species of mosses and found no phylogenetic support for a positive correlation between asexual reproduction and dioicous condition. This relationship emerged only when species were treated as independent data points, indicating that asexual reproduction may be not an adaptation to dioicous condition, but a trait shared among relative species. Moreover, dioicous species were generally larger than monoicous ones, suggesting a compensation of the low production of sporophytes and spores by increased size and life-span (Crawford et al. 2009).

The Largest Diversity of Asexual Structures Among all the Plants

Asexual reproduction *sensu lato* is so common in bryophytes that it has been used as a diagnostic character for some taxa (e.g., genera within Lejeuneaceae, Bastos 2008). In no other plant group the asexual reproduction is so important as in bryophytes (Frey and Kürschner 2011), for instance, it is estimated that about 15% and 17% of North-American and British mosses, respectively, produce at least one type of specialized asexual structure (Longton and Schuster 1983; Glime 2007). If these asexual structures have small sizes, being dispersed over long distances, they may contribute

considerably to the gene flow at both local and landscape scales (Pohjamo et al. 2006).

Among bryophytes the asexual reproduction *s.l.* is commonly divided in two basic types: (1) asexual reproduction *sensu stricto* and (2) clonal reproduction (cloning), including body fragmentation (Frey and Kürschner 2011). Therefore, asexual structures may be completely specialized or not. If they are able to detach from the parent shoot, being spatially or temporally dispersed, these asexual structures are recognized as asexual diaspores (During 2001). Many bryophyte species, especially dioicous ones, reproduce asexually exclusively, by regeneration from more or less specialized caducous structures (leaves, leaf tips, shoots, branches, and bulbils) and by the production of specialized asexual structures (gemmae, protonemal gemmae, and rhizoidal gemmae; Frey and Kürschner 2011 and references therein). Based on different studies, the main types of asexual structures known in bryophytes are summarized below (Longton and Schuster 1983; Imura 1994; Glime 2007; Frey and Kürschner 2011). For a detailed account of the diversity of asexual structures among bryophytes, see a recent review by Frey and Kürschner (2011).

Propagules

Propagules have a differentiated apical cell, and sometimes show also leaves and rhizoids. They comprise caducous tips of branches, branches and leaves, flagelliform branches and bulbils. Propagules differ from gemmae due to the apical cell, which originates a new shoot with no protonemal stage (Goffinet and Buck 2013).

Caducous shoot apices

These structures are little modified shoot tips, commonly deciduous along an abscission line. After the detachment from the mother plant, they may grow into a whole plant with rhizoidal development from the basal part, e.g., *Campylopus* spp., *Bryum argenteum* (Imura 1994; Frey and Kürshner 2011).

Caducous branches and branchlets (Cladia)

Caducous branches and branchlets are asexual propagules with normal-size and reduced leaves, respectively. They are found in leaf axils at branch tips and have an abscission line. Cladia are generally recognized as small branches with reduced leaves, e.g., *Dicranum flagellare*, *D. scoparium*, *Platygyrium repens*, *Pseudoeskeella nervosa*, *Cheilolejeunea oncophylla*, *Lejeunea laetevirens*, *L. cancellata*, *L. cardoti*, and *Microlejeunea epiphylla* (Schuster 1983; Bastos 2008; Frey and Kürshner 2011).

Caducous leaves

Normal-size vegetative leaves and specialized diminutive leaves (brood leaves), which detach from the parent shoot and sometimes have rhizoids or young plants on borders. When present, brood leaves are frequently clustered on an axis, e.g., *Hypopterygium didictyon*, *Pleurochaete squarrosa*, *Aulacomnium androgynum, Syntrichia laevipila*, *Campylopus fragilis, Dicranodontium longirostre, Dicranum montanum, Bazzania nudicaulis*, *Ceratolejeunea caducifolia, Cheilolejeunea adnata, C. decidua, Drepanolejeunea propagulífera*, *Lejeunea phyllobola*, *Rectolejeunea berteroana*, *R. emarginuliflora* and *Plagiochila corniculata* (Giordano et al. 1996; Bastos 2008; Frey and Kürshner 2011).

Fragments of leaves (and thallus)

Pieces of leaves, commonly tips and edges, where the leaf often breaks off, e.g., *Campylopus fragilis*, *Dicranum viride*, *D. tauricum*, *Tortella fragilis*, *Acrobolbus ciliatus*, *Frullania microphylla*, *Lejeunea elliottii,* and *Plagiochila caduciloba*. In the hornwort *Nothoceros aenigmaticus* (previously *Megaceros aenigmaticus*), thallus fragmentation supports the growth of geographically isolated male and female populations (Bastos 2008; Renzaglia et al. 2009; Vanderpoorten and Goffinet 2009; Frey and Kürshner 2011).

Bulbils

Small and multicellular, filamentous or spherical, caducous, budlike to thread-like branches, with reduced leaves, with short stalk, growing in leaf axils. They occur from one to several per leaf, e.g., *Bryum* spp., *Leptobryum pyriforme*, *Pohlia* spp. (Imura 1994; Frey and Kürshner 2011).

Gemmae

Gemmae, different from propagules, are asexual structures without an apical cell and germinate following a pattern that recapitulates the ontogeny of the whole plant. They are filamentous, thallose or globose structures, varying from one to usually many cells. Gemmae frequently have a stalk and are developed on different parts of the gametophyte (on leaf, thalli, on stem rhizoids, in leaf axils, endogenous, on protonema, and on specialized non-deciduous gemmiferous shoots, or thallose gemmae-bearing cups).

They are common in liverworts and mosses, e.g., *Blasia pusilla*, *Anastrophyllum hellerianum*, *Cavicularia densa*, *Lunularia cruciata*, *Marchantia* spp., *Metzgeria* spp., *Calymperes* spp., *Syrrhopodon* spp., *Dicranum flagellare* and *Tetraphis pellucida* (Cavers 1903; Kimmerer 1994; Bartholomew-Began

and Jones 2005; Pohjamo et al. 2006; Jones and Bartholomew-Began 2007; Frey and Kürshner 2011; Goffinet and Buck 2013).

Endogenous gemmae

They are produced inside an initial cell on the basal lamina or on the ventral side of the costa in mosses, e.g., *Grimmia torquata, G. trichophylla* and *Racomitrium vulcanicola*. In liverworts, ovoid or ellipsoidal 2-celled endogenous gemmae occur at leaf tips or margins, e.g., *Bazzania kokawana*, Fossombroniaceae, *Endogemma caespiticia* and *Riccardia* spp. (Glime 2007; Frey and Kürshner 2011; Goffinet and Buck 2013).

Protonemal gemmae

One to a few (12)-celled gemmae on chloronemal filaments, with abscission mechanisms consisting in a thin-walled abscission (tmema-) cells that break easily, releasing the gemmae. Protonemal gemmae occur mostly in acrocarpous, but also in pleurocarpous mosses, e.g., *Diphyscium foliosum*, *Dicranella heteromalla*, *Dicranoweisia cirrata*, *Dicranum montanum*, *D. tauricum*, *Tortula muralis*, *Orthodontium lineare*, *Schistostega pennata*, *Rhizomnium punctatum*, *Zygodon* spp., *Orthotrichum* spp., *Isopterygium elegans*, *Bryum* spp., *Mittenia* spp., *Orthotrichum obtusifolium*, *Oxyrrhynchium hians*, *Pseudotaxiphyllum elegans* and *Lepidopilum muelleri*. Most records of protonemal gemmae among mosses are from cultivation in laboratory. The role of these gemmae is apparently to increase the initial establishment, especially when sporophytes are rare (Duckett and Ligrone 1992; Duckett and Matcham 1995; Maciel da Silva et al. 2006; Pressel et al. 2007; Frey and Kürshner 2011).

Tubers (and Rhizoidal gemmae)

Tubers are spherical to ellipsoidal or pyriform structures, thick-walled and with an apical cell. They are usually reddish to dark brown and subterranean, vary from 10 to more than one hundred cells and are attached to rhizoids. Tubers are resistant to drought and can store many substances like lipids and proteins. Dark color suggests anti-herbivory compounds or a filter that avoids germination under high light conditions (Imura 1994; Duckett and Ligrone 1992; Frey and Kürshner 2011).

In mosses, two germination modes are recorded from rhizoidal tubers: (1) tubers develop directly into a leafy shoot (without protonemal phase), when the apical cell is reactivated or (2) tubers produce secondary protonemata and form moss plants indirectly, when the apical cell is not reactivated. These asexual structures are common among acrocarpous

mosses (e.g., Bryaceae, Dicranaceae, Ditrichaceae, Fissidentaceae, Pottiaceae, and *Grimmia pulvinata*), and are absent among pleurocarpous mosses (Risse 1987; Duckett and Ligrone 1992; Frey and Kürshner 2011).

Differences between rhizoidal tubers and rhizoidal gemmae are not clear in the bryological literature, and both terms are commonly used as synonyms. However, rhizoidal gemmae seem to lack an apical cell, and germinate into leafy shoots passing through a secondary protonema, e.g., *Dicranella staphylina*, *D. schreberana* and *Leptobryum pyriforme* (Risse 1987; Imura 1994; Duckett and Ligrone 1992). Both rhizoidal tubers and gemmae are common components of the asexual diaspore bank of bryophytes, surviving unfavorable conditions (During 2001).

In liverworts, tubers occur in *Fossombronia* spp., *Petalophyllum* spp. and *Sewardiella* spp. (Fossombroniales), *Geothallus tuberosus* spp. (Sphaerocarpales) and *Riccia* spp. (Ricciaceae), and are associated with adaptations to arid environments. In hornworts, they are common in *Phaeoceros* spp., and develop on apical parts of the thallus mostly during the dry season (Longton and Schuster 1983; Risse 1987; Imura 1994; Duckett and Ligrone 1992; Glime 2007; Frey and Kürshner 2011).

Some Reproductive Patterns and their Morphological and Evolutionary Consequences

In the three lineages of bryophytes several evolutionary processes resulted in reduction or simplification of gametophytic and sporophytic traits, reverse transitions of sexual systems, "abnormal" ploidy shifts and precocious development. Some of these processes may confer advantages during vegetative growth, reproduction and dispersal to short and long distances relative to habitats where species live and their life histories. Below, we present some of these patterns, which are directly or indirectly linked to reproduction in bryophytes.

Dioicy *vs*. Monoicy

The dioicy among bryophytes is considered a plesiomorphic character that is also present in the Charales and Coleochaetales algae, which are closely related to the embryophytes (Longton and Schuster 1983; Mishler 1988). However, this character has a complex history within the group, with at least 133 transitions between monoicy and dioicy among mosses (McDaniel et al. 2012). Moreover, reversals to dioicy are twice as frequent as the transitions to monoicy within this group. A possible explanation for the recurrent evolution of dioicy among mosses may be the sexual specialization. Sexual dimorphism is commonly found among dioicous species (Une 1984, 1985; Stark et al. 2001; Fuselier and McLetchie 2002; Hedenäs and Bisang 2011;

Pichonet and Gradstein 2012) and indicates that males and females may be subject to conflicting selective pressures. There are different optimal phenotypes for males, which release sperm, and for females, which nurture embryo and sporophyte until maturation, spore dispersal and senescence (McDaniel 2005; McDaniel et al. 2012). Regarding the liverworts, Devos et al. (2011) found that, at least in the *Radula*-clade (a group of leaf liverworts), the monoicy is a recent evolutionary acquisition associated to epiphytism, occurring six times independently, without reversions.

The evolution of the pollination in seed plants gave rise to selective pressures that favor genetic recombination (Bawa 1980; Bawa and Beach 1981), but in bryophytes the dependence of water for fertilization generates differences of fertilization likelihood vs. genetic recombination for monoicous and dioicous species (Longton and Schuster 1983; Longton 1992; 1997; Eppley et al. 2007). High fertilization rates and sporophyte production are generally associated with sexual reproduction. Among dioicous species this assumption is always true since out-crossing (i.e., crossing between two different gametophytes) is involved, but among monoicous species the fertilization commonly involves female and male genotypes from the same gametophyte, i.e., autofertilization or intragametophytic selfing (Crawford et al. 2009). Thus, in practical terms the sexual reproduction in monoicous species may, indeed, be a type of asexual reproduction. However, these species do not seem to be affected by inbreeding depression, since they rapidly purge recessive deleterious mutations through intragametophytic selfing (Eppley et al. 2007; Taylor et al. 2007). Conversely, in dioicous species, intergametophytic selfing can lead to sporophytic inbreeding depression (Taylor et al. 2007). There are evolutionary benefits and disadvantages involved in the reproduction of dioicous and monoicous bryophytes, and selective forces appear to maintain ratios of dioicy to monoicy in an almost unbiased way (compared to angiosperms) among the bryophyte species.

Neoteny

Neoteny is a type of paedomorphosis that leads to a retardation of somatic development relative to the normal onset of sexually mature features, resulting in the persistence of juvenile or pre-adult physical characteristics into adulthood. In bryophytes, the neoteny is generally expressed in the persistence of the protonema, which is more common in mosses than in liverworts. Protonemal neoteny is usually interpreted as an adaptation to growth in ephemeral or unstable substrate or habitats, such as twigs, living leaves and disturbed soil, where rapid maturation and completion of the life cycle are very important for survival (Schuster 1988; Gradstein et al. 2006). In mosses it is known in species of *Buxbaumia*, *Discelium*, *Ephemeropsis* and *Pogonatum*. In liverworts it occurs in *Metzgeriopsis*,

Protocephalozia ephemeroides and *Radula aguirrei* (note that this is an invalid name: www.tropicos.org) and *R. yanoella*. Other forms of neoteny, with no maintenance of the protonemal stage, are present in the liverworts *Myriocoleopsis*, *Aphanolejeunea*, *Cololejeunea*, *Chondriolejeunea*, *Metzgeriopsis*, *Aphanotropis*, *Calatholejeunea*, *Colura*, *Diplasiolejeunea*, *Macrocolura* and *Myriocolea* (Gradstein et al. 2006).

Life-history traits of liverworts, like monoicy, lobule inflation, imbricate leaves, cell wall ornamentation, presence of asexual propagules and neotenic features are suggested to be adaptations to epiphytism *s.l.* (including epiphylly—habitat on leaves; Schuster 1988; Gradstein et al. 2006). A recent study of epiphyllous liverworts has shown that just the last four traits are correlated with the common epiphylly in a cross-species comparison. In addition, after a phylogenetic comparison only the presence of asexual propagules emerged as a true adaptation for the epiphylly (Kraichak 2012). The high correlation between neoteny and epiphylly, in a cross-species analyses, is due to the sharing of neotenic features among closely related liverwort lineages (e.g., *Tuyamaella-Cololejeunea* clade), favoring the colonization of leaves. Therefore, the neoteny appears to be an exaptation instead of an adaptation to epiphylly (Kraichak 2012). However, the hypothesis of its evolution in response to epiphytism should not be rejected and needs more investigation.

Apospory and Apogamy

Apospory (development of a gametophyte from sporophyte tissue without meiosis, i.e., without spore formation) and apogamy (development of a sporophyte, usually from the vegetative cells of the gametophyte, without union of gametes or fertilization) have been rarely observed in nature (El-Saadawi et al. 2012), but have been induced experimentally with bryophytes in laboratory (Fig. 4; Chopra 1988; Chopra and Kumra 1988; Bell 1992; Cvetić et al. 2005; Goffinet et al. 2009).

Apospory is usually induced *in vitro* by factors as suitable temperature and light, sufficient humidity and lack of sugar in the medium. On the other hand, apogamy is favored by the opposite conditions such as low light intensity, increased sugar concentration in the medium, or growth regulators (e.g., indol acetic acid) at low concentrations (Hughes 1969; Chopra 1988; Chopra and Kumra 1988; Cvetić et al. 2005). Multiplication of a filamentous protonema (mosses) or a thallus (liverworts and hornworts) from sporophytic tissue is recorded mostly at controlled conditions in the laboratory (Lang 1901; Matzke and Raudzens 1968; Goffinet and Buck 2013).

Apospory is one of three way through which autopolyploidization—the doubling of the genome with out hybridization—occurs in bryophytes. The

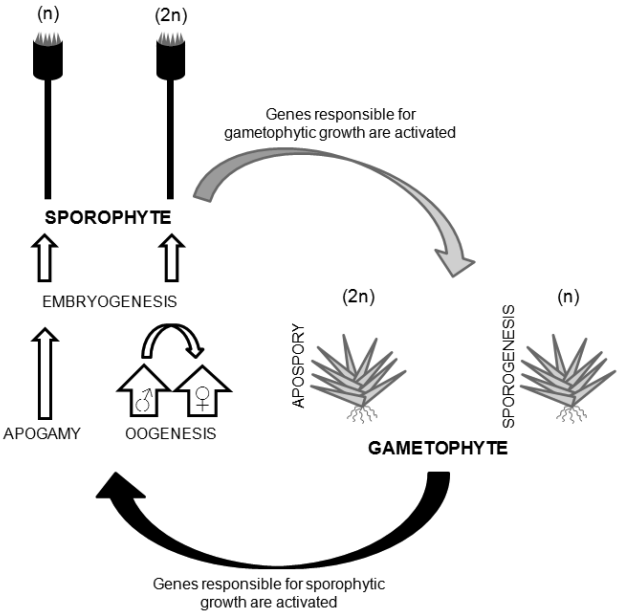

Figure 4. The life cycle of a moss with sexual, apogamous and aposporous events. Adapted from Bell (1992).

two others are diplospory and syndiplospory (for details see Rensing et al. 2013). After an apospory event, natural polyploids may be formed from reprogramming of vegetative sporophytic cells. Aposporous gametophytes, different from their haploid progenitors, may have larger gametangia and sexual cells, which are likely due to the duplicated genome. An apospory event commonly leads to a bisexual gametophyte with functional gametes, i.e., a bisexual gametophyte (Bell 1992; Rensing et al. 2013). Although the apospory is recognized like an abnormal process in the life cycle of bryophytes, it may be responsible for some events of diocy-monoicy transition at least in mosses, where this phenomenon is more common than in liverworts (Bell 1992).

Strong correlation between polyploidy and monoicous condition in mosses suggest that changes in chromosome number may alter the sexual system (Crawford et al. 2009). In the moss *Atrichum undulatum*, gametophytes with combined sexes were diploid or triploid, whereas gametophytes with separate sexes were haploid, diploid or triploid, suggesting that polyploidy has a role in the evolution of monoicy, but not necessarily always resulting in monoicy (Jesson et al. 2011).

Regarding the apogamous sporophytes, these generally have morphological modifications compared to "normal" sporophytes, including absence of stomata. Some abnormalities of apogamous sporophytes

are caused during sporophyte development without the protection of a calyptra, resulting in decrease or no production of offspring. Viability and germination of spores vary among species. When a sporophyte develops from the haplophase, spores are usually unable to germinate. Conversely, if sporophytes grow directly from sporophytic tissue (diplophase), spores are usually viable (Cvetić et al. 2005 and references therein). After spore germination, protonemata grow originating leafy gametophytes and, consequently, gametangia and viable gametes. Leafy gametophytes have large morphological variation, mostly due to mutations during sporogenesis (Chopra 1988; Chopra and Kumra 1988; Cvetić et al. 2005).

Gametangia Number

Some authors have suggested a decreasing pattern of gametangia number among bryophytes, in species from temperate to tropical zones and from basal to derived lineages. Non-tropical liverworts generally produce gynoecia (female branches) with a large number of archegonia (12–25 in *Cephalozia* and 25–50 in *Haplomitrium*), whilst in tropical liverworts, especially in the families Radulaceae, Jubulaceae and Lejeuneaceae, there is a strong trend to gametangia reduction, e.g., Lejeuneaceae are generally monogynous. Similarly, the number of antheridia tend to decrease from non-tropical taxa (4–6(–16) per bract in *Haplomitrium*, *Herbertus* and Schistochilaceae) to tropical families (with (1)2–3 in Radulaceae, 1 in Porellaceae, 1–2 in Lejeuneaceae and Jubulacaeae, e 1(1–2) or rarely more in Plagiochilaceae; Schuster 1988; Gradstein 1991). In the hornworts there is an evolutionary trend to decrease the antheridia number per antheridial chamber, e.g., from the maximum of 30–80 per chamber in the basal *Leiosporoceros* to one per chamber in Dendrocerotaceae (the most derived taxon in the hornworts) (Renzaglia et al. 2009). Moreover, the family Dendrocerotaceae, *Megaceros*, *Nothoceros* and *Dendroceros* are tropical genera. In future, a phylogenetic comparison should be used to analyze the real role of decreasing number of gametangia among bryophytes, and elucidate if this trait, indeed, is an adaptation to life in tropics.

Furthermore, the number of gametangia in bryophytes may influence fertilization chances and sporophyte output in each species, and apparently is associated to the sexual system. In general, for bryophytes, the ratio of male to female gametangia per sexual branch is considerably higher in the dioicous taxa (Une and Tateishi 1996; Glime 2007). Based on a literature survey and unpublished data, Maciel-Silva et al. (2012a) recorded a male-biased sex ratio of gametangia per sexual branch in dioicous bryophytes (♂ 77.79% and ♀ 22.21%; ♂:♀ ratio = 5.28), and in some degree in monoicous ones (♂ 54.75% and ♀ 42.25%; ♂:♀ = 1.77). The markedly biased sex ratio of gametangia in dioicous species compared to monoicous ones suggests

that dioicous species have strategies to increase out-crossing (Glime 2007; Maciel-Silva et al. 2012a).

Green Spores and Precocious Germination

Green (or chlorophyllous) spores are common among bryophytes and some ferns. Since spore colour is frequently related to spore longevity, green spores lose viability shortly compared to non-green spores (e.g., within a single day to a few months; Lloyd and Klekowski 1970; Pence 2000; Wiklund and Rydin 2004; Maciel da Silva et al. 2009a). Yellow and brown spores can last longer because they are protected against desiccation by thick walls and store nutrients in internal oil droplets (Mogensen 1981; Renzaglia et al. 2009).

Green spores are commonly associated to tropical taxa (e.g., liverworts: all Lejeuneaceae, *Radula*, *Plagiochila*; mosses: *Neckeropsis disticha*, *N. undulata*, *Octoblepharum albidum*, *Pyhhrobryum spiniforme,* and *Thamniopsis incurva*; hornworts: *Megaceros*, *Nothoceros*, and *Dendroceros*) (Schuster 1988; Renzaglia et al. 2009; Maciel da Silva et al. 2009 a, b; Maciel-Silva et al. 2014). In the hornworts, green spores are considered an apomorphic character, compared to yellow spores, occurring in three genera of the family Dendrocerotaceae (Renzaglia et al. 2009). Investigations with ultrastructural and phylogenetic approaches would clarify about the adaptive role of green spores among tropical species of bryophytes.

Green spores have fast germination, which in some species occurs inside the capsule (intracapsular germination) or still inside the spore wall (endosporic germination). Intracapsular germination is commonly recorded in tropical liverworts (especially epiphytes, e.g., Lejeuneaceae, *Frullania*, *Plagiochila*, *Porella*, and *Radula*; Schuster 1988; Thiers 1988), but appears rare among mosses. Kürschner (2004) recorded an example of intracapsular germination from the tropical moss *Brachymenium leptophyllum*, suggesting an achorous-strategy of life in which "*protonema dwells out of the capsule, forming immediately buds and juvenile gametophytes that establish near the mother plant ... new plants remain on the same phorophyte, increase the chance of re-establishment of the population at these favoured sites and lower the risk of extinction by long-range dispersal*".

Endosporic germination occurs after the first cell division of the spore, without the rupture of spore wall (exospore), and gives rise to a globose sporeling covered by intact exospore. This precocious germination is present in liverworts, mosses and hornworts (Nishida 1978; Schofield 1981; Nehira 1983; Thiers 1988; Schuette and Renzaglia 2010). Tropical and epiphytic hornworts of the genus *Dendroceros* have green multicellular spores (i.e., spores with precocious germination inside the capsule), which are large when dispersed and may have around 100 cells. Similarly, spores in the

liverwort *Pellia* have precocious germination inside the capsule, reaching 23–24 cells. Mosses of the genus *Andreaea* also have endosporic germination, but it occurs only after dispersal and at suitable conditions for germination (Nishida 1978; Nehira 1983; Schuette and Renzaglia 2010). Green spores associated to fast and precocious germination (intracapsular or endosporic) are suggested as a strategy adopted among different bryophyte taxa for desiccation tolerance and growth on epiphytic habitats, since the early stage of the gametophytes (protonema) remain protected by the capsule or exospore (spore wall).

Reproduction in Bryophytes: Now and Hereafter

Studies relative to reproduction *sensu lato* in bryophytes, including sexual and asexual cycles; reproductive phenology; diaspore development, dispersal and establishment; diaspore banks; reproductive effort and costs; evolution of sexual systems and trade-offs among life-history traits, have increased in number mostly during the last 30 years. To visualize publication trends on this topic, we carried out searches using the words' reproduction AND bryophytes' OR 'reproduction AND liverworts' OR 'reproduction AND hornworts' OR 'reproduction AND mosses' in the database of Web of Science (www.webofknowledge.com) from 1983 until 2013. We observed that studies directly or indirectly related to reproduction in bryophytes increased progressively from 1987, quickly reaching around 20 papers per year in 2001 and a maximum of 25 papers in 2011 (Fig. 5).

Assuming that all literature published is not available in the database of the Web of Science, and that there are studies written in languages other than English, we expect that these numbers are pretty higher. The increase of studies with bryophytes, especially linked to their reproduction, may be associated to improvements in microscopy technologies, informatics, geo-referencing, molecular biology, biochemistry and plant physiology. Students and researches world wide have realized the importance of these plants to understand relevant issues in climatic changes, nitrogen deposition, evolutionary developmental biology (evo-devo), phylogenetics and life-history theory of organisms.

Particularly in Brazil, studies on bryophyte reproduction started mostly in the last two decades (Oliveira and Pôrto 1998, 2001, 2002; Pôrto and Oliveira 2002; Bastos 2008; Alvarenga et al. 2009; Maciel-Silva and Válio 2011; Maciel-Silva et al. 2012a,b; Pôrto and Silva 2012; Maciel-Silva et al. 2013). Two explanations for this fact are: 1) the huge diversity of Brazilian ecosystems and consequently its large bryophyte species richness, which remain poorly known by scientists; 2) the significant increase in new bryologist training only in the last years. Brazilian ecosystems, which

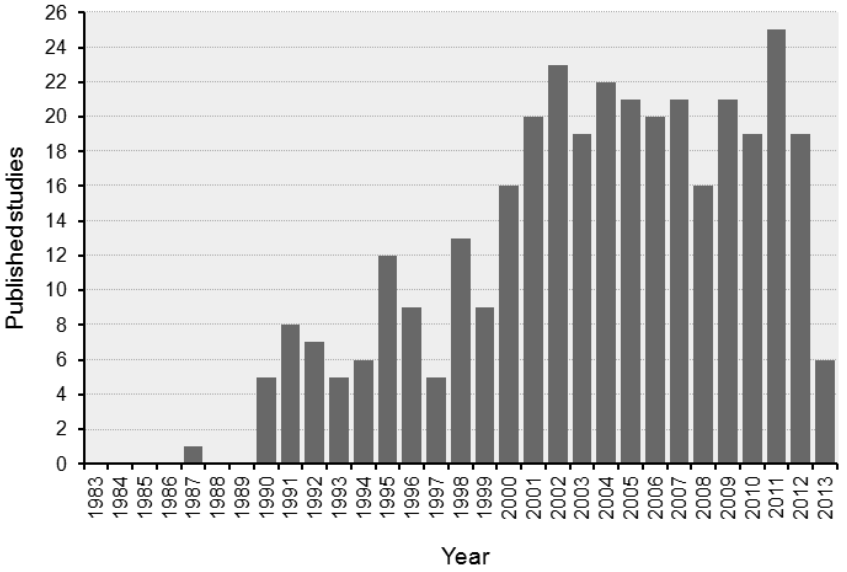

Figure 5. Studies published from 1983 to 2013 (May) based on database of Web of Science (www.webofknowledge.com), using the search topics ('reproduction AND bryophytes') OR ('reproduction AND liverworts') OR ('reproduction AND hornworts') OR ('reproduction AND mosses').

include tropical rainforests and dry forests, savannas, rock outcrops, mangrove, sandbanks, etc., have large potential to studies focusing on the reproductive biology of bryophytes.

Many species and ecosystems remain unexplored regarding the bryophytes and their reproductive biology, especially in the tropics, claiming for natural history studies that will identify and characterize interesting systems for research. However, it is also important that future researches walk hand in hand with other disciplines like evolutionary biology, biogeography and functional ecology, promoting a comparative framework of the reproductive patterns and processes among all plants and not only bryophytes.

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References

Allen, B.H. and R.E. Magill. 1987. Support of a distinct terminology for bryophyte sexuality. Taxon 36: 57–58.

Alvarenga, L.D.P., K.C. Pôrto and M.P.P. Silva. 2009. Relations between regional-local habitat loss and metapopulation properties of epiphyllous bryophytes in the Brazilian Atlantic forest. Biotropica 41: 682–691.

Anderson, L.E. 1980. Cytology and reproductive biology of mosses. *In*: R.J. Taylor and A.E. Leviton (eds.). The mosses of North America, Pacific Division, American Association for the Advancement of Science, San Francisco, pp. 37–76.

Andersson, K. 2002. Dispersal of spermatozoids from the splash-cups of *Plagiomnium affine*. Lindbergia 27: 90–96.

Bartholomew-Began, S. and N.L. Jones. 2005. Juvenile gametophyte development in the Blasiales (Marchantiophyta: Metzgeriidae). 1. Receptacular gemma/gemmaling ontogeny of *Blasia pusilla*. Bryologist 108: 497–505.

Bastos, C.J.P. 2008. Padrões de reprodução vegetativa em espécies de Lejeuneaceae (Marchantiophyta) e seu significado taxonômico e ecológico. Rev. Bras. Bot. 31: 309–315.

Bawa, K.S. 1980. Evolution of dioecy in flowering plants. Annu. Rev. Ecol. Syst. 11: 15–39.

Bawa, K.S. and J.H. Beach. 1981. Evolution of sexual systems in flowering plants. Ann. Mo. Bot. Gard. 68: 254–274.

Bell, P.R. 1992. Apospory and apogamy: implications for understanding the plant life cycle. Int. J. Plant Sci. 153: S123–S136.

Bisang, I. and J. Ehrlén. 2002. Reproductive effort and cost of sexual reproduction in female *Dicranum polysetum*. Bryologist 105: 384–397.

Bisang, I., J. Ehrlén and L. Hedenäs. 2006. Reproductive effort and costs of reproduction do not explain female-biased sex ratios in the moss *Pseudocalliergon trifarium* (Amblystegiaceae). Am. J. Bot. 93: 1313–1319.

Bisang, I. and L. Hedenäs. 2005. Sex ratio patterns in dioicous bryophytes re-visited. J. Bryol. 27: 207–219.

Bisang, I. and L. Hedenäs. 2013. Males are not shy in the wetland moss *Drepanocladus lycopodioides*. Int. J. Plant Sci. 174: 733–739.

Bowker, M.A., L.R. Stark, D.N. McLetchie and B.D. Mishler. 2000. Sex expression, skewed sex ratios, and microhabitat distribution in the dioecious desert moss *Syntrichia caninervis* (Pottiaceae). Am. J. Bot. 87: 517–526.

Budke, J.M., B. Goffinet and C.S. Jones. 2013. Dehydration protection provided by a maternal cuticle improves offspring fitness in the moss *Funaria hygrometrica*. Ann. Bot. 111: 781–789.

Cavers, P. 1903. On asexual reproduction and regeneration in hepaticae. New Phytol. 2: 121–133.

Chopra, R.N. 1988. *In vitro* production of apogamy and apospory in bryophytes and their significance. J. Hattori Bot. Lab. 64: 169–175.

Chopra, R.N. and P.K. Kumra. 1988. Biology of Bryophytes. Wiley Eastern Limited, New Delhi.

Crandall-Stotler, B., R.E. Stotler and D.G. Long. 2009. Morphology and classification of the Marchantiophyta. *In*: B. Goffinet and A.J. Shaw (eds.). Bryophyte Biology. Cambridge University Press, Cambridge, pp. 1–54.

Crawford, M., K.J. Linley and P.J. Garnock-Jones. 2009. Correlated evolution of sexual system and life-history traits in mosses. Evolution 63: 1129–1142.
Cronberg, N. 2012. Animal-mediated fertilization in bryophytes—parallel or precursor to insect pollination in angiosperms? Lindbergia 35: 76–85.
Cronberg, N., R. Natcheva and K. Hedlund. 2006. Microarthropods mediate sperm transfer in mosses. Science 313: 1255.
Crum, H. 2001. Structural diversity of bryophytes. University of Michigan Herbarium, Ann. Arbor.
Cvetić, T., M. Sabovljević, A. Sabovljević and D. Grubišić. 2005. *In vitro* culture and apogamy-alternative pathway in the life cycle of the moss *Amblystegium serpens*. Arch. Biol. Sci. 57: 267–272.
Devos, N., M.A.M. Renner, R. Gradstein, A.J. Shaw, B. Laenen and A. Vanderpoorten. 2011. Evolution of sexual systems, dispersal strategies and habitat selection in the liverwort genus *Radula*. New Phytol. 192: 225–236.
Duckett, J.G. and R. Ligrone. 1992. A survey of liberation mechanisms and germination patterns in mosses. J. Bryol. 17: 335–354.
Duckett, J.G. and H.W. Matcham. 1995. Gemmiferous protonemata: an overlooked dimension in the reproductive biology of mosses. Bull. Brit. Bryol. Soc. 65: 22–23.
Duckett, J.G., A.M. Schmid and R. Ligrone. 1998. Protonemal morphogenesis. *In*: J.W. Bates, N.W. Ashton and J.G. Duckett (eds.). Bryology for the twenty-first century. Leeds: Maney and British Bryological Society, pp. 223–246.
During, H.J. 2001. Diaspore banks. Bryologist 104: 92–97.
El-Saadawi, W.H. Shabbara and M. El-Faramawi. 2012. The second record of a natural apogamous moss sporophyte worldwide. Cryptogam. Bryol. 33: 185–190.
Eppley, S.M., P.J. Taylor and L.K. Jesson. 2007. Self-fertilization in mosses: a comparison of heterozygote deficiency between species with combined versus separate sexes. Heredity 98: 38–44.
Frey, W. and H. Kürschner. 2011. Asexual reproduction, habitat colonization and habitat maintenance in bryophytes. Flora 206: 173–184.
Fuselier, L. and D.N. McLetchie. 2002. Maintenance of sexually dimorphic preadult traits in *Marchantia inflexa* (Marchantiacae). Am. J. Bot. 89: 592–601.
Fuselier, L. and D.N. McLetchie. 2004. Microhabitat and sex distribution in *Marchantia inflexa*, a dioicous liverwort. Bryologist 107: 345–356.
Gemmell, A.R. 1950. Studies in the Bryophyta I. The influence of sexual mechanism on varietal production and distribution of British Musci. New Phytol. 49: 64–71.
Gemmell, A.R. 1952. Studies in the Bryophyta II. The distribution of the sexual groups of British mosses. New Phytol. 51: 77–89.
Giordano, S., F. Alfano, A. Esposito, V. Spagnuolo, A. Basile and R.C. Cobianchi. 1996. Regeneration from detached leaves of *Pleurochaete squarrosa* (Brid.) Lindb. in culture and in the wild. J. Bryol. 19: 219–227.
Glime, J.M. 2007. Bryophyte Ecology. Michigan Technological University/Botanical Society of America/International Association of Bryologist. Available at: <www.bryoecol.mtu.edu> [accessed 01 May 2013].
Goffinet, B. and W.R. Buck. 2013. The evolution of body form in bryophytes. Annu. Plant Rev. 45: 51–90.
Goffinet, B., W.R. Buck and A.J. Shaw. 2009. Morphology, anatomy, and classification of the Bryophyta. *In*: B. Goffinet and A.J. Shaw (eds.). Bryophyte Biology. Cambridge University Press, Cambridge, pp. 55–138.
Gradstein, S.R. 1991. Diversity and distribution of Asian Lejeuneaceae subfamily Ptychanthoideae. Trop. Bryol. 4: 1–16.
Gradstein, R. and J. Uribe. 2011. A synopsis of the Frullaniaceae (Marchantiophyta) from Colombia. Caldasia 33: 367–396.
Gradstein, S.R., S.P. Churchill and N. Salazar-Allen. 2001. Guide to the Bryophytes to Tropical America. Mem. New York Bot. Gard. 86: 1–577.

Gradstein, S.R., R. Wilson, A.L. Ilkiu-Borges and J. Heinrichs. 2006. Phylogenetic relationships and neotenic evolution of *Metzgeriopsis* (Lejeuneaceae) based on chloroplast DNA sequences and morphology. Bot. J. Linn. Soc. 151: 293–308.

Graham, L.E. 1996. Green algae to land plants: an evolutionary transition. J. Plant Res. 109: 241–251.

Hedenäs, L. and I. Bisang. 2011. The overlooked dwarf males in mosses—unique among green land plants. Perspectives in Plant Ecology, Evolution and Systematics. Perspectives in Plant Ecology, Evolution and Systematics 13: 121–135.

Herrnstadt, I. and G.J. Kidron. 2005. Reproductive strategies of *Bryum dunense* in three microhabitats in the Negev Desert. Bryologist 108: 101–109.

Hughes, J.G. 1969. Factors conditioning development of sexual and apogamous races of *Phascum cuspidatum* Hedw. New Phytol. 68: 883–900.

Imura, S. 1994. Vegetative diaspores in Japanese mosses. J. Hattori Bot. Lab. 77: 177–232.

Jesson, L.K., A.P. Cavanagh and D.S. Perley. 2011. Polyploidy influences sexual system and mating patterns in the moss *Atrichum undulatum* sensu lato. Ann. Bot. 107: 135–143.

Jones, N.L. and S. Bartholomew-Began. 2007. Juvenile gametophyte development in the Blasiales (Metzgeriidae). 2. Gemma/gemmaling ontogeny of *Cavicularia densa*. Bryologist 110: 453–464.

Kimmerer, R.W. 1994. Ecological consequences of sexual versus asexual reproduction in *Dicranum flagellare* and *Tetraphis pellucida*. Bryologist 97: 20–25.

Kraichak, E. 2012. Asexual propagules as an adaptive trait for epiphylly in tropical leafy liverworts (Lejeuneaceae). Am. J. Bot. 99: 1436–1444.

Kürschner, H. 2004. Intracapsular spore germination in *Brachymenium leptophyllum* (Müll.Hal.) A. Jaeger (Bryaceae, Bryopsida)—an achorous strategy. Nova Hedwigia 78: 447–451.

La Farge-England, C. 1996. Growth form, branching pattern, and perichaetial position in mosses: cladocarpy and pleurocarpy redefined. Bryologist 99: 170–186.

Laaka-Lindberg, S., T.A. Hedderson and R.E. Longton. 2000. Rarity and reproductive characteristics in the British hepatic Flora. Lindbergia 25: 78–84.

Lang, W.H. 1901. On Apospory in *Anthoceros laevis*. Ann. Bot. 15: 503–510.

Lloyd, R.M. and E.J. Klekowski Jr. 1970. Spore germination and viability in Pteridophyta: evolutionary significance of chlorophyllous spores. Biotropica 2: 129–137.

Longton, R.E. 1976. Reproductive biology and evolutionary potential in bryophytes. J. Hattori Bot. Lab. 41: 205–223.

Longton, R.E. 1990. Sexual reproduction in bryophytes in relation to physical factors of the environment. *In*: R.N. Chopra and S.C. Bhatla (eds.). Bryophyte Development: Physiology and Biochemistry. CRC Press, Boca Raton, Florida, pp. 139–166.

Longton, R.E. 1992. Reproduction and rarity in British mosses. Biol. Conserv. 59: 89–98.

Longton, R.E. 1997. Reproductive biology and life-history strategies. Adv. Bryol. 6: 65–101.

Longton, R.E. 2006. Reproductive ecology of bryophytes: what does it tell us about the significance of sexual reproduction? Lindbergia 31: 16–23.

Longton, R.E. and R.M. Schuster. 1983. Reproductive biology. *In*: R.M. Schuster (ed.). New Manual of Bryology. Hattori Botanical Laboratory, Nichinan, pp. 386–462.

Maciel da Silva, A.S., K.C. Pôrto and E.A. Simabukuro. 2006. Morfogênese protonemática de briófitas ocorrentes em Remanescentes de Floresta Atlântica do estado de Pernambuco, Brasil. Boletim do Instituto de Botânica 18: 213–227.

Maciel da Silva, A.S., K.C. Pôrto and E.A. Simabukuro. 2009a. Effect of light and water availability on spore germination and protonemal growth of the Neotropical moss *Thamniopsis incurva* (Pilotrichaceae). Cryptogam. Bryol. 30: 243–257.

Maciel da Silva, A.S., E.A. Simabukuro and K.C. Pôrto. 2009b. Effect of water availability on spore germination of the moss *Octoblepharum albidum* from Brazilian Atlantic Forest. J. Bryol. 31: 169–173.

Maciel-Silva, A.S., K.C. Pôrto and M.L. Coelho. 2013. Reproductive traits in the tropical moss *Octoblepharum albidum* differ between rainforest and coastal sites. J. Bryol. 35: 206–215.

Maciel-Silva, A.S., F.C.L. Silva, I.F.M. Válio. 2014. All green, but equal? Morphological traits and ecological implications on spores of three species of mosses in the Brazilian Atlantic forest. Anais da Academia Brasileira de Ciências. In press.
Maciel-Silva, A.S. and I.F.M. Válio. 2011. Reproductive phenology of bryophytes in tropical rain forests: the sexes never sleep. Bryologist 114: 708–719.
Maciel-Silva, A.S., I.F.M. Válio and H. Rydin. 2012a. Altitude affects the reproductive performance in monoicous and dioicous bryophytes: examples from a Brazilian Atlantic rainforest. AoB Plants 2012: pls016. doi:10.1093/aobpla/pls016 first published online June 15, 2012.
Maciel-Silva, A.S., I.F.M. Válio and H. Rydin. 2012b. Diaspore bank of bryophytes in tropical rain forests: the importance of breeding system, phylum and microhabitat. Oecologia 168: 321–333.
Manyanga, P., L. Söderström and T.A. Hedderson. 2011. Co-variation of life history characters in the family Lophoziaceae: a multivariate analysis. Bryologist 114: 583–594.
Matzke, E.B. and L. Raudzens. 1968. Aposporous diploid gametophytes from sporophytes of the liverwort *Blasia pusilla* L. Proc. Natl. Acad. Sci. USA 59: 752–755.
McDaniel, S.F. 2005. Genetic correlations do not constrain the evolution of sexual dimorphism in the moss C*eratodon purpureus*. Evolution 59: 2353–2361.
McDaniel, S.F., J. Atwood and J.G. Burleigh. 2012. Recurrent evolution of dioecy in bryophytes. Evolution 67: 567–572.
McLetchie, D. 1992. Sex ratio from germination through maturity and its reproductive consequences in the liverwort *Sphaerocarpos texanus*. Oecologia 92: 273–278.
Merced-Alejandro, A. and I. Sastre-de-Jesús. 2009. A developmental sequence for paraphyses in *Neckeropsis* (Neckeraceae). Bryologist 112: 342–353.
Mishler, B.D. 1988. Reproductive ecology of bryophytes. *In*: J. Lovett Doust and L. Lovett Doust (eds.). Plant reproductive ecology: patterns and strategies. Oxford University Press, New York, pp. 285–306.
Mishler, B.D. and M.J. Oliver. 1991. Gametophytic phenology of *Tortula ruralis*, a desiccation-tolerant moss, in the Organ Mountains of southern New Mexico. The Bryologist 94: 143–153.
Mogensen, G.S. 1981. The biological significance of morphological characters in bryophytes: The Spore. Bryologist 84: 187–207.
Nehira, K. 1983. Spore germination, protonema development and sporeling development. *In*: R.M. Schuster (ed.). New Manual of Bryology. The Hattori Botanical Laboratory, Nichinan, pp. 343–379.
Newton, M.E. 1972. Sex ratio differences in *Mnium hornum* Hedw. and *M. undulatum* Sw. in relation to spore germination and vegetative regeneration. Annals of Botany 36: 163–178.
Nishida, Y. 1978. Studies on the sporeling types in mosses. J. Hattori Bot. Lab. 44: 371–454.
Oliveira, S.M. and K.C. Pôrto. 1998. Reprodução sexuada em musgos acrocárpicos do estado de Pernambuco, Brasil. Acta Bot. Bras. 12: 385–392.
Oliveira, S.M. and K.C. Pôrto. 2001. Reproductive phenology of the moss *Sematophyllum subpinnatum* in a tropical lowland forest of north-eastern Brazil. J. Bryol. 23: 17–21.
Oliveira, S.M. and K.C. Pôrto. 2002. Population profile of *Bryum apiculatum* Schwaegr. in an Atlantic Forest remnant, Pernambuco, Brazil. J. Bryol. 24: 251–294.
Pence, V.C. 2000. Survival of chlorophyllous and nonchlorophyllous fern spores through exposure to liquid nitrogen. Am. Fern J. 90: 119–126.
Pichonet, A. and S.R. Gradstein. 2012. Male Dwarfism in the Genus *Dicranum* (Dicranaceae)—A Review. Cryptogam. Bryol. 33: 299–311.
Pohjamo, M., S. Laaka-Lindberg, O. Ovaskainen and H. Korpelainen. 2006. Dispersal potential of spores and asexual propagules in the epixylic hepatic *Anastrophyllum hellerianum*. Evol. Ecol. 20: 415–430.

Pôrto, K.C. and S.M. Oliveira. 2002. Reproductive phenology of *Octoblepharum albidum* (Bryopsida, Leucobryaceae) in a tropical lowland forest of north-eastern Brazil. J. Bryol. 24: 291–294.

Pôrto, K.C. and M.P.P. Silva. 2012. A frequência de reprodução sexuada afeta a distribuição de briófitas? Uma abordagem regional para a Floresta Atlântica nordestina. *In*: Anais do 63º Congresso Nacional de Botânica, Joinville, Santa Catarina, pp. 12–20.

Pressel, S., H.M. Matcham and J.G. Duckett, J.G. 2007. Studies of protonemal morphogenesis in mosses. XI. *Bryum* and allied genera: a plethora of propagules. J. Bryol. 29: 241–258.

Pursell, R.A. 1989. Notes on Neotropical *Fissidens* I, II, and III. I. The relationship of *F. leptophyllus*. II. the relationship of *F. obtusissimus*, stat. nov. III. the identity of *F. hornschuchii*. Bryologist 92: 523–528.

Reese, W.D. 1984. Reproductivity, fertility and range of *Syrrhopodon texanus* Sull. (Musci; Calymperaceae), a North American endemic. Bryologist 87: 217–222.

Reiner-Drehwald, M.E. 2006. Type studies on Neotropical Lejeuneaceae (Jungermanniopsida). *Cheilolejeunea* and *Lepidolejeunea*. Nova Hedwigia 83: 473–482.

Renner, S.S. and R.E. Ricklefs. 1995. Dioecy and its correlates in the flowering plants. Am. J. Bot. 82: 596–606.

Rensing, S.A., A.K. Beike and D. Lang. 2013. Evolutionary importance of generative polyploidy for genome evolution of haploid-dominant land plants. *In*: I.J. Leitch, J. Greilhuber, D. Jaroslav and W. Jonathan (eds.). Plant Genome Diversity Volume 2: Physical Structure, Behaviour and Evolution of Plant Genomes. Springer-Verlag Wien, pp. 295–305.

Renzaglia, K.S., J.C. Villarreal and R.J. Duff. 2009. New insights into morphology, anatomy, and systematic of hornworts. *In*: B. Goffinet and A.J. Shaw (eds.). Bryophyte Biology. Cambridge University Press, Cambridge, pp. 139–171.

Risse, S. 1987. Rhizoid gemmae in mosses. Lindbergia 13: 111–126.

Rohrer, J.R. 1982. Sporophyte production and sexuality of mosses in two Northern Michigan habitats. Bryologist 85: 394–400.

Rosenstiel, T.N., E.E. Shortlidge, A.N. Menlychenko, J.F. Pankow and S.M. Eppley. 2012. Sex-specific volatile compounds influence microarthropod-mediated fertilization of moss. Nature 489: 431–433.

Schofield, W.B. 1981. Ecological significance of morphological characters in the moss gametophyte. Bryologist 84: 149–165.

Schofield, W.B. 1985. Introduction to Bryology. Macmillan, New York.

Schuette, S. and Renzaglia, K.S. 2010. Development of multicellular spores in the hornwort genus *Dendroceros* (Dendrocerotaceae, Anthocerotophyta) and the occurrence of endospory in Bryophytes. Nova Hedwigia 91: 301–316.

Schuster, R.M. 1983. Evolution, phylogeny and classification of the Hepaticae. *In*: R.M. Schuster (ed.). New Manual of Bryology. The Hattori Botanical Laboratory, Nichinan, pp. 892–1070.

Schuster, R.M. 1988. Ecology, reproductive biology and dispersal of Hepaticae in the tropics. J. Hattori Bot. Lab. 64: 237–269.

Shaw, A.J. and J.F. Gaughan. 1993. Control of sex ratios in haploid populations of the moss, *Ceratodon purpureus*. Am. J. Bot. 80: 584–591.

Shimamura, M., T. Yamaguchi and H. Deguchi. 2008. Airborne sperm of *Conocephalum conicum* (Conocephalaceae). J. Plant. Res. 121: 69–71.

Singer, S.R. 2010. An overview of plant development. *In*: S.F. Gilbert. (ed.). Developmental Biology. Sinauer Associates, Inc. pp. 627–654.

Solli, I.M.S., L. Soderstrom, S. Bakken, K.I. Flatberg and B. Pedersen. 2000. Studies of fertility of *Dicranum majus* in two populations with contrasted sporophyte production. J. Bryol. 22: 3–8.

SpringerVideos. 2010. Airborne sperm of *Conocephalum conicum* (Conocephalaceae). Available at: <http://www.youtube.com/watch?v=ACbXWuR4KkQ> [accessed 13 May 2013].

Stark, L.R. 1983. Reproductive biology of *Entodon cladorrhizans* (Bryopsida, Entodontaceae). I. reproductive cycle and frequency of fertilization. Systematic Botany 8: 381–388.

Stark, L.R. 1986. The life history of *Forsstroemia trichomitria* (Hedw.) Lindb., an epiphytic moss. Lindbergia 12: 20–32.
Stark, L.R. 2002. Phenology and its repercussions on the reproductive ecology of mosses. Bryologist 105: 204–218.
Stark, L.R. and Brinda, J.C. 2013. An experimental demonstration of rhizautoicy, self-incompatibility, and reproductive investment in *Aloina bifrons* (Pottiaceae). Bryologist 116: 43–52.
Stark, L.R. and R.C. Castteter. 1995. Phenology of *Trichostomum perligulatum* (Pottiaceae, Bryopsida) in the Chihuahuan Desert. Bryologist 98: 389–397.
Stark, L.R. and C. Delgadillo. 2001. Rhizoautoicous *Aloina bifrons* in the Mojave Desert, a possible adaptation to increase spore production. Bryologist 104: 104–108.
Stark, L.R., D.N. McLetchie and S.M. Eppley. 2010. Sex ratios and the shy male hypothesis in the moss *Bryum argenteum* (Bryaceae). Bryologist 113: 788–797.
Stark, L.R., D.N. McLetchie and B.D. Mishler. 2001. Sex expression and sex dimorphism in sporophytic populations of the desert moss *Syntrichia caninervis*. Plant Ecology 157: 183–196.
Stark, L.R., D.N. McLetchie and B.D. Mishler. 2005. Sex expression, plant size, and spatial segregation of the sexes across a stress gradient in the desert moss *Syntrichia caninervis*. Bryologist 108: 183–193.
Stark, L.R., B.D. Mishler and D.N. McLetchie. 1998. Sex expression and growth rates in natural populations of the desert soil crustal moss *Syntrichia caninervis*. Journal of Arid Environments 40: 401–416.
Taylor, P.J., S.M. Eppley and L.K. Jesson. 2007. Sporophytic inbreeding depression in mosses occurs in a species with separate sexes but not in a species with combined sexes. Am. J. Bot. 94: 1853–1859.
Thiers, B.W. 1988. Morphological adaptations of the Jungermanniales (Hepaticae) to the Tropical Rainforest habitat. J. Hattori Bot. Lab. 64: 5–14.
Une, K. 1984. Sexual dimorphism in some Japanese species of *Macromitrium*. J. Hattori Bot. Lab. 56: 123–132.
Une, K. 1985. Sexual dimorphism in some Japanese of *Macromitrium* Brid. (Musci: Orthotrichaceae). J. Hattori Bot. Lab. 59: 487–513.
Une, K. 1986. Sexuality of the Japanese mosses. Hikobia 9: 339–344.
Une, K. and Y. Tateishi. 1996. Life cycle of *Physcomitrella patens* (Hedw.) B.S.G. subsp. *californica* (Crum & Anderson) Tan in Japan. Hikobia 12: 151–156.
Vanderpoorten, A. and B. Goffinet. 2009. Introduction to Bryology. Cambridge University Press, Cambridge.
Wiklund, K. and H. Rydin. 2004. Ecophysiological constraints on spore establishment in bryophytes. Func. Ecol. 18: 907–913.
Wyatt, R. 1982. Population ecology of bryophytes. J. Hattori Bot. Lab. 52: 179–198.
Wyatt, R. 1985. Terminology for bryophyte sexuality: toward a unified system. Taxon 34: 420–425.
Wyatt, R. 1994. Population genetics of bryophytes in relation to their reproductive biology. J. Hattory Bot. Lab. 76: 147–157.
Zander, R.H. 1984. Bryophyte sexual systems: -oicous versus -oecious. Bryol. Beitraege 3: 46–51.

4

The Cytological Studies of Oogenesis and Fertilization of Ferns

Cao Jian-Guo

ABSTRACT

The present paper reports some new advances on the sexual reproduction of ferns, including, mainly, the detailed processes of the formation of the archegonia, the egg development and fertilization. The archegonia of the ferns are derived from the archegonial initial cell under the growing point. The initial cell has dense cytoplasm and a large centrally placed nucleus. The initial cell gives rise to a tier of three cells, by two divisions, middle of which is the primary cell. The primary cell undergoes two unequal divisions, and forms a neck canal cell, a ventral cell and an egg cell. During maturation, the egg cell becomes progressively isolated from the adjacent cells by forming a separation cavity and an egg envelope. It is proved that the advanced ferns form a fertilization pore in the upper egg envelope. It is discovered that the ventral canal cell takes part in formation of the fertilization pore. The fertilization experiment indicated that the spermatozoid penetrate the egg through the fertilization pore. Immediate shrinkage of the egg at the moment of the sperm penetration and formation of a large sac blocking the fertilization pore are proposed to be used to prevent polyspermy.

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Subsequently, the fertilized egg undergoes nuclear fusion, digestion of the male organelles, rearrangement of the zygotic organelles and change of the cellular polarity of the zygote, rebuilding of the plasmalemma and the cell wall. Finally a functional zygote is formed.

Keywords: fern, oogenesis, fertilization, sexual reproduction

Introduction

Pteridophytes are spore-bearing vascular plants. They are considered to be transitional taxa between bryophytes and seed plants. Pteridophytes are usually divided into ferns (macrophyll pteridophytes) and fern allies (microphyll pteridophytes) according to the traditional viewpoint. Recent phylogenetic investigations revealed a basal dichotomy within vascular plants, separating the lycophytes from other vascular plants (the euphyllophytes) (Smith et al. 2006). Thus, the euphyllophytes comprise two clades: the spermatophytes (seed plants) and ferns (the monilophytes), which have about 9000 species, including horsetails, whisk ferns, and all eusporangiate and leptosporangiate ferns (Pryer et al. 2004). The ferns are divided into two categories according to the spore types: the homosporous ferns, which have homosporous spores, including all horsetails, whisk ferns, eusporangiate, and almost all the leptosporangiate ferns; and heterosporous ferns, which have large and small spores, and only include the Marsileaceae, Salviniaceae and Azollaceae. The ferns have independently-living gametophytes, also known as the prothallus. The gametophyte propagates by sexual reproduction, which includes spermatogenesis, oogenesis, fertilization and embryo development. The spermatogenesis has been studied in many aspects and with numerous species of ferns (Bell 1974, 1975; Bell et al. 1971; Bell and Duckett 1976; Doonan et al. 1986; Duckett 1973; Hoffman 1995; Myles and Hepler 1977; Kotenko 1990; Renzaglia and Garbary 2001). Spermatogenesis in ferns can usually be divided into two stages. The first stage is from the initial spermatogenous cell to the spermatocytes (spermatid mother cell); and the second stage is the differentiation of the spermatocytes. The differentiation of the spermatocytesis a complex process, which includes a multilayered structure (MLS) and microtubular ribbon (MTR) that are formed *de novo*; mitochondrial fusion and nuclear shaping. And finally a spiral spermatozoid is formed. However, the oogenesis and fertilization are less reported. The present paper reports our recent investigations on the oogenesis and fertilization of ferns.

Oogenesis

Formation of the archegonia and the egg cells

Archegonia of the ferns are usually produced on the lower surface just behind the apical notch of the gametophyte (Fig. 1A). So far, formation of the archegonia of many species, including *Ceratopteris thalictroides*, *Phymatosorus hainanensis*, *Adiantum flabellulatum* and *Pteridium aquilinum* var. *latiusculum* have been investigated (Yang et al. 2009; Cao et al. 2010a; Dai et al. 2010; Huang et al. 2011). These studies showed that the archegonia are derived from the initial cell under the lower surface just behind the apical notch of the gametophyte (Fig. 1B). The main features of the initial cell are dense cytoplasm and central placed nucleus in contrast to the somatic cells. The vacuoles in the initial cell are asymmetrically distributed. Large vacuoles are located in the lower part, but small vacuoles lie in the upper part of the cell (Cao et al. 2011). The chloroplasts in the initial cell, lacking well-developed lamellae and containing little starch, are usually smaller than those in the somatic cells. The initial cell forms three cells by two periclinal divisions (Fig. 1C). The upper cell becomes the neck jacket initial; the middle is the primary cell and the lower cell becomes the jacket cells in the future. The primary cell is a square with almost equivalent height and width. The nucleus is larger and the chromatin becomes more dispersed than those in the adjacent cells. Soon, the primary cell enlarges and its upper surface protrudes upwards (Fig. 1D). Before division of the primary cell, the neck initial cell divides into a rosette of four cells by two anticlinal divisions (Fig. 1D). The primary cell divides asymmetrically to form two cells. The

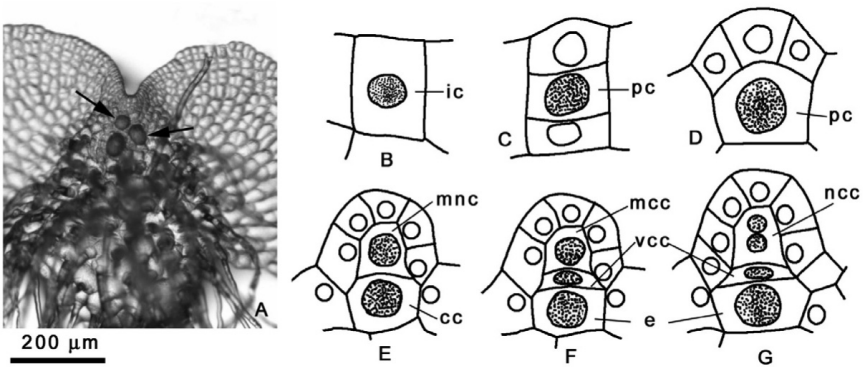

Figure 1. Gametophyte of *Pteridium aquilinum* var. *latiusculum* (A, arrow showing archegonia) and oogenesis (B-G)
B. initial cell; C, D. primary cell; E. central cell; F, G. young egg
cc, central cell; e, egg; ic, initial cell; mnc, mononucleate neck canal cell; ncc, neck canal cell; pc, primary cell; vcc, ventral canal cell.

cell towards the neck of the archegonium is a mononucleate neck canal cell (mnc) (Fig. 1E). The lower cell, obtaining more cytoplasm from its mother cell, is named as the central cell (cc) (Fig. 1E). The central cell also divides asymmetrically to form a small ventral canal cell (VCC) and a large egg cell, which possesses most of the cytoplasm (Fig. 1F). Soon after the egg is formed, the nucleus of the mononucleate neck canal cell divides into two, without cell wall formation between the two nuclei, which resulting in a binucleate neck canal cell (NCC) (Fig. 1G). Thus, an archegonium contains an axial row of three cells, i.e., the egg cell, the ventral canal cell, and the neck canal cell (Fig. 1G).

Development of the Egg Cell

Investigations of the egg development of the ferns are few in contrast to the spermatogenesis because of the simplicity of oogenesis. Oogenesis that has been investigated includes *Pteridium aquilinum* (Bell and Mühlethler 1962; Bell and Duckett 1976), *Histiopteris incisa* (Bell 1980), two species of *Osmunda* (Bao et al. 2003; Cao et al. 2012a), and *Dryopteris crassirhizoma* (Bao et al. 2005). These investigations revealed that the egg of advanced ferns is surrounded by a conspicuous extra egg membrane. This same structure has been regarded as a venter coat, covering the top of the egg, in the ferns *Athyrium filix-femina* (Fasciati et al. 1994) and *Ceratopteris richardii* (Lopez-Smith and Renzaglia 2008). The nuclear evaginations are formed during maturing of the egg development. Recently, we discovered a fertilization pore in the mature egg of the ferns *C. thalictroides*, *A. flabellulatum*, *Plagiogyria euphlebia*, and *P. aquilinum* var. *latiusculum* (Cao et al. 2009, 2010b, 2010c, 2011, 2012b). And the detailed development of oogenesis has been investigated with the mode species of *Ceratopteris thalictroides* and *Pteridium aquilinum* (Cao et al. 2010b, 2012b).

Young Egg Stage

The inner three cells are closely appressed to the wall of the archegonial jacket cells. There are well developed plasmodesmata between the egg and the VCC (Fig. 2A), but these are absent between the inner cells and the jacket cells. The nucleus of the new egg is spherical and contains two or more irregular nucleoli. Abundant vesicles, with a diameter of 0.5–1 μm, are distributed principally in the lateral side of the egg cytoplasm. Plastids, containing fewer starch grains and lamellae, lie closely to the nucleus. Mitochondria are distributed randomly throughout the cytoplasm of the egg. At this stage the organelles in the canal cells are similar to the egg.

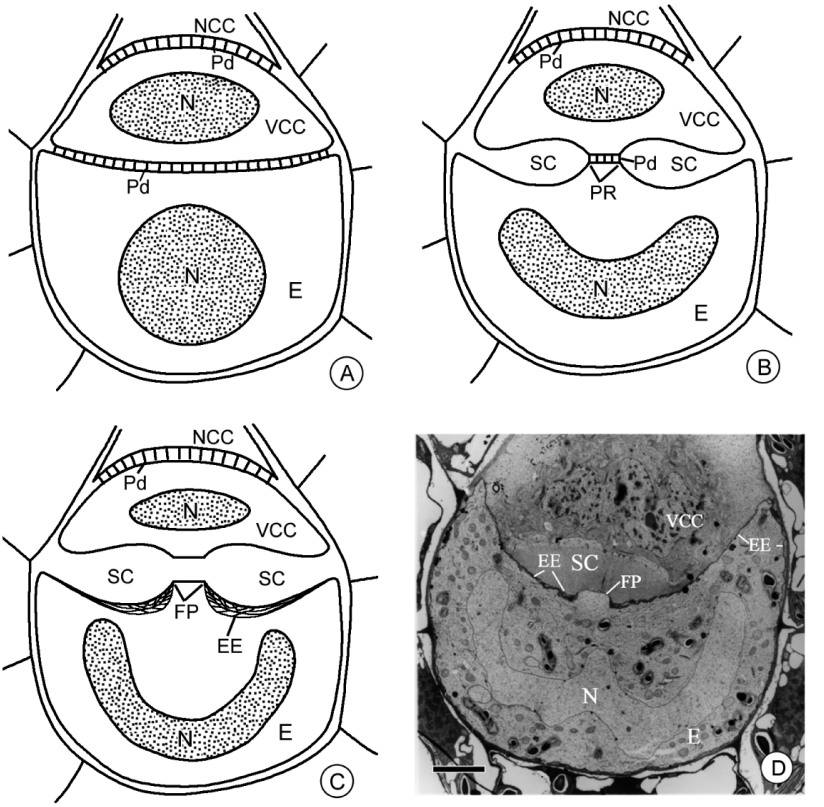

Figure 2. Schematic Diagram of the Egg Development (A-C) and Mature Egg (D) of *Ceratopteris thalictroides*
(A, Young egg; B, Middle stage of the egg; C, D, Matured egg)
E, egg; EE, egg envelope; FP, fertilization pore; N, nucleus; NCC, neck canal cell; Pd, plasmodesmata; PR, pore region; SC, separation cavity; VCC, ventral canal cell

Formation of the Separation Cavity

The first detectable change in oogenesis is the formation of a separation cavity, which forms initially around the periphery in the upper surface of the egg in *C. thalictroides*, *A. flabellulatum*, *P. euphlebia*, and *P. aquilinum* (Cao et al. 2010b, 2010c, 2011, 2012b). The plasmalemma of the egg is dissociated from the wall in the periphery. Simultaneously, the plasmodesmata disappear from the wall of the separation region. However, plasmodesmata still connect the egg and the VCC in the central region. Subsequently, the separation cavity expands centripetally and the connection region becomes correspondingly decreased. However, a pore region with a diameter

about 2–3 μm persistently connects the egg and the VCC. There are well developed plasmodesmata in the pore region (Fig. 2B). For the *P. euphlebia* and *P. aquilinum*, a temporary wall between the egg and the VCC becomes thickened except in the pore region during formation of the separation cavity. This wall always lies closely to the VCC (Cao et al. 2011, 2012b). The biological significance of the temporary cell wall may lie in the isolation of the egg cell and ensures the independent development of the sex cells (Cao et al. 2011). At this stage, the Golgi bodies in the egg cytoplasm increase in number, especially, in the upper side of the egg, which may take part in formation of the separation cavity.

Formation of the Egg Envelope and the Fertilization Pore

It is shown that an egg envelope is formed outside the mature egg in the advanced ferns, such as *P. aquilinum*, *H. incise*, *Dryopteris crassirhizoma*, *C. thalictroides*, *A. flabellulatum*, *P. euphlebia*, and *P. aquilinum* (Bell and Duckett 1976; Bell 1980; Cao et al. 2008, 2010c, 2011, 2012b). And the egg envelope on the upper surface of the egg is especially thick in contrast to the side and lower part of the egg. However, the primary fern Osmunda japonica has no typical egg envelope outside the mature egg (Cao et al. 2012a). In the *C. thalictroides* and *A. flabellulatum*, the egg envelope may be formed by the endoplasmic reticula, which are attached to the inner surface of the plasmalemma. And some lipid materials may take part in formation of the egg envelope (Cao et al. 2008, 2010c). But in *P. euphlebia* and *P. aquilinum*, formation of the egg envelope is accompanied by the decreasing of the amorphous materials in the separation cavity. So it is considered that the egg envelope is formed by amorphous materials depositing on the outer surface of the plasmalemma (Cao et al. 2011, 2012b). The thickness of the upper egg envelope increases from the periphery to the center and its maximum thickness reaches to about 0.5 μm.

During the formation of the egg envelope, the pore region still connects the egg cell and the VCC. It is striking that sheets of ER are not deposited on the inner surface of the pore region. Eventually, a fertilization pore is formed at the pore region (Fig. 2C, D). The only membrane covering the fertilization pore is plasmalemma. So far, the fertilization pore have been discovered in *C. thalictroides* (Cao et al. 2009), *A. flabellulatum* (Cao et al. 2010c), *P. euphlebia* (Cao et al. 2011), *P. aquilinum* (Cao et al. 2012b), *Coniogramme emeiensis* (Wang et al. 2012a), *Cibotium barometz* (Wang et al. 2012b). However, no fertilization pore was discovered in the primary fern *O. japonica* (Cao et al. 2012a). It possibly indicated that the fertilization pore is a derived structure in the advanced ferns, which may be in favor of preventing polyspermy.

Nuclear Behavior and Evagination

The nuclear behavior is noticeable. The rounded nucleus of the advanced ferns in the young egg becomes gradually cup-shaped and has a irregular surface (Cao et al. 2010b, 2010c, 2011, 2012b). The primitive fern *Osmunda* possesses a rounded nucleus (Cao et al. 2012a). Moreover, most advanced ferns produces obvious nuclear evaginations during the maturing of the egg, like that of the ferns *P. aquilinum* (Bell and Mülethaler 1962; Bell and Duckett 1976), *Dryopteris filix-mas* (Cave and Bell 1975), *Histiopteris incisa* (Bell 1980), *Dryopteris crassirhizoma* (Bao et al. 2005) and *Adiantum* (Cao et al. 2010c). In *Ceratopteris thalictroides*, the nucleus also becomes highly irregular, but it does not produce evaginations during oogenesis. It is suggested that the nuclear behavior and evaginations may have some significance in assessing the affiliation of the ferns. The advanced ferns are inclined to produce more complicated evaginations (Cao et al. 2010b, 2011, 2012b).

The Function of the Ventral Canal Cell in Oogenesis

The function of the VCC in oogenesis is less mentioned in previous reports. Our recent investigations of *C. thalictroides*, *A. flabellulatum*, and *P. euphlebia* showed that there is persistent connection of the egg and the VCC through the pore region, which suggests that the VCC plays an important role in oogenesis. The well-developed plasmodesmata in the pore region undoubtedly indicate informational and material communication between the egg cell and VCC, which consequentially influence the development of the egg envelope and the formation of the fertilization pore. It is suggested that the VCC can absorb materials from the egg through the plasmodesmata in *C. thalictroides* (Cao et al. 2010b). Or the VCC disturbs the activities of the endoplasmic reticula beneath the pore region, which leads to no formation of the egg envelop in *P. aquilimum* (Cao et al. 2012b). Finally a fertilization pore is developed from the connection region.

Fertilization and Zygote Development of the Fern

The fertilization and zygote development, including the approach of sperm to the egg, the fusion of gametes, and the prevention of polyspermy are poorly understood in ferns. The cytological processes, including the egg penetration, male nuclear decondensation, nuclear fusion, digestion of male organelles, rebuilding of plasmalemma and cell wall of zygote, are described in detail.

Fertilization and Preventing Polyspermy

The fertilization process occurs inside the archegonium of gametophytes. So we can not see how the sperm enter the egg directly. Through investigations of the fertilization of *C. thalictroides*, the detailed processes of fertilization and zygote development are descrived (Cao et al. 2010d). It is indicated that the fertilization pore is an entrance, through which the spermatozoid can penetrate the egg (Fig. 3A). After the spermatozoid enters the egg, the egg envelope is still intact, which indicated that the spermatozoid penetration is restricted exclusively to the fertilization pore. So far, such a fertilization experiment has not been reported in other ferns so far investigated.

How the egg prevent polyspermy is interesting. In our investigations on the fertilization of *C. thalictroides*, although several spermatozoids were observed in the cavity above the egg, only one of these is able to penetrate the egg. Penetration of the egg by more than one spermatozoid was not encountered in any of the specimens examined. The mechanism of preventing polyspermy may be attributable to the sac that containing numerous small vesicles, which are seen to block the fertilization pore persistently (Cao et al. 2009, 2010d). Perhaps, the shrinkage of the fertilized egg may also result in the pyknosis of the protoplasm, which may also contribute to prevent polyspermy.

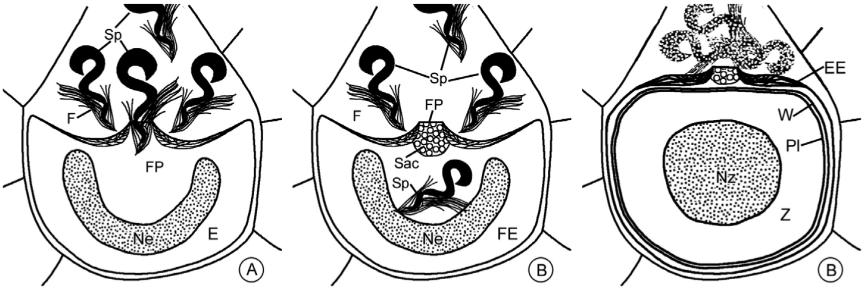

Figure 3. Schematic Diagram of Fertilization of *Ceratopteris thalictroides* (A, sperm penetration; B, preventing polyspermy; C, zygote rebuilding)
E, egg; EE, egg envelope; F, flagellum; FE, fertilized egg; FP, fertilization pore; Ne, nucleus of egg; Nz, nucleus of zygote; Pl, plasmalemma; Sp, sperm; W, wall; Z, zygote.

Features of the Fertilized Egg at Early Stage

As soon as the egg is fertilized, it shrinks markedly. The volume of the fertilized egg decreases to almost one-half that of the unfertilized egg. The protoplasm of the fertilized egg becomes dense and opaque. The organelles, particularly the mitochondria and endoplasmic reticula of the egg and most of the motile apparatus of the fertilizing spermatozoid, including the

flagella, microtubular ribbon, multilayered structure, and mitochondrion, are scarcely identifiable. Only the starch grain-containing plastids remain prominent in the egg cytoplasm. The egg envelope is still intact except in the region of the fertilization pore where the plasmalemma has been broken up after the spermatozoid enters the egg. At approximately 10–20 min after fertilization, the fertilized egg begins to increase in volume. The original obscure organelles in the cytoplasm become clear gradually.

Male Nuclear Decondensation and Nuclear Fusion

At approximately 20 min after fertilization, a conspicuous feature of the male nucleus is that the MTr is detaching itself from the male chromatin. Some times, the mitochondrion is observed lying between the MTr and the male chromatin. It seems that the mitochondrion is stripping off the MTr from the male chromatin (Cao et al. 2010d). Simultaneously, the male chromatin decondenses continuously and eventually transforms into a fibrous form. When the male nucleus becomes dissociated from the MTr, no envelope is observed at the surface of the male chromatin. Approximately 30 min after fertilization, an envelope reappears around the male chromatin. The electron-clear space can be observed in the male nucleus.

At approximately 45 min after fertilization, the gametic nuclei begin to fuse with each other. The manner of nuclear fusion of the ferns is interesting, because it involves a helical male nucleus fusing with a highly irregular egg nucleus. Bell (1975) suggested that the anterior end of the male nucleus first fuses with the female nucleus. Fasciati et al. (1994) considered that the anterior parts of the spermatozoid cytoskeleton served as a guiding structure inside which the male nucleus twists into the egg nucleus like a corkscrew. Lopez-Smith and Renzaglia (2008) indicated that the spermatozoid defines a circular space within which the sperm nucleus progressively fuses with and enters the egg nucleus. But in *C. thalictroides*, the nuclear fusion is possibly in a different manner. The ultrastructural observation suggests the female nucleus fuses actively with the male nucleus. The anterior end of the female nuclear protrusions seems to approach and wrap the male nucleus (Cao et al. 2010d). However, the nuclear behaviors during nuclear fusion need a further investigation.

Digestion of Male Organelles

The male organelles of the spermatozoid that entered the cytoplasm of the egg include microtubular ribbon, flagellum, male mitochondrion. It is generally considered that the male organelles are digested finally. Our investigations about *C. thalictroides* provide a detailed degeneration process of the male organelles (Cao et al. 2010d). The different organelles

are digested at different time of zygote development. The membranes outside the flagella and the MTr are digested shortly after fertilization. The microtubules are digested about 3–6 h after fertilization. Our observations show that the male mitochondria are digested between 6 and 9 h after fertilization. ERs and vacuoles possibly participate in the digestion of the male organelles. It is often observed that the male organelles are enveloped by ER in the early stages.

Rebuilding of a Functional Zygote

At approximately 9 h after fertilization the nucleus becomes to be round or elliptical. The male organelles cannot be detected in any forms. However, the rebuilding of the functional zygote needs still more than 24 hours (Cao et al. 2010d). During this time, the zygote undergoes complex cytological changes. Firstly, the organelles in the zygote undergoes rearrangement and polarity of the zygote is rebuilt. The zygote at the early stage of the zygote development showed a conspicuous vertical polarity. Vacuoles lie in the upper part of the zygote at early stage. However, the zygote changes its vertical polarity into a horizontal polarity by the rearrangement of the organelles. Vacuoles migrate to one lateral side of the zygote and the non-vacuole organelles move into the opposite side of the zygote. This may be important for the first division, which is always vertical to the gametophyte. The cell toward the notch becomes the stem polarity and the cell toward the posterior part of the gametophyte becomes the root polarity.

Secondly, functional zygote rebuilding depends on the formation of the plasmalemma. The egg cell loses its plasmalemma and cell wall when the egg envelope and separation cavity has been formed. However, a new plasmalemma is rebuilt inside the extra egg membrane before the zygotic division. It is possible that ERs participate in the formation of the plasmalemma, since ERs are frequently observed in the periphery of the zygote and paralleling to the surface of the egg. Terasaki and Jaffe (1991) also suggested that ER may be comparable to the plasmalemma in complexity of function. Soon after the formation of the plasmalemma, a layer of cell wall deposits between the newly-formed plasmalemma and the extra egg membrane. The rebuilding of plasmalemma and the cell wall undoubtedly represents the formation of the first functional cell of the sporophyte.

References

Bao, W.M., J.G. Cao and S.J. Dai. 2003. Ultrastructure of oogenesis in *Osmunda cinnamomea* var. *Asiatica*. Acta Botanica Sinica 45: 843–851.

Bao, W.M., Q. He, Q.X. Wang et al. 2005. Ultrastructure of Oogenesis in Dryopteris crassirhizoma Nakai. Journal of Integrative Plant Biology 47: 201–213.

Bell, P.R. 1974. The origin of the multilayered structure in the spermatozoid of *Pteidium aquilinum*. Cytobiologie 8(2): 203–212.
Bell, P.R. 1975. Observations on the male nucleus during fertilization in the fern *Pteridium aquilinum*. J. Cell Sci. 17: 141–153.
Bell, P.R. 1980. Nucleocytoplasmic interaction during maturation of the egg of the fern *Histiopteris incisa* (Thunb.). J. Smith. Annal of Botany 45: 475–481.
Bell, P.R. and J.G. Duckett. 1976. Gametogenesis and fertilization in *Pteridium*. Bot. J. Linn. Soc. 73: 47–78.
Bell, P.R., J.G. Duckett and D.G. Myles. 1971. The occurrence of a multilayered structure in the motile sperm of *Pteridium aquilinum*. J. Ultrastructre Res. 34: 181–189.
Bell, P.R. and K. Mühlethaler. 1962. The fine structure of the cells taking part in oogenesis in *Pteridium aquilinum* (L.) Kuhn. J. Ultrastruct. Res. 7: 452–466.
Cao, J.G., N.Y. Yang and Q.X. Wang. 2008. Observations on the Formation and Ultrastructure of the Egg Membrane in the fern *Ceratopteris thalictroides* (Parkeriaceae). Acta Botanica Yunnanica 30(5): 543–548.
Cao, J.G., N.Y. Yang and Q.X. Wang. 2009. Ultrastructure of the mature egg and fertilization in the fern *Ceratopteris thalictroides* (L.) Brongn. Journal of Integrative Plant Biology 51: 243–250.
Cao, J.G., W.J. Huang and Q.X. Wang. 2010a. Microstructural observation on the development of gametophytes and oogenesis in the fern *Adiantum flabellulatum* L. Act. Bot. Boreal Occident Sin 30: 0645–0651.
Cao, J.G., Q.X. Wang and W.M. Bao. 2010b. Formation of the fertilization pore during oogenesis of the fern *Ceratopteris thalictroides*. Journal of Integrative Plant Biology 52(6): 518–527.
Cao, J.G., Q.X. Wang, X.L. Dai and J.G. Duckett. 2010c. Ultrastructural observations of oogenesis in the fern *Adiantum flabellulatum* L. (Adiantaceae). American fern journal 100: 93–102.
Cao, J.G., Q.X. Wang, N.Y. Yang and W.M. Bao. 2010d. Cytological events during zygote formation of the fern *Ceratopteris thalictroides*. Journal of Integrative Plant Biology 52(3): 254–264.
Cao, J.G., X.L. Dai and Q.X. Wang. 2011. Archegonial development and oogenesis of the fern *Plagiogyria euphlebia* and their phylogenetic significance. American fern journal 101: 231–240.
Cao, J.G., X.F. Dai and Q.X. Wang. 2012a. Cytological features of oogenesis and their evolutionary significance in the fern *Osmunda japonica*. Sexual Plant Reproduction 25: **61–69.**
Cao, J.G., X.L. Dai and Q.X. Wang. 2012b. Ultrastructural and cytochemical studies on oogenesis of the fern *Pteridium aquilinum*. Sex. Plant Reprod. 25: 147–156.
Cave, C.F. and P.R. Bell. 1975. Evidence for the association of acyl transferases with the production of nuclear evaginations in maturing eggs of the fern *Dryopteris filix-mas*. Histochemistry 44: 57–65.
Dai, X.F., N. Jiang, J.G. Cao and Q.X. Wang. 2010. Primary study on the archegonium formationand oogenesis in the fern *Phymatosorus hainanensis*. Bulletin of Botanical Research 30(4): 411–415.
Doonan, J.H., C. Lloyd and J.G. Duckett. 1986. Anti-tubulin antibodies locate the blapharoplast during spermatogenesis in the fern *Platyzoma microphyllum* R.BR: A correlated immunofluorescence and electron-microscopic study. J. cell Sci. 81: 243–265.
Duckett, J.G. 1973. An ultrastructural study of the differentiation of the spermatozoid of *Equisetum*. J. Cell Sci. 12: 96–129.
Fasciati, R., J. Schneller, Jenni et al. 1994. Fertilization in the fern *Athyrium filix–femina* (Pterophyta): II. Ultrastructure. Cryptogamic Botany 4: 356–367.
Hoffman, J.C. and K.C. Vaughn. 1995. Post-translational tubulin modifications in spermatogeneous cells of the Pteridophyte *Ceratopteris richardii*. Protoplasma 186: 169–182.

Huang, W.J., J.G. Cao and Q.X. Wang. 2011. Microstructural Observations on the Development of Gametophytes and Oogenesis in the Fern *Pteridium aquilinum* var. *latiusculum*. Bulletin of Botanical Research 31(2): 159–163.

Kotenko, J.L. 1990. Spermatogenesis in a homosporous fern, *Onoclea sensibilis*. Am. J. Bot. 77: 809–825.

Lopez-Smith, R. and K. Renzaglia. 2008. Sperm cell architecture, insemination, and fertilization in the model fern, *Ceratopteris richardii*. Sexual Plant Reproduction 21: 153–167.

Myles, D.G. and P.K. Hepler. 1977. Spermiogenesis in the Fern *Marsilea*: Microtubules, Nuclear Shaping, and Cytomorphogenesis. J. Cell Sci. 23: 57–83.

Pryer, K.M., E. Schuettpelz, P.G. Wolf, H. Schneider, A.R. Smith and R. Cranfill. 2004. Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. Amer. J. Bot. 91: 1582–1598.

Renzaglia, K.S. and D.J. Garbary. 2001. Motile male gametes of land plants: diversity, development and evolution. Critical Reviews in Plant Sciences 20: 107–213.

Smith, A.R., K.M. Pryer, E. Schuettpelt et al. 2006. A classification for extant ferns. Taxon 55: 705–731.

Terasaki, M. and L.A. Jaffe. 1991. Organization of the Sea Urchin Egg Endoplasmic Reticulum and its Reorganization at Fertilization. Journal of Cell Science 114: 929–940.

Wang, G., H. Yang, Q.X. Wang and J.G. Cao. 2012a. Development of Gametophyte and Oogenesis in the Fern *Coniogramme emeiensis*. J. Act. Bot. Boreal Occident Sin 32(2): 0263–0269.

Wang, G., H. Yang, Q.X. Wang and J.G. Cao. 2012b. Development of eggs of the fern *Cibotium barometz*. Journal of Tropical organisms 3(4): 361–364.

Yang, N.Y., J.G. Cao and Q.X. Wang. 2009. Formation and Development of Archegonium in the Fern *Ceratopteris thalictroides* (L.) Brongn. Journal of Wuhan Botanical Research 27(6): 569–573.

5

Pollen-ovule Interactions in Gymnosperms

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ABSTRACT

After pollen is delivered to ovules via wind or insects, there are complex interactions between pollen and ovules prior to fertilization in seed plants. These interactions are integral in the functional reproductive biology of seed plants. Among extant gymnosperms there are diverse pollen delivery mechanisms that bring pollen into the ovule, where it germinates. Pollination mechanisms can be classified into types according to presence and absence of pollination drops, and by pollen grain and/or ovular modifications. Although most pollination mechanisms of gymnosperms can be grouped into four pollen capture mechanisms (PCMs) that describe pollen-ovule interactions, there are a few gymnosperms in which ovules neither capture the pollen directly, nor are they the site of germination. We classify these mechanisms as extra-ovular capture and germination (ECGs). In most gymnosperm genera, pollen grains germinate in pollination drops—liquid secretions containing minerals, carbohydrates and proteins, including active enzymes. Once germinated, pollen interacts primarily with the nucellus. The fossil record of seed plants suggests now extinct modes of pollen-

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ovule interactions. We provide a phylogenetic framework to show that pollination drops were probably a fundamental part of pollination in the earliest seed plants. To this we have added recent research to shed light on the evolutionary, developmental and biochemical aspects of pollen-ovule interactions. Pollination drop biochemistry offers a particularly rich area of exploration. Ongoing and future research in pollen ovule interactions are expected to provide new insights into this aspect of gymnosperm reproductive biology.

Keywords: gymnosperm, pollination drop, pollen germination, nucellus, proteins

Introduction

Pollen released from male cones of gymnosperms is delivered to ovules either by the wind (anemophily) or by insects (entomophily). From this moment until fertilization, pollen will have many interactions with the ovule. The capture of pollen, that will eventually produce sperm for fertilization, is described as a Pollination Mechanism. Pollination mechanisms are diverse among the extant lineages of both anemophilous and entomophilous gymnosperms. The variation in the structure of pollen and ovules are considered to be reproductive adaptations (Tomlinson et al. 1997; Doyle 2013). There is also a significant diversity in biochemical constituents of liquids associated with pollination capture, e.g., pollination drops (Fig. 1; Gelbart and von Aderkas 2002).

As drops withdraw, pollen is transported into the interior of the ovule. This retreat of the drop is either caused by evaporation, active retraction

Figure 1. Three ovules of *Ginkgo biloba* at time of pollination. Fully exuded drop at top, partially exuded drop at bottom, and ovule with no drop at left. Scale bar = 1 mm.

Color image of this figure appears in the color plate section at the end of the book.

of the drop, or by a combination of both active and passive processes (Mugnaini et al. 2007). Among modern gymnosperms, pollen grains inside the ovule germinate and produce outgrowths that penetrate the nucellus. In the case of Cycadales and *Ginkgo biloba* L., this outgrowth is haustorial in nature, but in all other modern gymnosperms (Gnetales and conifers *sensu lato*) the outgrowth is a more or less linear pollen tube, lacking or only having minimal haustorial side branches, which delivers non-motile sperm to the eggs. Pollen tubes, whether haustorial or linear, grow into the ovule nucellus. The time from pollination until fertilization shows substantial variability among different groups of modern gymnosperms that have been studied (Willson and Burley 1983; Williams 2012). The shortest time from pollination to fertilization among gymnosperms is found in *Ephedra* L., a gnetophyte; its pollen germinates, the pollen tube grows through the nucellus and releases male gametes, all within a day (El-Ghazaly 1998; Williams 2009). However, in most gymnosperms, this period is much longer. In conifers, pollination to fertilization is two weeks in *Picea* A. Dietr. (Runions and Owens 1999a,b), two months in *Pseudotsuga* Carrière (von Aderkas and Leary 1999a), and over a year in *Pinus* L. (Owens et al. 2005). These long periods of pollen growth within the nucellus require coordination of pollen and ovule development. Delivery of male gametes to briefly receptive eggs requires more complex physiological and molecular mechanisms than, for example, pollen capture.

Of the three stages of pollen-ovule interaction that we discuss in this chapter, some have received much more attention than others. For example, studies of first contact of pollen with ovules (pollen capture) are the most common type of study, as this stage is easier to study than the prolonged and complex interactions of microgametophytes growing within ovular tissues. The advantage to these numerous descriptive pollen capture studies is that they allow more comparisons and inferences to be made. In contrast, there are few experimental studies, e.g., wind tunnel experiments on ovule capture (Niklas 1982; Niklas and Kyaw 1982), or the physiological study of pollination drop withdrawal (Xing et al. 2000; Mugnaini et al. 2007; Leslie 2010; Jin et al. 2012). Interactions between pollen (including the microgametophyte), and nucellus are the least studied. It is not well-known whether there is an influence of the nucellus on pollen growth, or of the pollen on ovule development.

In this chapter, these various interactions will be outlined in three parts: i. pollination capture mechanisms, ii. germination, and iii. nucellus-pollen interactions. Where we have sufficient comparative evidence, e.g., pollination mechanisms, we will provide a guide to the evolutionary history of pollen-ovule interactions through deep-time, including both extant and extinct gymnosperms. We outline these evolutionary, developmental and

biochemical aspects of pollen-ovule interactions, with a view towards identifying hypotheses for future lines of research and providing new insights into this key group of seed plants.

Pollination Mechanisms

Pollen can enter the ovule in one of two ways: i. pollen is captured by the ovule, or ii. pollen germinates outside the ovule and produces a pollen tube that grows towards, and then into, the nucellus. The first we will call a *pollen capture mechanism (PCM)*, the second an *extra-ovular capture and germination (ECG)*. These syndromes can be broadly considered as pollination mechanism sub-types.

Pollen Capture Mechanisms (PCMs)—Extant Taxa

Of these two general classes of pollination mechanisms, PCMs are the most common type. They are found in the only species of *Ginkgo*, and all species of Gnetales and Cycadales. PCMs also are found in most of the Pinaceae and Podocarpaceae, though there are some notable exceptions that will be discussed later. There are four general types of PCMs: i. pollination drop capture and delivery with non-saccate pollen, ii. non-drop capture, i.e., physical entrapment of saccate pollen, followed by drop-mediated transport into the ovule, iii. non-drop capture of saccate pollen by inverted ovules with scavenging pollination fluid, and iv. non-drop capture of non-saccate pollen followed by physically-mediated pollen delivery into the ovule followed some weeks later by a drop.

The first PCM, pollination drop capture and delivery with non-saccate pollen, is the most widespread (Tomlinson 2012). A secreted drop appears at the entrance to the ovule. The drop persists, depending on species, for days or weeks. Wind- or pollinator-borne pollen is captured by the drop and sinks into the interior of these typically upright ovules (Xing et al. 2000). *Ginkgo*, as well as all extant Cycadales and Gnetales have this mechanism (Gelbart and von Aderkas 2002). Additionally, all members of some conifer families capture pollen in this way (Cupressaceae, Taxaceae, Sciadopityaceae, and Cephalotaxaceae).

The second PCM initially traps pollen on paired micropylar extensions. The pollen is modified with two hemispheric projections called sacci that promote buoyancy in pollination drops. Windborne pollen grains adhere to lipid microdrops on the micropylar extension surfaces. Soon afterwards, a pollination drop is secreted from the ovule that flows into the space between the flaps, filling it and liberating the pollen. The saccate pollen float upwards

into the downward-oriented ovule via the micropyle. This mechanism is found in some Pinaceae, e.g., most species of *Picea* (Doyle and Kane 1943) and *Pinus* (Owens et al. 1981), Cedrus Mill. (Takaso and Owens 1995), and Tsuga mertensiana (Bong.) Carrière (Owens and Blake 1983). Additionally, Abies Mill could be classified as having PCM ii, but no pollination drop has yet been observed for this genus (Singh and Owens 1982).

The third PCM is restricted to Podocarpaceae, in which there are genera that have both inverted ovules and saccate pollen (Tomlinson 1994; Tomlinson et al. 1991, 1997). Unlike most other pollination drops, these are involved in pollen scavenging. These ovules produce pollination drops that do not form a hemispheric drop because the entrance to the ovule as well as the nearby surrounding bract surfaces have little wax and are hydrophilic. As a consequence the drop spreads along the surfaces adjacent to the ovule. Saccate pollen captured by the liquid eventually float upwards into the ovule, or are drawn into the ovule as the drop retracts. Over a number of days, the pollination drop is repeatedly secreted and retracted, scavenging pollen grains from the surfaces adjacent to the ovule.

In the fourth PCM, pollen is trapped on hairs found on two flap-like integumentary extensions at the ovule's entrance. The hairs synchronously collapse inwards, pushing the pollen grains further into the micropyle (Barner and Christianson 1962). The pollen grain swells, and stays in this state for approximately two months (Takaso and Owens 1994). Pollen grains only form tubes after a drop is secreted (Said et al. 1991). Because of the substantial delay in secretion, this drop has been called the post-pollination prefertilization drop to differentiate it from a pollination drop (i.e., PCM i), although they both share functions such as transport of pollen and induction of pollen germination (von Aderkas and Leary 1999a,b). Like the second mechanism, this one is also restricted to the Pinaceae, in particular to *Pseudotsuga* and *Larix* Mill. (Doyle and O'Leary 1935a).

Extra-ovular Capture and Germination (ECG)—Extant Taxa

Unlike PCMs, extra-ovular capture and germination (ECG) is rare among extant gymnosperms. This type of pollination mechanism, found in several families, has no drop at any point in the process. In all cases, pollen tubes germinate outside the ovule, growing towards, and then into, the nucellus. This is in contrast to some conifer pollen, e.g., *Pinus,* which can germinate readily in high humidity conditions outside of ovules, but fails to fertilize the ovule because the pollen tubes are unable to find the ovule entrance. In the case of conifers with ECGs, pollen must find either the nucellus and/or the micropyle opening. The distance that tubes are able to grow and

still reproduce successfully, i.e., reach the nucellus, varies among species. In ECGs, how pollen tubes are attracted to the nucellus is not known; the expectation is that there is form of chemotaxis present.

The ovules of *Saxegothea* Benth. & Hook.f. (Podocarpaceae) have a wide micropyle with a protruding nucellar beak (Doyle and O'Leary 1935b; Tison 1908). Air-borne pollen that lands on the bract, the ovule exterior, or on the nucellus will germinate. This is similar to what is seen in Araucariaceae.

In *Agathis australis* (D. Don) Lindl. and in the genus *Araucaria* Juss. (Araucariaceae), pollen lands on the bract surface at a distance from the micropyle or on the ovule. It germinates and the tube grows along towards the micropyle until it reaches the nucellus, which, typical of species in the family, extends out of the micropyle (Owens et al. 1995).

Pollen of *Tsuga pattoniana* Engelm. (Pinaceae), captured on micropylar extensions of the integument will germinate and tubes grow into the micropyle, entering the ovule interior (Doyle and Kane 1943). *Tsuga heterophylla* Sarg. pollen behaves like that of *Araucaria*. Its spiny pollen grains are trapped in cuticle hairs on the bract surface, sometimes at substantial distances from an ovule entrance. Pollen germinates and the pollen tubes seek the micropylar entrance to the ovule (Doyle and O'Leary 1935a).

Fossils and Interpretation of PCM Evolution

The major lineages of extant seed plants, angiosperms, *Ginkgo*, Gnetales, Pinaceae, Cycadales, and non-pinaceous conifers represent only a small portion of the diversity of seed plant lineages that first arose in the late Devonian (Rothwell and Serbet 1994; Hilton and Bateman 2006; Doyle 2013). Thus, meaningful interpretations of origins and evolutionary changes in pollen-ovule interactions require the inclusion of extinct seed plants. A seminal paper by Doyle (1945), established various evolutionary models of how pollination mechanisms may have evolved over time, mainly using transformational series with key fossils as ancestors to illustrate a model for the origin of modern diversity. Recently, Tomlinson (2012) further developed these ideas, incorporating temporal aspects of seed plant reproductive syndromes established by Robert Brown (1844), including fossil and modern taxa. Pollination drops are generally accepted to be a fundamental aspect of the evolution of PCMs in gymnosperms, and pollination drops play key roles in pollen-ovule interactions. However, preservation of fossil pollination drops is rare (Rothwell 1977), and as a result, much of the evolutionary inferences regarding drops rely on a synthesis of phylogeny, modern biology, and over a century of paleobotanical research.

We used the phytochrome gene duplication rooting of seed plants (Mathews et al. 2010) as well as the sister-group relations of the 6 major

extant seed plant lineages based on various DNA sequences (Graham and Iles 2009) as a backbone constraint for the phylogenetic analysis of a morphological matrix of seed plants that includes fossils (Doyle 2008). We produced 10 trees of 348 steps, using the program TNT (Goloboff et al. 2000), arriving at the same 10 trees using both the ratchet as well as typical TBR heuristic searches. Mapping the presence of a pollination drop on the phylogeny using parsimony reconstruction (Maddison and Maddison 2010) suggests that the occurrence of pollination drops was probably fundamental to PCMs of the earliest seed plants, not just in crown-group seed plants/ modern lineages (Fig. 2).

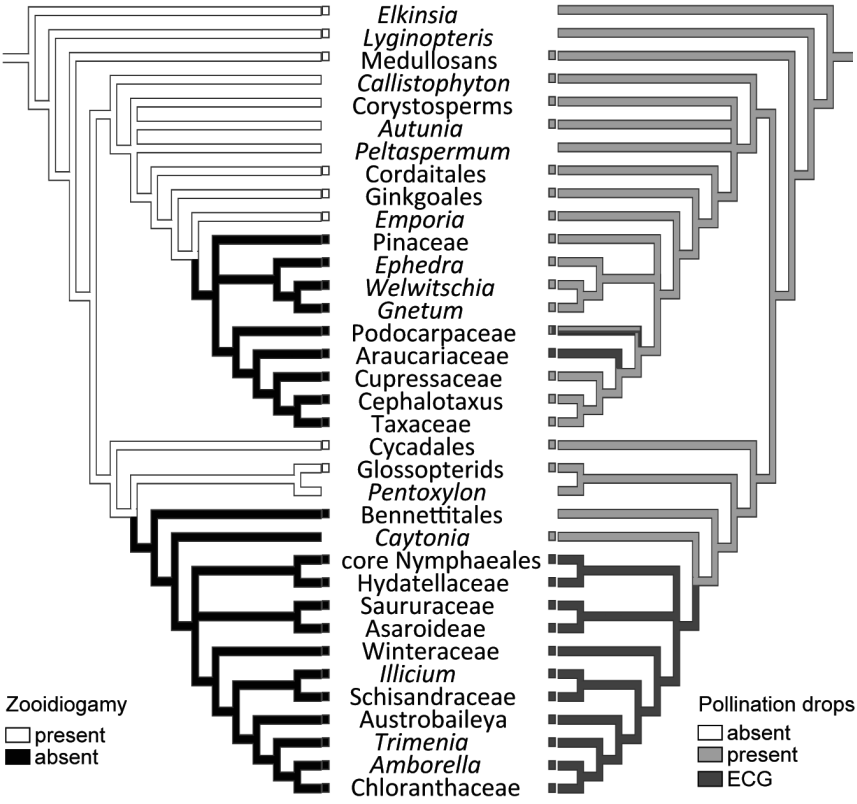

Figure 2. Mirror trees showing the parsimony based character mapping on the strict consensus of 10 trees; parsimony model for characters is unordered. Left, mapping of zoodiogamy presence and absence; fossil taxa with prepollen scored as present. Right, mapping of pollination drop absence, presence, or occurrence of ECG (extra-ovular capture and germination); fossil taxa with saccate pollen scored as present.

Color image of this figure appears in the color plate section at the end of the book.

Inference of ancestral pollination drops is further supported by using the presence of saccate pollen in fossil taxa as a proxy for the presence of pollination drops. Although virtually all modern PCMs with saccate pollen have a pollination drop, they all have an intervening non-drop based pollen capture mechanism (i.e., PCMs ii and iii). The evolution of saccate pollen is considered part of an evolutionary syndrome that would have included inverted ovules at the time of pollination (Doyle 1945; Runions et al. 1999; Leslie 2008). However, there are no known modern gymnosperms with a simple case of inverted ovules with exuded pollination drops that serve as both the pollen capture and transport mechanism (i.e., PCM i); this might be expected to have occurred in the evolution of PCMs that include saccate pollen. Making the assumption that there is a pollination drop present if a species has saccate pollen, has been part of evolutionary interpretations of PCMs since Doyle (1945). Thus, for the scoring of presence of pollination drops in fossils, we score each taxon as having a drop if it has saccate pollen grains.

Medullosans had large pollen grains that could not have been wind transported (Niklas 1983; Schwendemann 2007). The medullosan pollen organ *Parasporotheca* Dennis and Eggert (1978) was found with *Parasporites*-type pollen *in situ*. *Parasporotheca* pollen have saccus-like structures, suggesting that species of the genus may have had pollination drops and that *Parasporotheca*-bearing plants had evolved to having inverted ovules at, or around, the time of pollen capture. Thus we score Medullosans as having pollination drops, with the assumption that saccate pollen evolves in species with existing drops.

Glossopterids are scored as having pollination drops and having zoodiogamy. Species in this genus had saccate pollen (Ryberg et al. 2012a), supporting the scoring of droplet presence in the lineage. Nishida et al. (2003, 2004) observed flagellated sperm within a pollen chamber. Most of the Paleozoic gymnosperms associated with coniferophytes and conifer evolution have saccate pollen. Some species or all species may be saccate, these details are succinctly summarized by Doyle (2010).

Inferring ancestral PCMs without information from the fossil record is probably misleading since logically there would be several evolutionary steps between PCM i and any other pollen capture mechanism. For example, several PCMs are only found in Pinaceae and nowhere else, such as those with saccate pollen and non-drop primary pollen capture, a minimum of two trait changes from PCM i. The fossil record of Pinaceae begins in the Lower Cretaceous (131–129 million years ago; Ryberg et al. 2012b), relatively recent in comparison to the oldest known fossil gymnosperms ca. 300 million years (Clarke et al. 2011). Determination of PCMs from the paleobotanical record would aid in inference of how extinct seed plants shifted pollination

syndromes, but is rare. An additional complication of inferring PCM evolution in deep-time is that several pollination mechanisms that have been inferred from the fossil record have no modern analogue such as that of the Mesozoic cheirolepidiaceous conifer *Alvinia* (Kvaček 2000; Labandeira et al. 2007; Labandeira 2010), in Medullosales (Stewart 1951; Niklas1983), although many PCMs reconstructed from fossils appear to be more or less modern (e.g., the Paleozoic conifer *Otovicia*; Kerp et al. 1990).

Since the discovery of swimming sperm in *Cycas* L. and *Ginkgo* L. (Hirase 1896a,b; Ikeno 1896), the prevailing thinking is that free swimming sperm is a pleisiomorphic trait of seed plants. This is also logical since all free-sporing/seed-free land plant lineages have free swimming sperm. Thus, given that the earliest seed plant lineages had nonsaccate pollen, this suggests that some version of PCM i and zoodiogamy, may have been present in the earliest gymnosperms. If this is the case, then pollination drops could have performed the dual function of pollen capture and delivery into the ovule interior (i.e., PCM i), but may have also provided the medium for swimming sperm to reach and fertilize the egg (Fig. 2). This idea is not new to paleobotanical research (e.g., Brongniart 1881; Renault 1887; Stewart 1951), but is interesting in that such interpretations remain reasonable in light of modern phylogenetic hypotheses. Further support from the fossil record comes from rare observations of microgametophytes, such as two probable sperm in pollen enclosed in a medullosan pollen chamber (Stewart 1951), pre-germination microgametophytes in pollen of *Idanothekion*, Callistophytales (Millay and Eggert 1974), and direct observation of cycad-like top-shaped sperm in the pollen chamber of *Homevaleia gouldii* H., a Glossopterid (Nishida et al. 2003, 2004).

Hydrasperman reproduction, found in *Elkinsia* Rothwell related plants (Elkinsiales) and in Lyginopteridales probably had a variant of PCM i, but with possible zoodiogamy directly within pollination drops (Fig. 2). These groups, along with medullosans, and several extinct gymnosperms had pre-pollen, in which microspores have proximal germination. Proximal is in relation to the meiotic mark, the face that is toward the meiotic divisions (Rudall and Bateman 2007). For the purpose of character mapping, we score all fossil taxa with prepollen as zoodiogamous. All modern pollen has distal or non-proximal germination (Poort et al. 1996). Zoodiogamy from haustorial microgametophytes is found in modern Cycadales and *Ginkgo*, and their position in the seed plant phylogeny suggests that at least late stem seed-plants were also zoodiogamous (Fig. 2). Thus the ancestral seed plant PCM probably had no modern analogue. These early gymnosperms would have had non-saccate pollen, delivered by wind that was captured by pollination drops on erect ovules, and had pre-pollen which germinated in pollination drops that may have directly released free-swimming sperm.

Pollination Drops as a Medium for Germination

Pollination drops serve as the medium for pollen germination in most extant gymnosperms. Pollen germination can occur soon after pollination. In Cycadales, pollen germinates in the residual droplet contained in the pollination chamber (Choi and Friedman 1991). Anhaeusser (1953) was able to germinate pollen of *Taxus* in isolated pollination drops. In *Ephedra*, pollen germinates in the droplet within hours of pollen capture (Williams 2009, 2012; El-Ghazaly 1998). In other cases, pollination and germination are separated by a number of weeks, yet the drop still acts as the trigger for germination. In both *Pseudotsuga* and *Larix*, pollen is captured and brought into the ovule by stigmatic hairs. Only weeks later, when the post-pollination pre-fertilization drop is released does pollen germination occur (Said et al. 1991; Takaso and Owens 1996). *In vitro* studies of pollen germination support the idea that specific biochemical components are required for pollen germination (Brewbaker and Kwack 1963; Nygaard 1977; Dehoux and Pham Thi 1980). Many taxa have slower pollen tube growth rates *in vivo* versus *in vitro* (Williams 2012) which suggests that there are components in pollination drops that mediate/control germination. This leads one to the question, what is contained in pollination drops that promotes and/or moderates germination rates? A range of organic and mineral compounds has been identified in pollination drops of gymnosperms. Together these constituents create the biological environment in which pollen germinates.

Sugars are present in the drops of conifers (McWilliam 1958; Ziegler 1959), Cycadales (Tang 1987), *Gnetum* L. (Kato et al. 1995), *Welwitschia* Hook.f. (Carafa et al. 1992), and *Ephedra* (Ziegler 1959; Bino et al. 1984a,b). It is likely that sugars are present in *Ginkgo* drops as well. Glucose, fructose and sucrose were identified in a number of conifers (McWilliam 1958; Ziegler 1959; Seridi-Benkaddour and Chesnoy 1988) and Cycadales (Tang 1993). Other sugars, such as mannitol (Mugnaini et al. 2007), galactose (Carafa et al. 1992), xylose and melezitose (von Aderkas et al. 2012) have also been identified. Sugars are also present in some conifers as polymers containing arabinose, galactose, glucose, mannose, and rhamnose (Seridi-Benkaddour and Chesnoy 1988). Total sugar concentrations vary between groups. For conifers, total sugar concentrations between 1–2% have been found (McWilliam 1958). Other gymnosperms have higher concentrations: 10% for *Ephedra* (Ziegler 1959); 4–14% for cycads (Tang 1993); 3–15% for *Gnetum* (Kato et al. 1995; Nepi et al. 2009).

Drop sugars have potential roles in pollen germination. They could provide a source of energy for pollen. Monosaccharides are taken up and used by pollen during germination *in vitro* to support the growth of the pollen tube and the accumulation of polysaccharide storage molecules

(Nygaard 1977). Additionally, sugars could be involved in mechanisms of osmotic regulation, such as at higher concentrations to provide an osmotic environment that inhibits microbial growth. Total sugar concentration varies greatly between groups, and specific pollen types may have optimal osmotic conditions for germination, thus providing germination specificity in different osmotic environments, a probable adaptation. Sugar concentration and composition have been observed to be controlled by enzymes present in the drop, in some taxa. A functional invertase is present in *Pseudotsuga*, breaking down sucrose into glucose and fructose (von Aderkas et al. 2012) thus affecting proportions among these sugars.

Proteomic studies have revealed that pollination drops of conifers and *Welwitschia* contain a number of proteins. These include xylosidases, invertases, aspartyl proteases, peroxidases, serine-carboxypeptidases, and galactosidases (Poulis et al. 2005), thaumatin-like proteins (Wagner et al. 2007; O'Leary et al. 2007), and chitinases (Poulis 2004; Wagner et al. 2007). Additional pollination drop proteins found in cuppresaceous conifers include a glucanase-like protein, a glycosyl hydrolase, glucan 1,3-ß-glucosidases, a ß-D-glucan exohydrolase, subtilisin-like proteinases (Wagner et al. 2007). Several arabinogalactan proteins occur in *Taxu*s x *media* Rehder, discovered using immunohistology (O'Leary et al. 2004).

Drop proteins likely play an active role in pollen germination. Like sugars, their presence may alter the osmotic environment of the drop (Wagner et al. 2007). If broken down to free amino acids, they may also provide a source of nutrients for germinating pollen by supplying key components for protein synthesis within pollen tubes as they grow (Zhang et al. 1982). *In vitro*, externally supplied free amino acids have been observed to increase pollen tube growth and development (Dehoux and Pham Thi 1980). Proteases present in the drop are the expected driver of free amino acid concentrations (Poulis et al. 2005). Other active enzymes may also affect germination. Xylosidases and galactosidases could loosen the pollen cell wall by cleaving xyloglucans, a group of hemicelluloses that support the cellulose microfibrils of the cell wall (Poulis et al. 2005). This would help prime the pollen wall for tube emergence. Additional proteins, such as chitinases (Coulter et al. 2012) and thaumatin-like proteins may function to inhibit microbial growth (O'Leary et al. 2007).

Mineral components are also present in pollination drops and are known to affect pollen germination and growth. Calcium has been found in *Taxus baccata* L. (Fujii 1903), *Larix* and *Pseudotsuga* (von Aderkas et al. 2012). Application of calcium in pollen germination media was a key discovery for development of culture methods (Brewbaker and Kwack 1963). *In vitro*, calcium is required for pollen tube elongation in Norway spruce (Lazarro et al. 2005). Calcium sustains pollen viability and increases the percentage of pollen grains producing pollen tubes in *Pseudotsuga* (Fernando et al.

1997). Calcium-regulating proteins have been identified in pollen grains of *Pinus yunnanensis* Franch. (Gong et al. 1993) and *Cryptomeria japonica* D. Don (Yokota et al. 2004), suggesting an active role for calcium during pollen germination in conifers.

Pollination drops are complex mixtures of organic and mineral compounds in which germination takes place in many gymnosperms. There is evidence that pollination drops provide *genus*-specific conditions, unique enough to provide a form of pre-zygotic selection (von Aderkas et al. 2012), which includes pollen-nucellus interactions.

Pollen-Nucellus Interactions

Among modern gymnosperms, after germination of pollen within the drop, or without a drop as in ECGs, pollen tubes produced from the distal sulcus grow into the nucellus. In the siphonogamous gymnosperms (Gnetales and conifers), the tube acts as a conduit for delivering the sperm to the egg. In the zoodiogamous gymnosperms (Cycadales and *Gingko*), the tube functions mainly in the transfer of nutrients from the nucellus to the developing male gametophyte, and motile sperm are released from the swollen, proximal end of the pollen (Rudall and Bateman 2007). Among fossil seed-plants, pollen tubes are only known in a single case in Callistophytales (Rothwell 1972, 1981) and in *Williamsonia* Carruthers, Bennettitales (Stockey and Rothwell 2003). It is not known if prepollen (with proximal germination) produced pollen tubes.

Tube morphology is variable among lineages, perhaps as a consequence of variable function (Friedman 1993). In *Gingko* and Cycadales, tubes form branches, which facilitate the uptake of nutrients by increasing the surface area through which exchange occurs. In *Gingko*, tubes are highly branched and slender, and grow between the cells of the nucellus (intercellular growth; Friedman 1987, 1993). In contrast, the tubes of Cycadales are wide, and may be unbranched or form small side branches. Growth can be intercelluluar or tubes may penetrate the nucellus cells (intracellular growth). In either case, cells adjacent to the tube are degraded by tube growth and possibly by enzymes emitted by the tube; hydrolases and acid phosphatases have been found in the intine of several cycads (Pettit 1977, 1982). Degradation may increase the availability of nutrients for absorption (Pettit 1977, 1982; Choi and Friedman 1991), although mechanisms of absorption are unknown.

The proximal end of the pollen does not branch. Instead, it swells inside the pollination chamber just above the archegonia. The spermatogenous cell, which remains inside the proximal end of the pollen, divides to form the sperm. At maturity, the proximal end bursts, and releases the mobile gametes that swim through the fluid-filled pollination chamber to the

archegonia (Friedman 1987). Ultimately, one sperm nuclei will fuse with the egg nuclei after entry of the sperm into the archegonia via the neck cells.

In the extant, siphonogamous conifers and the Gnetales, the tube is usually unbranched, and grows intercellularly, more or less unbranched, and in a direct path through the nucellus towards the archegonia. *In vitro* studies suggest that a chemical signal from the female gametophyte may attract the pollen tube (Dumont-Beboux et al. 1998). Each tube penetrates an archegonium, bursts, and releases two sperm, one of which will fuse with the egg (von Aderkas et al. 2012). In *Gnetum* and *Welwitschia*, sperm are released into surface cells of the megagametophyte which seem to be able to function as eggs, even though the cells do not have obvious anatomical modifications seen in eggs and archegonia in other gymnosperm groups (Singh 1978; Fernando et al. 2010). As the tube grows, adjacent cells are often damaged, although less so than what is observed in Cycadales. Cell damage may result from the physical impact of the passing tube, or may be induced by enzymes released by the tube, in a manner similar to that proposed in Cycadales (Friedman 1993; Pettit 1985). It may also be a result of programmed cell death induced by the pollen tube (Hiratsuka et al. 2002). The tube grows more easily through space created by damaged cells, and may absorb nutrients released by cell break-down (Fernando et al. 2005a).

Pollen tube branching may occur in some conifers. In *Pinus contorta* Douglas many short branches form during the first year of cone development (Owens et al. 1981). In *Podocarpus*, the tube grows in a straight line, then branches near the female gametophyte, enlarging to form a disk-like structure over the neck cells; small side branches grow through the megaspore membrane and nucellus (Wilson and Owens 1999). In *Araucaria* and *Agathis*, tube branches form after penetration of the megagametophyte (Owens et al. 1995). Although nutrient absorption and tube branches may occur in some siphonogamous taxa, sperm transfer is viewed as the primary tube function. Sperm travel through the tube to reach the archegonia. In contrast, sperm do not enter the haustorial tube in *Ginkgo* or Cycadales (Rudall and Bateman 2007).

The ovule may influence the growth and development of the pollen, and pollen selection may occur before fertilization (Gelbart and von Aderkas 2002; Takaso and Owens 1994). In angiosperms, it is well-known that pollen selection occurs as the pollen grows through carpellary tissues to reach ovules. Self-incompatibility (SI) reactions are well understood (for review, Takayama and Isogai 2005), but selection mechanisms that act on pollen from foreign species and genera also exist (e.g., de Nettancourt 2001). In gymnosperms, selection was widely thought to occur mainly during embryo development; the over-expression of lethal alleles causes abortion of some embryos (Williams 2008).

In self-pollinated ovules, several studies suggest that low seed set may sometimes be linked to the failure of pollen tube growth in the nucellus, for example: *Larix* (Kosiński et al. 1986), *Abies* (Kormutak et al. 1999), *Picea* (Runions et al. 1998), and *Thuja* (Owens et al. 1990). However, statistical analyses did not support this finding in any of the studies, and several of the studies did not include data to support this claim. Other studies have looked for and not found evidence of self-incompatibility (Orr-Ewing 1956; Plym-Forshell 1974). Thus, further studies should explore self-incompatibility across gymnosperms before general conclusions can be drawn.

Pollen growth and development in some Pinaceae is dependent on whether the pollen is conspecific or heterospecific with respect to the ovule: *Pinus* (Stockwell 1939; Buchholz 1944; McWilliam 1959; Hagman 1975; Fernando et al. 2005b), *Picea* (Mikkola 1969), and *Pseudotsuga* and *Larix* (von Aderkas et al. 2012). Several of the studies had little or no data to support this observation, but others did have ample data that were supported by statistical analyses. McWilliam (1959) performed interspecific crosses using four species of *Pinus*, and found that growth of foreign pollen was halted in the nucellus or at the time of germination. In reciprocal crosses between *Larix* x *marschlinsii* Coax and *Pseudotsuga menziesii* (Mirb.) Franco, von Aderkas et al. (2012) found that foreign pollen germinated less frequently than conspecific pollen, and that growth of foreign pollen was interrupted at various subsequent stages of development. Fernando at al. (2005b) performed reciprocal crosses between *Pinus lambertiana* Douglas and *P. monticola* Douglas, and found that pollen tube growth aborted in the nucellus of one cross, whereas in the other, male and female gametes did not fuse. In contrast, one study examined the *in vitro* interaction between pollen tubes and female gametophytes of four different Pinaceae genera, and found that no barriers to the entry of foreign pollen tubes into the female gametophyte exist (Dumont-Beboux et al. 1998). Further studies that examine these phenomena in a wider range of taxa and at various stages of pollen development are needed.

Not only can the ovule affect the growth and development of pollen, but pollen can also affect ovule growth and development. In many extant taxa, ovule development is dependent on pollination and subsequent pollen tube growth. Requiring pollination for further ovule development has been observed in *Ginkgo biloba* (Nakao et al. 1998), *Pinus* (Owens et al. 2005), *Picea* (Dogra 1967; Owens and Blake 1984), *Thuja* (Owens et al. 1990), and *Juniperus* (Ortiz et al. 1998). Thus it is likely that there is a signal from developing pollen that is required for continued ovule development; if pollination and germination does not occur, the ovule will abort. In other taxa, ovule development continues whether or not pollination occurs, but instead abortion of ovules occurs if fertilization does not occur (e.g., *Pseudotsuga*, Owens et al. 1991).

The interdependency of pollen growth and development with that of the nucellus indicates that a strong feedback exists between the male and female reproductive tissues. Interactions between the tissues are probably mediated by the exchange of chemical signals, but no information regarding such mechanisms is known. In angiosperms a diversity of molecules, from proteins to ions, are known to be involved in pollen-ovule interactions. It is unclear what types of molecules may serve as signaling molecules in gymnosperms. The pollination drop contains a suite of proteins, some of which may influence pollen growth and development. These proteins originate from the nucellus, thus it is reasonable to assume that such proteins may also be active within the nucellus as well as the drop. Arabinogalactan proteins (AGPs) are known to function in pollen tube guidance in angiosperms (Cheung et al. 1996). AGPs are present in pollen tube walls of all gymnosperms, and also in the cell walls of the nucellus of some taxa (Fernando et al. 2010; O'Leary et al. 2004), and may be involved in signaling. Multiple proteins are released from pollen, which may also be involved in communication (Pettit 1982, 1985).

Current studies are exploring the molecular mechanisms that underlie pollen and ovule growth and development as well as the factors that affect these processes, but more work is needed. These studies, coupled with research that identifies molecules involved in pollen-ovule signaling will lead to a more complete understanding of pollen-ovule interactions in gymnosperms.

References

Anhaeusser, H. 1953. Keimung und Schlauchwachstum des Gymnosperm enpollensunterbesonderer Berücksichtigung des Wuchsstoff problems. Beotr. Biol. Pfl. 29: 297–338.

Barner, H. and H. Christianson. 1962. The formation of pollen, pollination mechanism, and the determination of the most favourable time for controlled pollination in Pseudotsuga menziesii. Silvae Genet. 11: 89–102.

Bino, R.J., A. Dafni and A.D.J. Meeuse. 1984a. Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forsk (=*E. alte* C.A. Mey), with some notes on *Ephedra campylopoda* C.A. Mey .1. Aspects of the entomophilous syndrome. P. K. Ned. Akad. C. Biol. 87: 1–13.

Bino, R.J., N. Devente and A.D.J. Meeuse. 1984b. Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forsk (=*E. alte* C.A. Mey), with some notes on *Ephedra campylopoda* C.A. Mey .2. pollination droplets, nectaries, and nectarial secretion in *Ephedra*. P. K. Ned. Akad. C. Biol. 87: 15–24.

Brewbaker, J.L. and B.H. Kwack. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Am. J. Bot. 50: 859–865.

Brongniart, A.T. 1881. Recherchessur les graines fossil essilicifiées Imprimerie Nationale, Paris.

Brown, R. 1844. On the plurality and development of the embryos in the seeds of Coniferae. Ann. Mag. Nat. Hist. May XLIV: 1–7.

Buchholz, J.T. 1944. The cause of sterility in cross-pollinations between certain species of pines. Am. J. Bot. 31: 2s.

Carafa, A.M., G. Carratu and P. Pizzolongo. 1992. Anatomical observations on the nucellar apex of *Welwitschia mirabilis* and the chemical composition of the micropylar drop. Sex. Plant Reprod. 5: 275–279.

Cheung, A.Y., X.Y. Zhan, H. Wang and H.M. Wu. 1996. Organ-specific and agamous-regulated expression and glycosylation of a pollen tube growth-promoting protein. PNAS 93: 3853–3858.

Choi, J.S. and W.E. Friedman. 1991. Development of the pollen tube of *Zamia furfuracea* (Zamiaceae) and its evolutionary implications. Am. J. Bot. 78: 544–560.

Clarke, J.T., R.C. Warnock and P.C. Donoghue. 2011. Establishing a time-scale for plant evolution. New Phytol. 192: 266–301.

Coulter, A., B.A.D. Poulis and P. vonAderkas. 2012. Pollination drops as dynamic apoplastic secretions. Flora 207: 482–490.

Dehoux, E. and A.T. Pham Thi. 1980. Influence de quelques acides aminés libres de l'ovulesur la croissanceet le développement cellulaire *in vitro* du tube pollinique chez *Juniperus communis* (Cupressaceae). Physiol. Plantarum 50: 6–10.

de Nettancourt, D. 2001. Incompatibility and incongruity in wild and cultivated plants. Springer, Berlin.

Dennis, R.L. and D.A. Eggert. 1978. *Parasporotheca*, *gen. nov.*, and its bearing on the interpretation of the morphology of permineralized medullosan pollen organs. Bot. Gaz. 139: 117–139.

Dogra, P. 1967. Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in Pinaceae. Stud. Forest Suec. 45: 8–97.

Doyle, J.A. 2008. Integrating molecular phylogenetic and paleobotanical evidence on origin of the flower. Int. J. Plant Sci. 169: 816–843.

Doyle, J.A. 2010. Function and evolution of saccate pollen. New Phytol. 188: 7–9.

Doyle, J.A., 2013. Phylogenetic analyses and morphological innovations in land plants. *In*: B.A. Ambrose and M.D. Purugganan (eds.). Annual Plant Reviews vol. 45 The Evolution of Plant Form. Wiley-Blackwell, Hoboken, pp. 1–50.

Doyle, J. 1945. Developmental lines in pollination mechanisms in the Coniferales. Sci. Prop. Roy. Dublin Soc. 24: 43–62.

Doyle, J. and A. Kane. 1943. Pollination in *Tsuga pattoniana* and in species of *Abies* and *Picea*. Sci. Proc. Roy. Dublin Soc. 23: 57–70.

Doyle, J. and M. O'Leary. 1935a. Pollination in *Tsuga*, *Cedrus*, *Pseudotsuga*, and *Larix*. Sci. Proc. Roy. Dublin Soc. 21: 191–204.

Doyle, J. and M. O'Leary. 1935b. Pollination in *Saxegothaea*. Sci. Proc. Roy. Dublin Soc. 21: 175–179.

Dumont-BeBoux, N., M. Weber, Y. Ma and P. von Aderkas. 1998. Intergeneric pollen-megagametophyte relationships of conifers *in vitro*. Theor. Appl. Genet. 97: 881–887.

El-Ghazaly, G., J. Rowley and M. Hesse. 1998. Polarity, aperture condition and germination in pollen grains of Ephedra (Gnetales). Plant Systemat. Evol. 213: 217–231.

Fernando, D.D., J.N. Owens, P. von Aderkas and T. Takaso. 1997. *In vitro* pollen tube growth and penetration of female gametophyte in Douglas fir (*Pseudotsuga menziesii*). Sex. Plant. Reprod. 10: 209–216.

Fernando, D.D., M.D. Lazzaro and J.N. Owens. 2005a. Growth and development of conifer pollen tubes. Sex. Plant Reprod. 18: 149–162.

Fernando, D.D., S.M. Long and R.A. Sniezko. 2005b. Sexual reproduction and crossing barriers in white pines: the case between *Pinus lambertiana* (sugar pine) and *P. monticola* (western white pine). Tree Genet. Genomes 1: 43–150

Fernando, D.D., C.R. Quinn, E.D. Brenner and J.N. Owens. 2010. Male gametophyte development and evolution in extant gymnosperms. Int. J. Plant Repro. Biol. 4: 47–63.

Friedman, W.E. 1987. Growth and development of the male gametophyte of *Ginkgo biloba* within the ovule (*in vivo*). Am. J. Bot. 74: 1797–1815.

Friedman, W.E. 1993. The evolutionary history of the seed plant male gametophyte. Trends Ecol. Evol. 8: 15–21.

Fujii, K. 1903. Über die Bestäubungstropfen der Gymnospermen, Ber. Dtsch. Bot. Ges. 21: 211–217.
Gelbart, G. and P. von Aderkas. 2002. Ovular secretions as part of pollination mechanisms in conifers. Ann. Forest Sci. 59: 345–357.
Goloboff, P., S. Farris and K. Nixon. 2000. TNT (Tree analysis using New Technology) ver. 1.1 Published by the authors, Tucumán, Argentina.
Gong, M., Z.H. Yang and T.H. Tsao. 1993. Isolation and characterization of calmodulin and a novel calcium-binding protein calpollenin from *Pinus yunnanensis* pollen. Plant Sci. 89: 5–12.
Graham, S.W. and W.J. Iles. 2009. Different gymnosperm outgroups have (mostly) congruent signal regarding the root of flowering plant phylogeny. Am. J. Bot. 96: 216–227.
Hagman, M. 1975. Incompatibility in forest trees. Phil. Trans. Roy. Soc. Lond. B Biol. Sci. 188: 319–332.
Hilton, J. and R.M. Bateman. 2006. Pteridosperms are the backbone of seed-plant phylogeny. J. Torrey Bot. Soc. 133: 119–168.
Hirase, S. 1896a. Spermatozoid of *Ginkgo biloba*. (In Japanese) Bot. Mag. Tokyo 10: 171.
Hirase, S. 1896b. On the spermatozoid of *Ginkgo*. (In Japanese) Bot. Mag. Tokyo 10: 325–328.
Hiratsuka, R., Y. Yamada and O. Terasaka. 2002. Programmed cell death of *Pinus* nucellus in response to pollen tube penetration. J. Plant Res. 115: 141–148.
Ikeno, S. 1896. Spermatozoiden von *Cycas revoluta*. (In Japanese) Bot. Mag. Tokyo 10: 367–368.
Jin, B., L. Zhang, Y. Lu, D. Wang, X. Jiang, M. Zhang and L. Wang. 2012. The mechanism of pollination drop withdrawal in *Ginkgo biloba* L. BMC Plant Biol. 12: 59.
Kato, M., T. Inoue and T. Nagamitsu. 1995. Pollination biology of *Gnetum* (Gnetaceae) in a lowland mixed dipterocarp forest in Sarawak. Am. J. Bot. 82: 862–868.
Kerp, J.H.F., R.J. Poort, H.A.J.M. Swinkels and R. Verwer. 1990. Aspects of Permian paleobotany and palynology. 9. Conifer-Dominated Rotliegend Floras from the Saar-Nahe Basin (Late Carboniferous Early Permian, Sw Germany) with Special Reference to the Reproductive-Biology of Early Conifers. Rev. Palaeobot. Palyno. 62: 205–248.
Kormutak, A. 1999. Self-incompatibility in silver fir (*Abies alba*). Biologia 54: 101–105.
Kosiński, G. 1986. Megagametogenesis, fertilization, and embryo development in *Larix decidua*. Can. J. Forest Res. 16: 1301–1309.
Kvaček, J. 2000. *Frenelopsis alata* and its microsporangiate and ovuliferous reproductive structures from the Cenomanian of Bohemia (Czech Republic, Central Europe). Rev. Palaeobot. Palyno. 112: 51–78.
Labandeira, C.C. 2010. The pollination of mid Mezozoic seed plants and the early history of long-proboscid insects. Ann. Mo. Bot. Gard. 97: 469–513.
Labandeira, C.C., J. Kvaček and M.B. Mostovski. 2007. Pollination drops, pollen, and insect pollination of Mesozoic gymnosperms. Taxon 56: 663–695.
Lazzaro, M.D., L. Cardenas, A.P. Bhatt, C.D. Justus, M.S. Phillips, T.L. Holdaway-Clarke and P.K. Hepler. 2005. Calcium gradients in conifer pollen tubes; dynamic properties differ from those seen in angiosperms. J. Exp. Bot. 56: 2619–2628.
Leslie, A.B. 2008. Interpreting the function of saccate pollen in ancient conifers and other seed plants. Int. J. Plant Sci. 169: 1038–1045.
Leslie, A.B. 2010. Flotation preferentially selects saccate pollen during conifer pollination. New Phytol. 188: 273–279.
Maddison, W.P. and D.R. Maddison. 2010. Mesquite: A modular system for evolutionary analysis, version 2.74. Website http://mesquiteproject.org [accessed April 2012].
Mathews, S., M.D. Clements and M.A. Beilstein. 2010. A duplicate gene rooting of seed plants and the phylogenetic position of flowering plants. Phil. Trans. Roy. Soc. Lond. B Biol. Sci. 365: 383–395.
McWilliam, J.R. 1958. The role of the micropyle in the pollination of *Pinus*. Bot. Gaz. 120: 109–117.
McWilliam, J.R. 1959. Interspecific incompatibility in *Pinus*. Am. J. Bot. 46: 425–433.

Mikkola, L. 1969. Observations on interspecific sterility in *Picea*. Ann. Bot. Fenn. 6: 285–339.
Millay, M.A. and D.A. Eggert. 1974. Microgametophyte development in the Paleozoic seed fern family Callistophytaceae. Am. J. Bot. 61: 1067–1075.
Mugnaini, S., M. Nepi, M. Guarnieri, B. Piotto and E. Pacini. 2007. Pollination drop in *Juniperus communis:* Response to deposited material. Ann. Bot. 100: 1475–1481.
Nakao, Y., A. Tateishi, K. Kawase, T. Ogata, S. Shiozaki and S. Horiuchi. 1998. Seed set of *Ginkgo biloba* L. as related to pollination and its optimum pollination time. J. Jpn. Soc. Hortic. Sci. 67: 753–758.
Nepi, M., P. von Aderkas, R. Wagner, S. Mugnaini, A. Coulter and E. Pacini. 2009. Nectar and pollination drops: how different are they? Ann. Bot. 104: 205–219.
Niklas, K.J. 1982. Simulated and empiric wind pollination patterns of conifer ovulate cones. PNAS 79: 510–514.
Niklas, K.J. 1983. The influence of Paleozoic ovule and cupule morphologies on wind pollination. Evolution 37: 968–986.
Niklas, K.J. and T.P.U. Kyaw. 1982. Pollination and air-flow patterns around conifer ovulate cones. Science 217: 442–444.
Nishida, H., K.B. Pigg and J.F. Rigby. 2003. Swimming sperm in an extinct Gondwanan plant. Nature 422: 396–397.
Nishida, H., K.B. Pigg, K. Kudo and J.F. Rigby. 2004. Zooidogamy in the Late Permian genus *Glossopteris*. J. Plant Res. 117: 323–328.
Nygaard, P. 1977. Utilization of exogenous carbohydrates for tube growth and starch sythesis in pine pollen suspension cultures. Physiol. Plantarum 39: 206–210.
O'Leary, S.J.B., C. Joseph and P. von Aderkas. 2004. Origin of arabinogalactan proteins in the pollination drop of *Taxus x media*. Aust. J. For. Sci. 121: 35–46.
O'Leary, S.J.B., B.A.D. Poulis and P. von Aderkas. 2007. The identification of two thaumatin-like proteins (TLPs) in the pollination drop of hybrid yew that may play a role in pathogen defence during pollen collection. Tree Physiology 27: 1649–1659.
Orr-Ewing, A. 1956. An investigation into the effects of self pollination on *Pseudotsuga menziesii*. Ph.D. thesis. University of British Columbia, British columbia, Canada.
Ortiz, P.L., M. Arista and S. Talavera. 1998. Low reproductive success in two subspecies of *Juniperus oxycedrus* L. Int. J. Plant Sci. 159: 843–847.
Owens, J.N. and M.D. Blake. 1983. Pollen morphology and development of the pollination mechanism in *Tsuga heterophylla* and *Tsuga mertensiana*. Can. J. Bot. 61: 3041–3048.
Owens, J.N. and M.D. Blake. 1984. The pollination mechanism of Sitka spruce (*Picea sitchensis*). Can. J. Forest Res. 62: 1136–1148.
Owens, J.N., S.J. Simpson and M. Molder. 1981. Sexual reproduction of *Pinus contorta*. 1. pollen development, the pollination mechanism, and early ovule development. Can. J. Forest Res. 59: 1828–1843.
Owens, J.N., A.M. Colangeli and S.J. Morris. 1990. The effect of self-, cross- and no pollination on ovule, embryo, seed, and cone development in western red-cedar (*Thuja plicata*). Can. J. Forest Res. 20: 66–75.
Owens, J.N., A.M. Colangeli and S.J. Morris. 1991. Factors affecting seed set in Douglas-fir (*Pseudotsuga menziesii*). Can. J. Bot. 69: 229–238.
Owens, J.N., G.L. Catalano, S.J. Morris and J. Aitken-Christie. 1995. The reproductive biology of Kauri (*Agathis australis*). I. pollination and prefertilization development. Int. J. Plant Sci. 156: 257–269.
Owens, J.N., J. Bennett and S. L'Hirondelle. 2005. Pollination and cone morphology affect cone and seed production in lodgepole pine seed orchards. Can. J. Forest Res. 35: 383–400.
Pettit, J.M. 1977. Detection in primitive gymnosperms of proteins and glycoproteins of possible significance in reproduction. Nature 266: 530–532.
Pettit, J.M. 1982. Ultrastructural and immuno-cytochemical demonstration of gametophytic proteins in the pollen-tube wall of the primitive gymnosperm *Cycas*. J. Cell Sci. 57: 189–213.

Pettit, J.M. 1985. Pollen-tube development and characteristics of the protein emission in Conifers. Ann. Bot. 56: 379–397.

Plym-Forshell, C. 1974. Seed development after self pollination and cross pollination of Scots pine *Pinus sylvestris*. Stud. Forest Suec. 118: 1–37.

Poort, R.J., H. Visscher and D.L. Dilcher. 1996. Zoidogamy in fossil gymnosperms: The centenary of a concept, with special reference to prepollen of late Paleozoic conifers. PNAS 93: 11713–11717.

Poulis, B.A.D. 2004. Safe sex in Douglas-fir. Ph.D. Thesis. University of Victoria, Canada.

Poulis, B.A.D., S.J.B. O'Leary, J.D. Haddow and P. von Aderkas. 2005. Identification of proteins present in the Douglas-fir ovular secretion: an insight into conifer pollen selection and development. Int. J. Plant Sci. 166: 733–739.

Renault, B. 1887. Note sur le genre *Aetheotesta*. Mem. Soc. Hist. Nat. Saône-et-Loire.

Rothwell, G.W. 1972. Evidence of pollen tubes in Paleozoic pteridosperms. Science 175: 772–774.

Rothwell, G.W. 1977. Evidence for A pollination-drop mechanism in Paleozoic pteridosperms. Science 198: 1251–1252.

Rothwell, G.W. 1981. The Callistophytales (Pteridospermopsida)—reproductively sophisticated Paleozoic gymnosperms. Rev. Palaeobot. Palyno. 32: 103–121.

Rothwell, G.W. and R. Serbet. 1994. Lignophyte phylogeny and the evolution of spermatophytes —a numerical cladistics analysis. Syst. Bot. 19: 443–482.

Rudall, P.J. and R.M. Bateman. 2007. Developmental bases for key innovations in the seed-plant microgametophyte. Trends Plant Sci. 12: 317–326.

Runions, C.J. and J.N. Owens. 1998. Evidence of pre-zygotic self-incompatibility in a conifer. *In*: S.J. Owens and P.J. Rudall (eds.). Reproductive Biology. Royal Botanic Gardens, Kew, pp. 255–264.

Runions, C.J. and J.N. Owens. 1999a. Sexual reproduction of Interior spruce (Pinaceae). I. pollen germination to archegonial maturation. Int. J. Plant Sci. 160: 631–640.

Runions, C.J. and J.N. Owens. 1999b. Sexual reproduction of Interior spruce (Pinaceae). II. fertilization to early embryo formation. Int. J. Plant Sci. 160: 641–652.

Runions, C.J., K.H. Rensing, T. Takaso and J.N. Owens. 1999. Pollination of *Picea orientalis* (Pinaceae): saccus morphology governs pollen buoyancy. Am. J. Bot. 86: 190–197.

Ryberg, P.E., G.W. Rothwell, R.A. Stockey, H. Jason, G. Mapes and J.B. Riding. 2012a. Reconsidering relationships among stem and crown group Pinaceae: oldest record of the genus *Pinus* from the Early Cretaceous of Yorkshire, United Kingdom. Int. J. Plant Sci. 173: 917–932.

Ryberg, P.E., E.L. Taylor and T.N. Taylor. 2012b. The first permineralized microsporophyll of the Glossopteridales: *Eretmonia macloughlinii* sp. nov. Int. J. Plant Sci. 173: 812–822.

Said, C., M. Villar and P. Zandonella. 1991. Ovule receptivity and pollen viability in Japanese larch (*Larix leptolepis* Gord.). Silvae Genetica 40: 1–6.

Schwendemann, A.B., G. Wang, M.L. Mertz, R.T. McWilliams, S.L. Thatcher and J.M. Osborn. 2007. Aerodynamics of saccate pollen and its implications for wind pollination. Am. J. Bot. 94: 1371–1381.

Seridi-Benkaddour, R. and L. Chesnoy. 1988. Secretion and composition of the pollination drop of *Cephalotaxus drupacea* (gymnosperm, Cephalotaxaceae). *In*: M. Cresti, P. Gori and E. Pacini (eds.). Sexual reproduction in higher plants. Springer Verlag, Berlin, pp. 345–350.

Singh, H. 1978. Embryology of gymnosperms. Spezieller Teil, Bd. 10 T.2 Gerbrüder Borntraeger, Berlin.

Singh, H. and J.N. Owens. 1982. Sexual reproduction in grand fir (*Abies grandis*). Can. J. Bot. 60: 2197–2214

Stewart, W.N. 1951. A New *Pachytesta* from the Berryville Locality of Southeastern Illinois. Am. Midl. Nat. 46: 717–742.

Stockey, R.A. and G.W. Rothwell. 2003. Anatomically preserved *Williamsonia* (Williamsoniaceae): evidence for bennettitalean reproduction in the Late Cretaceous of Western North America. Int. J. Plant Sci. 164: 251–262.

Stockwell, W. 1939. Preembryonic selection in the pines. J. Forest 37: 541–543.

Takaso, T. and J.N. Owens. 1994. Effects of ovular secretions on pollen in *Pseudotsuga menziesii* (Pinaceae). Am. J. Bot. 81: 504–513.

Takaso, T. and J.N. Owens. 1995. Pollination drop and microdrop secretions in *Cedrus.* Int. J. Plant Sci. 156: 640–649.

Takaso, T. and J.N. Owens. 1996. Postpollination-prezygotic ovular secretions into the micropylar canal in *Pseudotsuga menziesii.* J. Plant Res. 109: 147–180.

Takayama, S. and A. Isogai. 2005. Self-incompatibility in plants. Ann. Rev. Plant Biol. 56: 467–489.

Tang, W. 1987. Insect pollination in the cycad *Zamia pumila* (Zamiaceae). Am. J. Bot. 74: 90–99.

Tang, W. 1993. Nectar-like secretions in female cones of cycads. Cycad Newsletter 16: 10–13.

Tison, A. 1908. Le nucelle stigmatifère et al pollinisation chez le *Saxe-Gothea conspicua.* Compt. Rend. Sce. L'Acad. Science (Paris) 147: 137–139.

Tomlinson, P.B. 1994. Functional morphology of saccate pollen in conifers with special reference to Podocarpaceae. Int. J. Plant Sci. 155: 699–715.

Tomlinson, P.B. 2012. Rescuing Robert Brown—the origins of angio-ovuly in seed cones of conifers. Bot. Rev. 78: 310–334.

Tomlinson, P.B., J.E. Braggins and J.A. Rattenbury. 1991. Pollination drop in relation to cone morphology in Podocarpaceae: a novel reproductive mechanism. Am. J. Bot. 78: 1289–1303.

Tomlinson, P.B., J.E. Braggins and J.A. Rattenbury. 1997. Contrasted pollen captures mechanisms in Phyllocladaceae and certain Podocarpaceae (Coniferales). Am. J. Bot. 84: 214–223.

von Aderkas, P. and C. Leary. 1999a. Micropylar exudates in Douglas fir—timing and volume of production. Sex. Plant Reprod. 11: 354–356.

von Aderkas, P. and C. Leary. 1999b. Ovular secretions in the micropylar canal of larches (*Larix kaempferi* and *L.* x *eurolepis*). Can. J. Bot. 77: 531–536.

von Aderkas, P., M. Nepi, M. Rise, F. Buffi, M. Guarnieri, A. Coulter, K. Gill, P. Lan, S. Rzemieniak and E. Pacini. 2012. Post-pollination prefertilization drops affect germination rates of heterospecific pollen in Larch and Douglas-fir. Sex. Plant Reprod. 25: 215–225.

Wagner, R.E., S. Mugnaini, R. Sniezko, D. Hardie, B. Poulis, M. Nepi, E. Pacini and P. von Aderkas. 2007. Proteomic evaluation of gymnosperm pollination drop proteins indicates highly conserved and complex biological functions. Sex. Plant Reprod. 20: 181–189.

Williams, C.G. 2008. Selfed embryo death in *Pinus taeda*: a phenotypic profile. New Phytol. 178: 210–222.

Williams, J.H. 2009. *Amborella trichopoda* (Amborellaceae) and the evolutionary developmental origins of the angiosperm progamic phase. Am. J. Bot. 96: 144–65.

Williams, J.H. 2012. Pollen tube growth rates and the diversification of flowering plant reproductive cycles. Int. J. Plant Sci. 173: 649–661.

Willson, M.F. and N. Burley. 1983. Monographs in population biology Vol. 19. Mate choice in plants tactics mechanisms and consequences. Princeton University Press, Princeton, New Jersey.

Wilson, V.R. and J.N. Owens. 1999. The reproductive biology of Totara (*Podocarpus totara*) (Podocarpaceae). Ann. Bot. 83: 401–411.

Xing, S.P., Z.K. Chen, Y.X. Hu, F. Zhou and J.X. Lin. 2000. Ovule development, formation of pollination drop and pollination process in *Taxus chinensis* (Taxaceae). Acta Bot. Sin 42: 126–132.

Yokota, E., T. Ohmori and S. Muto. 2004. 21-kDa polypeptide, a low-molecular-weight cyclophilin, is released from pollen of higher plants into the extracellular medium *in vitro*. Planta 218: 1008–1018.

Zhang, H.Q., A.F. Croes and H.F. Linskens. 1982. Protein-synthesis in germinating pollen of Petunia—role of proline. Planta 154: 199–203.

Ziegler, H. 1959. Über die Zusammensetzung des "Bestäubungstropfens" und den Mechanismus seiner Sekretion, Planta 52: 587–599.

6

Determination of Sex Expression in Cycads

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ABSTRACT

Cycads, be it extinct or extant are unique among gymnosperms and plants in general due to their absolute dioecious nature. Apart from the living cycads, no bisexual cycad cones or individuals have ever been found in the fossil record. This dioecy can be correlated with occurrence of sex chromosome having unequal length but occasional sex change as encountered in cycads compounds the problem of explaining the sexuality. Whatever factor behind the sex expression, distinguishing the maleness or femaleness at an early vegetative stage is virtually impossible for cycads. This poses serious problems in raising a population prior undertaking a proper conservation strategy either through *in situ* or *ex situ* means. The present review, hence aims to address the classical and state-of-art molecular methods of sex determination in cycads at pre sporangial stage. Since genome information of cycads is scanty, most of the PCR (Polymerase Chain Reaction) based approaches adopt anonymous markers, which later on are usually converted to more informative SCAR (Sequence

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Characterized Amplified Region) markers. Even more reliable marker for sex determination in cycads at pre sporangial stage will plausibly emanate from a technique like Methylation—Sensitive Amplification Polymorphism (MSAP), which considers the epigenetic mechanism, especially DNA methylation since perturbation of environmental cue leads to occasional sex change.

Keywords: Cycads, cytological and molecular techniques, pre-sporangial stage, sex determination

Introduction

Gymnosperms include the earliest living lineages with innovations of a greatly reduced male gametophyte (pollen), pollination as well as seeds, but with extremely large genome. Recent molecular phylogenetic studies indicate that the five major lineages of extant gymnosperms (cycads, *Ginkgo*, Gnetales, Pinaceae, and all other conifers) form a monophyletic group that is sister to angiosperms, although an alternate placement of Gnetales as sister to the angiosperms cannot be unambiguously rejected. Cycads represent the likely sister lineage to all other extant gymnosperms and are consistently included in comparative developmental and molecular systematic studies. As a basal lineage, cycads provide exemplars to help ascertain the generalized gymnosperm reproductive features from which flowering plant morphology and genetic controls were likely modified (http://www.greenbac.org/tree.html: The green plant BAC library project).

Charles Darwin hypothesized that ancestral lineages were hermaphroditic (each individual produces both functional female and male gametes) and that dioecy evolved as a derived condition (Darwin 1873). In plants and animals, evolution from hermaphroditism to strict dioecy almost certainly occurred via an intermediate stage that involved individuals who were both hermaphroditic and either functional females or males (Darwin 1873; Charlesworth and Guttman 1999; Gorelick 2003). These intermediate stages are referred to as gynodioecy (functional females) or androdioecy (functional males). However, in spite of being an ancient lineage (existing for 275 to 300 million years in near modern form), hermaphrodite sexual morph was never found in cycads and dioecy appears to be quite a primitive trait among cycads. No bisexual cycad cones or individuals have ever been found in the fossil record (Gorelick and Osborne 2007) though occurrence of bisporangiate cones has been reported in conifers (Flores-Renteria et al. 2011). The only instances of anything other than strict dioecy that have ever been seen in cycads are those examples of sex change (Osborne and Gorelick 2007).

The living cycads can be divided into three families; Cycadaceae, Stangeriaceae and Zamiaceae which consisted of 11 genera, 297 species and sub-species. Since all cycads are dioecious, they appear to possess little if any sexual dimorphism as far as vegetative structures is concerned. Therefore, one can hardly distinguish the sexes of a species by visual inspection unless their reproductive organs are present. This poses serious problems in raising a population prior undertaking a proper conservation strategy either through *in situ* or *ex situ* means since many of the cycads, including *Zamia,* have already been placed in the endangered red list category (Roy et al. 2012). Attempt towards artificial pollination leading to recovery of large number of viable seeds is, hence, the only way to date, for scaling up the already diminishing population of these threatened plants, for which substantial number of donor plants of both sexes is a prerequisite.

In the backdrop of this, the present review is aimed to address the classical and state-of-art methods of sex determination in cycads, which are a popular area of research among experts in various fields, including plant molecular biology, agriculture, horticulture, ecology and environmental protection.

Ecological Approach: Sex and Population Differences

Four natural populations of *Cycas micronesica* growing under differing ecological conditions were surveyed over a 4 year period to assess the response of juveniles before and after the introduction of the cycad aulacaspis scale insect (*Aulacaspis yasumatsui* Takagi) and to test the hypothesis that monopodial ovulate plants are taller than pollen-producing plants with equivalent diameter (D). Height (H), D, and leaf and stem tip numbers were recorded for 297 ovulate and 186 pollen-producing ("male" and "female", respectively) plants and a total of 493 juveniles (n=976). Among the 483 adults, mean female plant H and D did not differ significantly from those of male plants. However, population-specific differences in mean plant size were observed; i.e., female plants achieve greater H and have significantly smaller leaf and stem tip numbers than do their male counterparts with equivalent increases in D. Population differences, however, were not statistically significant for juvenile plants (Niklas and Marler 2008).

Cytological Techniques towards Identification of Sex Chromosome

In many animals (but only in a handful of plants), sex chromosome of different lengths can be identified. For example, in humans, the Y chromosome is much shorter than the X chromosome. Evolution of sex chromosomes is usually explained by a population genetic model

known as Muller's ratchet (Griffin et al. 2002; Gorelick 2003). One of the prerequisites for Muller's ratchet for sexual organisms is that the haploid stage of the life cycle should be largely secluded from selection. This is the case in most animals, which generally have small and short-lived gametes. Plants, on the other hand, have large multi-celled and long-lived haploid stages (gametophytes), with cycads being extreme in this regard. Cycad gametophytes are enormous; occupying much of the volume of what becomes a seed following fertilization. Female cycad gametophytes are also long-lived; sometimes more than a year passes between pollination and fertilization (Norstog and Nicholls 1997). Male gametophytes are also large and complex compared with other plants. Although there are no data concerning whether cycad haploid stages express most of the genes expressed by their diploid stages, the structural complexity, size and age of cycad gametophytes indicate that they should be largely immune from the Muller's ratchet. Therefore, the chance of cycads (and most other plants) developing unequal length of sex chromosome is remote (Gorelick 2005). Another argument for cycads not having unequal length sex chromosome is that occasional sex change does occur in cycads and it could only be possible if large chromosomal rearrangements had taken place, which is virtually impossible (Gorelick and Osborne 2007).

However, contrary to the aforesaid rationale, the study of Sangduen et al. (2007), encompassing three species of *Cycas* and *Zamia* of Nong Nooch Tropical Botanical Garden, Thailand, revealed that all the three species of both *Cycas* and *Zamia* have an equal chromosome number in each species, namely 2n = 2x = 22 in *Cycas* and 2n = 2x = 16 in *Zamia*. The karyotype formulae of *Cycas* varied into 3 groups: 12 M + 8 SM + 2 A, 10 M + 8 SM + 2 A and 12 M + 8 SM + 2 T and of *Zamia* were 12 M + 4 SM. Nevertheless, of all the three *Cycas* species studied, the karyotype of female and male plants could be distinguished. The *Zamia* evidence, however, was further complicated as all three species had the same chromosome number and karyotype pattern as well. Only *Z. pumila* could be differentiated between male and female plants (Sangduen et al. 2007). Hence, concepts and/or reports on identification of sex at pre-sporangial stage through cytology or karyomorphological study seems to be plausibly conflicting.

The recent elegant review on sex chromosomes in land plants (Ming et al. 2011); however, is a proponent of unequal length of sex chromosomes in cycads. The hypothesis of six stages of plant sex chromosome evolution as proposed by them have placed the candidature of *Cycas revoluta* in the fifth stage where severe degeneration of the Y chromosome has caused the loss of function for most genes, and loss of nonfunctioning Y chromosome sequences resulting in a shrinking of the Y chromosome. They have hypothesized that some sex chromosome systems might not have undergone this phase of shrinking but instead kept expanding and

degenerating until a complete loss of the Y chromosome had taken place. In either case, a small portion of the Y chromosome has continued to meiotically pair with the X chromosome allowing proper disjunction. Since there were no known angiosperm sex chromosomes at this stage, they (Ming et al. 2011) are of opinion that the gymnosperm species *Cycas revoluta* having heteromorphic sex chromosomes with a reduced Y chromosome has played the intermediary stage (Fig. 1).

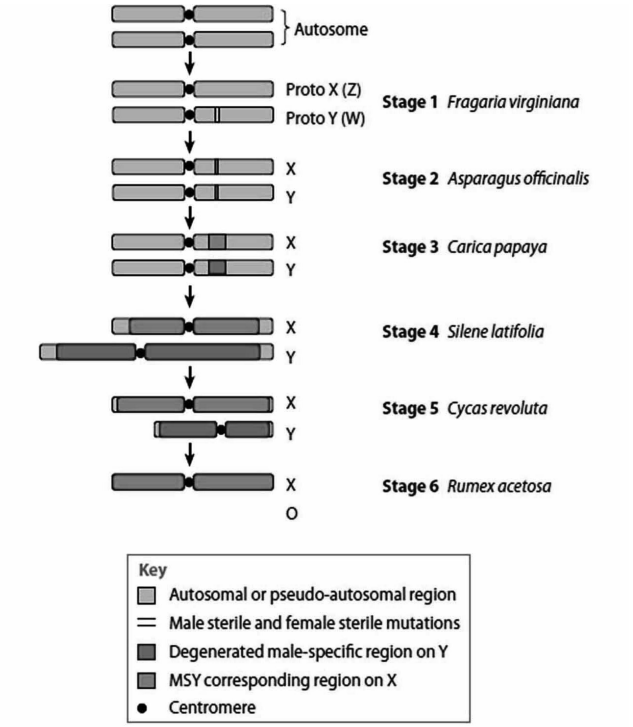


Figure 1

The six stages of sex chromosome evolution. Stage 1: Unisexual mutation of two sex determination genes with complementary dominance. Stage 2: Suppression of recombination between the two sex determination genes and YY genotype is viable. Stage 3: Suppression of recombination spread to neighboring regions and a small male-specific region of the Y chromosome (MSY) region evolved. YY genotype is not viable. Stage 4: The MSY expands in size and degenerates in gene content via accumulation of transposable element insertions and intrachromosomal rearrangements. The X and Y chromosomes become heteromorphic. Stage 5: Severe degeneration of the Y chromosome. Deletion of nonfunctional DNA sequences results in reduction of Y-chromosome size. Stage 6: Suppression of recombination spreads to the entire Y chromosome. The Y chromosome is lost, and X-to-autosome ratio sex determination system has evolved (Ming et al. 2011).

Color image of this figure appears in the color plate section at the end of the book.

More refined and state-of-art technique like FISH (Fluorescence *in situ* Hybridization) as attempted by Tagashira and Kondo (2001), who have studied the chromosome phylogeny of *Zamia* and *Ceratozamia* by rDNA analysis through FISH may throw some light in this direction.

Theoretically, at least, it can be postulated that there must have an epigenetic control behind this conflict regarding the existence of sex chromosome having unequal length. Cycads appear to have retained an ancestral form of dioecy, with the sex of an individual being determined by cytosine methylation down regulating genes responsible for production of gametes or sex chromosomes. Cycads have thereby retained the phenotypic plasticity to change sex via removing methylation in the face of large environmental perturbations. It is not obvious how many other plants have retained this plasticity in sex determination. It does not appear that any genetic assimilation of the epigenetic mechanism of sex determination has occurred in cycads. However, such canalization of dioecy may have been unnecessary because cycads can not revert to a hermaphrodite condition via allopolyploidy. Finally, it appears that cycads are immune from Muller's ratchet because they have haploid stages that express most of the genes expressed in their diploid stages. Micro array studies could be used to test this inference of immunity from Muller's ratchet on the large size, complexity and longevity of cycad gametophytes (Gorelick and Osborne 2007).

Molecular Marker Techniques for Sex Determination in Pre Sporangial Stage

If the epigenetic basis of sex expression is conceived in case of cycads, then it is the outcome of environmental perturbation mediated phenotypic plasticity, which is defined as the ability of an organism to change its phenotype in response to changes in the environment. However, while looking for an ideal marker for sex determination in pre sporangial stage, the markers should be completely independent of environmental conditions and should be detected at virtually any stage of plant development. The DNA based molecular markers *per se* probably satisfy all the essential criteria of 'true and full proof' markers. These markers are based on naturally occurring polymorphisms in DNA sequences (i.e., base pair deletions, substitutions, additions or patterns). There are various methods to detect and amplify these polymorphisms so that they can be used for genetic analysis. The DNA based molecular markers are superior to other forms of markers because they are relatively simple to detect, abundant throughout the genome, completely independent of environmental conditions and can be detected at virtually any stage of plant development. There are five conditions that characterize a suitable molecular marker (Gupta and

Varshney 1999): 1) Must be polymorphic 2) Co-dominant inheritance 3) Randomly and frequent distribution throughout the genome 4) Easy and cheap to detect 5) Reproducible. Consequently, these markers have been used for several different applications including: germplasms characterization, characterization of transformants, study of genome organization, phylogenic analysis and genetic vis-à-vis sex diagnostics. Almost all of the molecular markers are either based on DNA-DNA hybridization or polymerase chain reaction principle or sometimes a combination of both. Some of these important markers, which have been used in sex determination, particularly in case of gymnosperms are as follows.

RAPD

The discovery of RAPD or Random Amplified Polymorphic DNA (Williams et al. 1990) based genetic marker in the beginning of 1990s probably revolutionized the application of till then sophisticated Polymerase Chain Reaction technique. The instant popularity of this technique was probably due to its simplicity since RAPD analysis does not involve hybridization/ autoradiography or high technical expertise. It uses one or sometimes two short arbitrary primers (usually 8–10 bases) to amplify anonymous stretches of DNA which are then separated and visualized usually by simple agarose gel electrophoresis. Many different fragments are normally amplified using each single primer; the technique has, therefore, proved to be a fast method for detecting polymorphisms. Furthermore, the requirement of only tiny quantities of target DNA and relatively easy procurement of the different series of random primers from the commercial manufacturer made the unit costs per assay quite low. Suddenly all the field of Biology jumped to it and to the Population Geneticists it was initially probably the perfect technique they were looking for so long. However, the theoretical and practical limitations of this technique soon became quite evident.

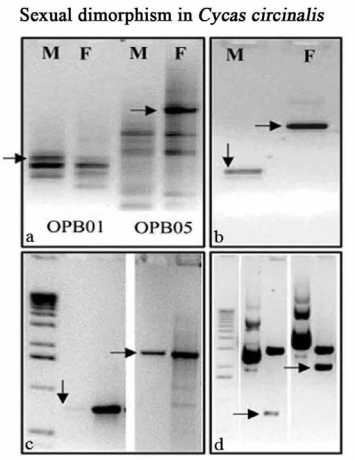
From the practical point of view it was observed that RAPD does suffer from sensitivity to changes in PCR conditions resulting in changes to some of the amplified fragments. Considering theoretically, it was understood that RAPD has problems of co-migration: and often it was found that result interpretation became problematic since it was difficult to predict whether same RAPD derived band is same DNA fragment and whether one band is solely one fragment. This is because the type of gel electrophoresis used, while able to separate DNA quantitatively (i.e., according to size), can not separate equal-sized fragments qualitatively (i.e., according to base sequence). Finally, the dominant nature of the RAPD markers was found to be another bottleneck for distinguishing homozygotes from heterozygotes thus proving this system quite unsuitable in Marker Assisted Selection particularly at the stage of F_1 hybrids.

In spite of the above shortcomings, the most popular markers for sex determination in plants surprisingly include RAPD (Milewicz and Sawicki 2013) and gymnosperms vis-à-vis cycads are no exception to that. The reason probably is simple since in the absence of any specific genome information in most of the non model plants and particularly the gymnosperms, the workers have to adopt this fast yet random technique to look for polymorphism between plants of expressed sex and later try to correlate their findings in population of unknown or yet to be expressed sex (i.e., at pre sporangial stage).

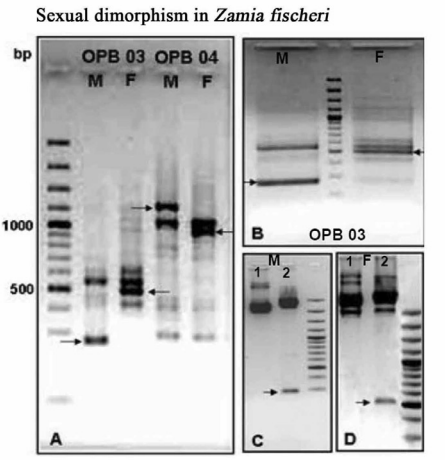
The group of the present reviewers has successfully used RAPD technique for detecting sex related marker in *Cycas circinalis* and observed readily distinguishable sexual dimorphism of this Gymnosperm in members of contrasting sex within population (Gangopadhyay et al. 2007). Of the RAPD fingerprints generated from a number of random primers, the profiles of primers OPB 01 and OPB 05 were noteworthy, since they represented one male-specific (686 bp) and another female specific (2048 bp) band respectively (Fig. 2) (Gangopadhyay et al. 2007). Sequencing of these two cloned DNA fragments, followed by BLASTX searching, revealed maximum homology with reverse transcriptase of *Ginkgo biloba* (score 69.3 bits; NCBI accession no. AAY87195) in case of male-specific DNA fragment (NCBI accession DQ386640, dated 22.02.2006), while the female-specific DNA fragment did not result in any significant match.

Unique RAPD derived polymorphism was also detected by the present group between male and female *Zamia fischeri* plants (Roy et al. 2012). The RAPD profiles of both OPB03 and OPB04 primers showed one male and one female-specific DNA fragments in each of the cases (Fig. 2). The molecular mass of male and female specific fragments in case of OPB03 was 294 and 534 kb, while those were 1320 and 1015 kb respectively in case of OPB04. The specific DNA fragments of both male and female samples of OPB03 were eluted out from gel, cloned and subsequently sequenced. Sequencing of these two cloned DNA fragments, followed by BLASTN searching, revealed homology with *Araucaria angustifolia* (a conifer) clone AAng27 micro satellite sequence (maximum identity 83%; GenBank accession no. AY865591) in case of male specific DNA fragment (GQ141708), while the female specific DNA fragment (GQ141709) did not result in any relevant homology with the available database.

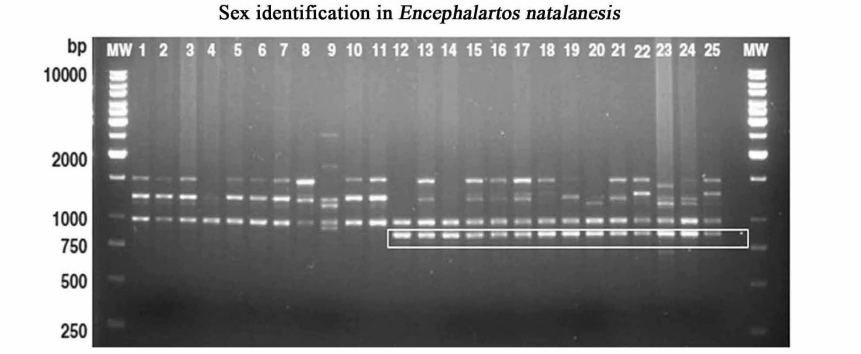
Similar RAPD based experimental approach was undertaken for sex identification in *Encephalartos natalensis*, another cycad (Prakash and Staden 2006). Initially, the workers used 140 primers to amplify the bulk DNA of five individuals each of known male and female sexuality. While a high degree of polymorphism was observed in the amplification profiles of male and female plants, only primer OPD-20 generated a specific band (~850 bp) in female DNA (Fig. 2). To validate this observation, this primer was



Cloning of RAPD derived male (M) - and female (F)- specific bands of *Cycas circinalis*. **a,** RAPD profiles. b, Purified elutedbands. **c**, Purified bands with representative colony PCR products. **d,** Purified plasmids from clones and their respective restriction enzyme (EcoR1) digested fragments showing inserts (all bands of interest are marked with arrows) (Gangopadhyay et al. 2007).



RAPD derived male (M) and female (F) specific bands of *Zamia fischeri* Miq. (A) Polymorphism in RAPD profiles using primers OPB 03 and OPB 04; (B) purified eluted bands of OPB 03 primer (M and F); (C,D) purified plasmids (Lane 1) from the clones (primer OPB 03) and their respective restriction enzyme (EcoR1) digested fragments (Lane 2) showing the inserts (all bands of interest are arrow marked) (Roy et al. 2012).

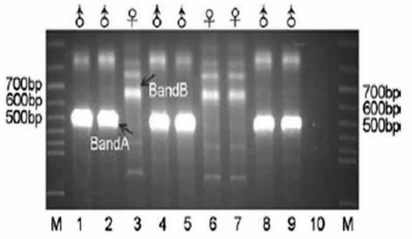


Sex identification in *Encephalartos natalanesis* : PCR amplification profiles of 25 individuals collected from the Durban Botanical Garden, Durban. Lanes 1–11 represent male individuals; Lanes 12–25 are of female individuals. The DNA band indicated by a white box represents the unique female sex-linked band (Prakash and Staden 2006).

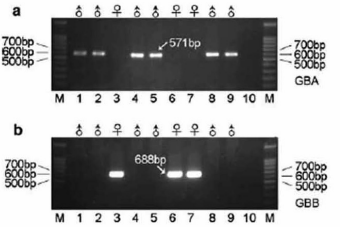
Fig. 2.

Figure 2. contd.

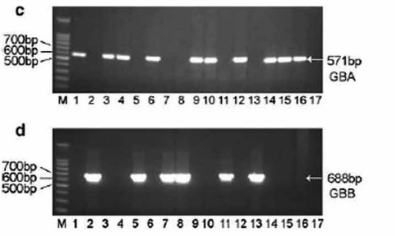
Development of RAPD derived SCAR marker in *Ginkgo biloba*



RAPD different amplification patterns obtained with S10 primer in individuals of six male (♂) and three female (♀). Band A was linked to the male individual and Band B was linked to the female individual. (Liao et al. 2009).



SCAR analysis carried out, respectively with GBA and GBB primers from the six male (♂) and three female(♀) samples. **a** Showed GBA primers amplified a single 571 bp band in male samples but not in the female sample. **b** Revealed GBB primers generated a 688 bp band only in the female individuals. (Liao et al. 2009).



SCAR analysis carried out, respectively with GBA and GBB primers from 16 sex-unknown *Ginkgo biloba* samples. **c** Indicated the samples were male which could be amplified a single 571 bp band by GBA primers and the female could be not. **d** Revealed the individuals that could generate a 688 bp band were female and the male could be not. GBA and GBB orimers obtained a consistent conclusion. (Liao et al. 2009).

re-tested with 69 individuals of *E. natalensis*. The ~850 bp DNA band was present in all 38 female individuals tested and it was consistently absent in all 31 male plants tested.

An even greater endeavor was undertaken by Ling et al. (2003) while searching for a sex-associated RAPD marker in the living fossil, *Ginkgo biloba*. The workers screened one thousand and two hundred random decamers and landed up to a single 682 bp RAPD marker, which appeared to be maleness associated after scoring a staggering figure of 8,372 amplicons.

SCAR

Though RAPD is the first choice to look for sex related polymorphism in many plants including gymnosperms but due to some inherent shortcomings of this technique, adoption of next level of marker is often recommended (Milewicz and Sawicki 2013). Conversion of RAPD polymorphic band into SCAR or Sequence Characterized Amplified Region marker though technically demanding but greatly enhances the reliability of the marker. Longer (20–30 bp) and more specific primers for these markers facilitate amplification of the desirable sequence, and they would guarantee repeatability of measurement results. Furthermore, development of sex-linked SCAR markers support sex differentiation even of a single individual—the sex-specific band appears or does not. For other marker systems, which generate the whole band patterns, it would be difficult to identify a gender without the need of comparing the band patterns for both sexes.

Endeavor in this direction has resulted in conversion of RAPD marker into female sex specific co dominant SCAR marker, which has provided a possibility of identifying the sex of *Cycas tanqingii* before sexual maturation, which is very important for *in situ* or *ex situ* conservation (Jing et al. 2007).

There are further issues in SCAR marker development. Ideally, a researcher should use one or two different SCAR markers which create products of different length in males and females in the same amplification. Sex-linked markers for *Ginkgo biloba* were determined in line with the above method. SCAR markers generated products with the length of 571 bp for males and 688 bp for females (Fig. 2) (Liao et al. 2009). Annealing temperature differed for both primer pairs. Situations such as those encountered in the study of *Ginkgo biloba* happen rarely. Even if the markers of both sexes are found in the same species, they are rarely discovered by the same research team, and their identification is a laborious process, leaving aside the luck or chance factor (Milewicz and Sawicki 2013).

AFLP

Described as the most full-proof of all molecular marker techniques, AFLP or Amplified Fragment Length Polymorphism (Vos et al. 1995) involves the

following steps: Digestion of DNA with two specific restriction enzymes, one frequent cutter and the other rare cutter; ligation of oligonucleotide 'adapters' to the ends of each fragment ensuring amplification of only the fragments, which have been cut by both frequent and rare cutters; designing of primers from the known sequences of the adapters plus 1–3 selective nucleotides, which extend into the fragment sequences; PCR followed by visualization of fragments in gel after autoradiography. The high number of bands eases analysis by providing better chance of polymorphism. Though theoretically sound but this time, skill and cost intensive technique is hardly suitable for evaluating large number of individuals of a population effectively. Hence, report of use of AFLP in determining sex of gymnosperm is relatively scarce. Only one report of identification of three female and one male specific AFLP polymorphism is available in *Ginkgo biloba* (Wang et al. 2001).

Micro satellites (SSR)

Micro satellite loci or simple sequence repeats (SSR) exhibit high levels of variability because of differences in the number of repeated units. The high allelic diversity and abundance of micro satellites in the eukaryotic genome make these co dominant molecular markers popular for detailed genetic studies as genetic diversity and genetic structure (Chase et al. 1996). Unfortunately, plausibly due to complexity of most of the gymnosperm genome as well as paucity of research materials in case of endangered ones, research is yet to be flourished in this direction unlike angiosperm crop plants. Only one report of development of twenty-nine species-specific and highly polymorphic micro satellite loci is available in *Araucaria angustifolia* from a genomic library enriched for AG/TC repeats (Schmidt et al. 2007). Future endeavor to link those SSRs with sex loci may throw some light in early detection of sex in gymnosperms.

Functional Genomics in Sex Determination

A promising functional genomics approach was undertaken by Zhang et al. (2002) to understand the molecular mechanisms controlling development of sexual characters in *Cycas edentate*. Their attempt towards cloning genes expressing differentially in male or female reproductive organs culminated in a novel gene, named Fortune-1 (Ft-1), with enhanced expression in male reproductive organs. The 593-base-pair Ft-1 cDNA was predicted to encode a 77-amino-acid protein, and exists as a single copy gene in the *C. edentata* genome. Ft-1 expression was enhanced in male cones, including the cone axis, microsporophylls and microsporangia, but was reduced in ovules and undetectable in megasporophylls. The secondary structure prediction and

homology search of Ft-1 protein showed that it has a helix–loop–helix motif, and predictably it was without any homologue in the database indicating paucity of basic research work in non model, rare plant materials.

Conclusion

Since sex is the queen of problems in evolutionary biology (Bell 1982), understanding the molecular factor(s) behind sex expression has immense importance both in basic and applied research. Despite the growing body of research, the mechanism of sex determination in many plant species remains unexplained, and this is even truer in case of gymnosperms and cycads to be precise. Though the search for molecular sex-linked markers paves the way for future scientific discoveries, on the other hand, sex-linked markers alone do not explain the molecular mechanism of sex determination in dioecious plants, but the number of markers, their sequence structure and homology between sequences characteristic of males and females provide a certain venture point for studies into sex determination mechanisms. Still, it appears that there remains a large gap between the theoretical understanding and the technique/marker of choice for early sex determination (in pre sporangial condition) that will be helpful for breeding program prior undertaking a proper conservation strategy. If an epigenetic control behind the conflict regarding the existence of sex chromosome having unequal length is finally envisaged on the backdrop of occasional sex change in cycads, then a proper technique, which considers the epigenetic mechanism, especially DNA methylation has to be adopted for future research towards sex determination in cycads. In this regard adoption of technique like Methylation-Sensitive Amplification Polymorphism (MSAP) seems to be promising, which has been first tried for sex determination in some cycads (Kanchanaketu et al 2007). The attempt of their work using modified AFLP technique using isochizomer enzyme (MspI and HpaII) or MSAP was carried out to assess the pattern of cytosine methylation in both sexes of *Cycas* and *Zamia*. Using seven pairs of primers, a total of 364 bands, some of which showing sex-specific were produced and classified into three groups. The first group was non-polymorphic markers, whereas the second group was chosen from the results of differentiation ability of MspI and HpaII to cut the methylated sequences, but sex-different markers were still not obtained. Markers in the third group were methylation-sensitive and they also showed some polymorphic patterns between the two sexes. Their end suggestion that sex in cycads may be associated with DNA methylation is probably justified, which however, warrants further studies in this direction with state-of-art techniques to reach a final conclusion.

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References

Bell, G. 1982. The Masterpiece of Nature: The Evolution and Genetics of Sexuality, University of California Press, Maynard.

Charlesworth, D. and D.S. Guttman. 1999. The evolution of dioecy and plant sex chromosome systems. *In*: C.C. Ainsworth (ed.). Sex determination in plants. Bios Scientific Publishers. Oxford, UK, pp. 25–49.

Chase, M.R., C. Moller, R. Kesseli and K.S. Bawa. 1996. Distant gene flow in tropical trees. Nature 383: 398–399.

Darwin, C.R. 1873. On the males and complemental males of certain cirripedes, and rudimentary structures. Nature 8: 431–432.

Flores-Renteria, L., A. Vázquez-Lobo, A.V. Whipple, D. Piñero, J. Márquez-Guzmán and C.A. Domínguez. 2011. Functional bisporangiate cones in *Pinus johannis* (Pinaceae): Implications for the evolution of bisexuality in seed plants. American J. Bot. 98: 130–139.

Gangopadhyay, G., S.K. Roy, K. Ghose, R. Poddar, D. Basu and K.K. Mukherjee. 2007. Sex detection of *Carica papaya* and *Cycas circinalis* in pre-flowering stage by ISSR and RAPD. Curr. Sc. 92: 524–526.

Gorelick, R. 2003. Evolution of dioecy and sex chromosomes via methylation driving Muller's ratchet. Biol. J. Linn. Soc. 80: 353–368.

Gorelick, R. 2005. Theory for why dioecious plants have equal length sex chromosome. Amer. J. Bot. 92: 979–984.

Gorelick, R. and R. Osborne. 2007. Evolution of dioecy and sex chromosomes in cycads. Mem. New York Bot. Gard. 97: 326–334.

Griffin, D.K., S.C. Harvey, R. Campos-Ramos, L.J. Ayling, N.R. Bromage, J.S. Masabanda and D.J. Penman. 2002. Early origins of the X and Y chromosomes: lessons from tilapia. Cytogenet. Genome Res. 99: 157–163.

Gupta, P.K. and R.K. Varshney. 1999. Molecular markers for genetic fidelity during micro propagation and germplasm conservation. Curr. Sci. 76: 1308–1310.

Jing, J.Z., H. Jin, D.L. Li, X.K. Chen and Y. Zhang. 2007. RAPD and SCAR molecular markers linked to the sexuality of cycads (*Cycas tanqingii* D.Y. Wang). Chinese Journal of Biotech. Sheng Wu Gong Cheng Xue Bao (in Chinese) 23: 1097–1101.

Kanchanaketu, T., V. Hongtrakul and N. Sangduen. 2007. Analysis of Sex Determination in Some Cycads Using Methylation-Sensitive Amplification Polymorphism (MSAP). Kasetsart J. (Nat. Sci.) 41: 641–650.

Liao, L., J. Liu, Y. Dai, Q. Li, M. Xie, Q. Chen, H. Yin, G. Qiu and X. Liu. 2009. Development and application of SCAR markers for sex identification in the dioecious species *Ginkgo biloba* L. Euphytica 169: 49–55.

Ling, J., Y. Rui-Lin, L. Mao-Xue and S. Chen. 2003. Identification of a sex-associated RAPD marker in *Ginkgo biloba*. Acta Bot. Sin 45: 742–747.

Milewicz, M. and J. Sawicki. 2013. Sex-linked markers in dioecious plants. Plant Omics J. 6: 144–149.

Ming, R., A. Bendahmane and S.S. Renner. 2011. Sex Chromosomes in Land Plants. Ann. Rev. Plant Biol. 62: 485–514.

Niklas, K.J. and T.E. Marler. 2008. Sex and population differences in the allometry of an endangered cycad species *Cycas micronesica* (Cycadales). Int. J. Plant Sci. 169: 659–665.

Norstog, K.J. and T.J. Nicholls. 1997. The biology of the cycads. Cornell University Press, Ithaca, NY.

Osborne, R. and R. Gorelick. 2007. Sex change in cycads-Cases, causes, and chemistry. Mem. New York Bot. Gard. 97: 335–345.

Prakash, S. and J. Staden. 2006. Sex identification in *Encephalartos natalensis* (Dyer and Verdoorn) using RAPD markers. Euphytica 152: 197–200.

Roy, S.K., G. Gangopadhyay and K.K. Mukherjee. 2012. Determination of sex in *Zamia fischeri* Miq., an endangered gymnosperm. Int. J. Biodiversity Conservation 4: 287–293.

Sangduen, N., M. Toahsakul and V. Hongtrakul. 2007. Karyomorphological Study of Some Selected Cycads. Au J. T. 11: 1–6.

Schmidt, A.B., A.Y. Ciampi, M.P. Guerra and R.O. Nodari. 2007. Isolation and characterization of microsatellite markers for *Araucaria angustifolia* (Araucariaceae). Mol. Ecol. Notes 7: 340–342.

Tagashira, N. and K. Kondo. 2001. Chromosome phylogeny of *Zamia* and *Ceratozamia* by means of Robertsonian changes detected by fluorescence *in situ* hybridization (FISH) technique of rDNA. Plant Syst. Evol. 227: 145–155.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Friters, J. Pot, J. Paleman, M. Kuiper and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. Nuclei Acids Res. 23: 4407–4414.

Wang, X.M., W.Q. Song, S. Liu, X.L. Li and R.Y. Chen. 2001. AFLP markers related to sex in dioecious plant, *Ginkgo biloba* L. Acta Scientiarum Naturalium Universitatis Nankaiensis 34: 5–9.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531–6535.

Zhang, P., K.-H. Pwee1, T.W.T. Hugh and P.K. Prakash. 2002. Cloning and characterization of Fortune-1, a novel gene with enhanced expression in male reproductive organs of *Cycas edentate*. Mechanisms of Development 114: 149–152.

7

Storage Lipids in Developing and Germinating Pollen Grain of Flowering Plants

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ABSTRACT

In plants, storage lipids serve as energy source and provide carbon equivalents for periods of active metabolism. They can be stored in different sporophytic organs and tissues usually in the form of triacylglycerols (TAGs), which are non-polar and can be stored in a nearly anhydrous form. Plants accumulate the storage lipids in specialized organelles called oil bodies (OBs), lipid bodies, lipid droplets or oil globules. Mature pollen grain of oleaginous plants present copious oil bodies in the vegetative cytoplasm. However, little is known about the behavior, breakdown and role of these cellular structures in processes directly connected to sexual plant reproduction. Up to now, data on storage lipids biology in pollen were rather few and fragmentary. The aim of this review is to sum up and verify the current knowledge on pollen grain OBs as well as to expose the significance of further studies on physiological and molecular nature of storage lipids in reproductive biology of angiosperms.

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Keywords: lipids mobilization, oil bodies, pollen development, pollen germination, storage lipids

Introduction

Lipids are major and vital cellular constituents. In plants, stored lipids reserves play an important role in the life cycle by providing carbon and energy equivalents for periods of active metabolism (Graham 2008; Murphy 2012). For most eukaryotes, the preferred storage compounds are lipids in the form of triacylglycerols (TAGs), which are non-polar and can be stored in a nearly anhydrous form. Plants accumulate the storage lipids in specialized organelles called oil bodies (OBs), lipid bodies, lipid droplets or oil globules (Murphy 2012). These organelles have been found in different sporophytic plant organs and tissues, like oil seeds and oleaginous fruits (Huang 1994) as well as in cells of both, male (Piffanelli et al. 1998; Rodríguez-García et al. 2003; Zienkiewicz et al. 2010) and female gametophyte (Wu et al. 1999; Jiang et al. 2009). However, despite the obvious presence of OBs in pollen grains of different species (Fig. 1) little is known about the behaviour, breakdown

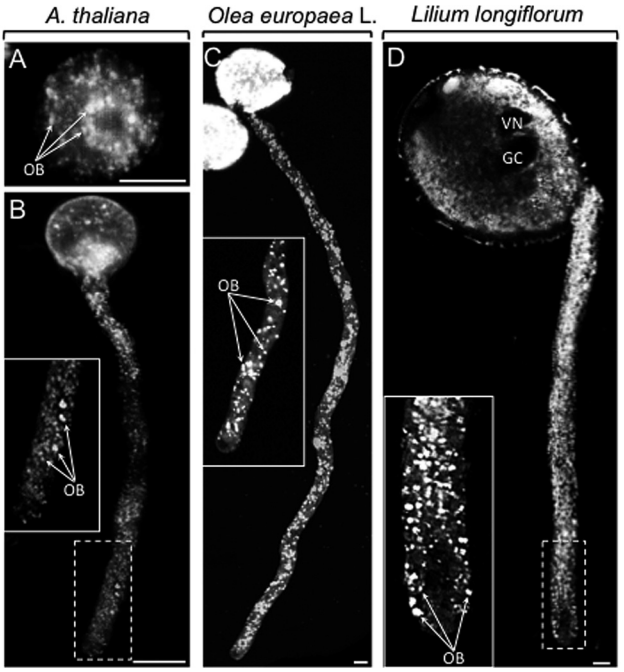

Figure 1. Nile red staining of OB (bright spots in the cytoplasm) in mature and germinating pollen of *Arabidopsis thaliana* (A and B) and in pollen tube of olive (*Olea europaea* L.) (C), and lily (*Lilium longiflorum*) (D). Numerous OBs fills up the cytoplasm of mature pollen grain (A). During *in vitro* pollen germination OBs are visible in the cytoplasm along the whole length of the pollen tube (B-D). Bar = 10 µm.

and role of these cellular structures in processes directly connected to sexual plant reproduction.

So far, data on storage lipids biology in pollen were rather few and fragmentary. Recent studies of our group strongly improved our knowledge on behaviour and mobilization of pollen OBs and proved the high importance of storage lipids during sexual processes in higher plants. Thus, the major goal of this report is to sum up and verify the current knowledge on pollen OBs as well as to expose the significance of further studies on physiological and molecular nature of storage lipids in reproductive biology of angiosperms.

The Biogenesis and Structure of Pollen Oil Bodies—Facts, Models and Theory

Formation of pollen OBs is correlated with the kinetics of gene expression and protein synthesis of enzymatic markers of lipid biosynthesis (Piffanelli et al. 1997). More than five-fold increase in the TAGs levels was demonstrated in developing pollen of *Brassica napus* from the first until the second pollen mitosis (Piffanelli et al. 1997). These events were temporally connected with OBs accumulation in pollen cytoplasm and with high expression of four lipid biosynthesis genes *SAD*, *EAR*, *CYB5* and *ACP,* encoding stearoyl ACP desaturase, enoyl ACP desaturase, cytochrome b_5, and acyl carrier protein, respectively (Piffanelli et al. 1997). In mature *Brassica napus* pollen fatty acid composition of the intracellular membrane and OBs membrane was found to be similar, which may reflect their shared fatty acid biosynthesis (Piffanelli et al. 1997, 1998).

The biogenesis of seed OBs and their constituents TAGs, PLs and oleosins occurs in specialized endoplasmatic reticulum (ER) microdomains and involves acyl-editing of fatty acyl chains within the nitrogenous phospholipids of the ER (Hsieh and Huang 2004). In the pollen grain the intracellular membrane systems is characterized by extensive proliferation of ER and vesicles during maturation and expansion of the cytoplasm in the vegetative cell (Piffanelli et al. 1998). Previously, it was suggested that ER as well as vacuoles and Golgi are linked to the accumulation of compounds necessary for pollen development and pollen tube growth (Rodríguez-García and Fernández 1990; McCormick 1993; Yamamoto et al. 2003). Indeed, highly dilated rough ER (rER) surrounding OBs was observed before and after anthesis in *Arabidopsis* pollen (Yamamoto et al. 2003). Similarly in the developing olive pollen OBs were often found in the direct contact with rER cisternae (Fig. 2) (Rodríguez-García and Fernández 1990; Zienkiewicz et al. 2011). These data support the significant role of ER cisternae in the formation of pollen OBs and suggest common mechanism of OBs ontogeny in different plants tissues.

The current model of OBs structure have risen from intensive studies in seeds of several species, mainly those in oilseeds (Tzen and Huang 1992; Tzen et al. 1993; Murphy 2001; Purktova et al. 2008; Tzen 2012). The presence of OBs across a wide range of organisms and their highly conserved molecular composition, suggest rather universal structure of these organelles in higher plants. Pollen OBs as well as seed OBs are spherical organelles with a size usually ranging from 0.1 to 2.5 µm and consist of a TAG matrix, surrounded by a single layer of phospholipids (PLs) with a few embedded unique proteins (Piffanelli et al. 1997; Jiang et al. 2007; Zienkiewicz et al. 2010) (Fig. 3).

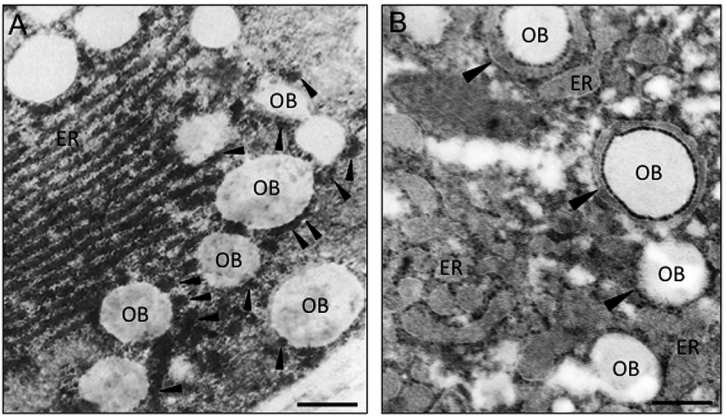

Figure 2. Spatial relationship between ER structures and oil bodies during olive pollen development. A) In the vegetative cell of young pollen OBs are often in close connexion with ER cisternae (arrowheads). B) At the mature pollen stage, OBs are often surrounded by dilated ER cisternae in the vegetative cytoplasm (arrowheads). ER, endoplasmatic reticulum; OB, oil body. Bar = 1 µm.

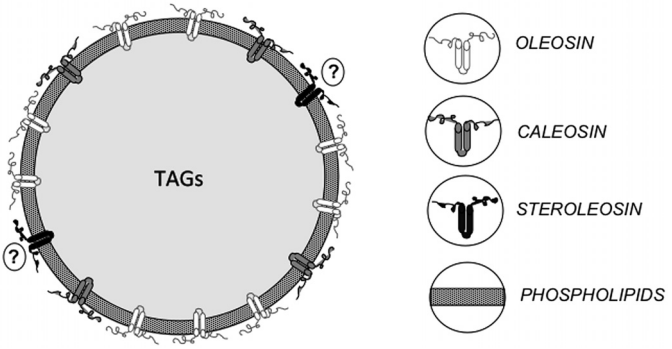

Figure 3. Structural model of pollen oil body. The TAG matrix is surrounded by a monolayer of phospholipids in which a set of a few unique proteins are embedded. Oleosin and caleosin were identified in pollen OBs, however the presence of pollen-specific steroleosin still remains unconfirmed (question mark).

Oleosins are the major proteins associated with seed OBs and are classified in two distinct classes, H- and L-(high and low molecular weight) oleosins (Tzen and Huang 1992). The H-form differs from the L-form by having insertion of 18 residues in their C-terminal domain. Most oleosins are relatively small proteins with molecular weight ranging from 15 to 26 kDa depending on the isoforms and plant species (Huang 1996; Murphy 1993). All oleosins have three domains: a hydrophilic N-terminal domain, a hydrophobic central domain containing 'proline knot' motif in its center, and a hydrophilic C-terminal domain (Tzen et al. 1993; Abell et al. 2004). Oleosins play an essential role in the stability of seed OBs, preventing coalescence of OBs during seed desiccation (Leprince et al. 1998). It was demonstrated that the size of OBs is correlated with oleosin content in seeds and might be regulated by oleosins (Ting et al. 1996; Siloto et al. 2006; Shimada et al. 2008).

In addition to seeds, oleosin has been found in tapetum and pollen of different species (Ross and Murphy 1996; Alché et al. 1999; Kim et al. 2002; Jiang et al. 2008). Putative oleosin isoforms were found in the pollen of rapeseed (Roberts et al. 1995) and a novel group of oleosins was identified in *Arabidopsis* pollen (Kim et al. 2002). A unique oleosin was found as the major protein of lily pollen OBs (Jiang et al. 2007). Sequence alignment showed that insertion of 18 residues in the C-terminal domain of seed H-oleosins is absent in the lily and *Arabidopsis* pollen oleosins. Moreover, phylogenetic tree analysis indicated that lily pollen oleosin of gametophytic origin is different from oleosins found in seed oil bodies and tapetum (sporophytic) and might represent a pollen-specific oleosin (Jiang et al. 2007).

Oleosin-like proteins or oleo-pollenins are a family of proteins whose members are highly expressed in tapetal cells of anthers (Robert et al. 1994; Alché et al. 1999; Ross and Murphy 1996; Murphy 2001). Oleo-pollenins initially contain the N-terminal domain (oleosin-like domain) that is similar to the central hydrophobic domain of seed oleosin (Murphy 2001). These proteins are associated with tapetal lipid droplets via their oleosin-like domain until the tapetal cells undergo apoptosis. At this point, the oleosin-like domain is removed by a specific peptidase to form the mature protein, pollenin, which is transferred to the outer wall of the pollen grains (Ting et al. 1998). Pollenins are the most abundant proteins in the pollen coat and are required for rapid hydration of *Arabidopsis* pollen grain (Mayfield and Preuss 2000).

In addition to oleosin, two minor proteins namely caleosin and steroleosin have been identified in seed OBs fraction (Frandsen et al. 1996; Lin et al. 2002; Lin and Tzen 2004). Caleosins belong to a large gene family found ubiquitously in higher plants and in several lipid-accumulating fungi, such as *Neurospora crassa* and *Aspergillus nidulans* (Murphy 2001). All caleosins contain, a calcium-binding site known as helix-loop-helix EF-

hand motif capable of binding a single calcium atom, a central hydrophobic region with a potential lipid-binding domain, and a C-terminal region including several conserved protein phosphorylation sites (Frandsen et al. 1996; Naested et al. 2000). Caleosin is located on the OBs surface or associated with ER-subdomain (Naested et al. 2000). This protein potentially contributes to OBs stability and might be involved in signal transduction via calcium binding or phosphorylation/dephosphorylation in processes such a membrane expansion, lipid trafficking or OBs biogenesis and degradation (Chen et al. 1999; Poxleitner et al. 2006). Moreover, it was demonstrated that caleosin possess peroxigenase activity, suggesting its involvement in phytooxylipin biosynthesis and biotic and abiotic stress response (Hanano et al. 2006; Kim et al. 2011). Caleosins in monocot seed OBs contain an additional N-terminal appendix of approximately 40–70 residues, therefore are larger than those in dicotyledonous seed OBs (Liu et al. 2005; Chen et al. 2012). Recently, a unique caleosin isoform distinct from that in seed OBs has been identified in OBs from pollen of lily and olive (*Olea europaea* L.) (Jiang et al. 2008; Zienkiewicz et al. 2010, 2011). However, olive pollen caleosin, similar to seed caleosins co-localize with ER structures, is able to bind calcium *in vitro* and shows similar structural conformation in OBs membrane like its seed counterpart (Zienkiewicz et al. 2011). Thus, despite different molecular structure, seed and pollen caleosins seem to have rather conserved functions in OBs formation and stabilization.

A second minor OBs-associated protein is steroleosin. These proteins contain a small N-terminal OBs anchoring domain and a large soluble sterol-binding dehydrogenase domain that belongs to a super-family of pre-signal proteins (Lin et al. 2002; Tzen et al. 2003). Sterol-binding dehydrogenases are implicated in signal transduction in different plant tissues and in the seed it is suggested that they specifically facilitate mobilization of OBs during germination (Lin et al. 2002). So far no steroleosin have been found in OBs from generative tissues of higher plants.

Storage Lipids Behaviour during Pollen Development

The major lipid-accumulating organs of flowers are the anthers, where pollen development occurs. The anther consists of the meiotic cells (microspores or pollen grain) at the centre, surrounded by the tapetum and by the anther wall somatic layers (sporophytic tissues) namely, from outside to inside, epidermis, endothecium and middle layers (Goldberg et al. 1993). The anther tapetum plays a secretory role in sporogenesis and is involved in pollen wall and pollen coat formation (Scott et al. 2004; Zhu et al. 2008).

Pollen development consists of two major phases: microsporogenesis and microgametogenesis (McCormick 1993). This process begins when pollen mother cells (PMC) produce a tetrad of haploid microspores after meiosis, which are encased in a callose (β-1, 3-glucan) wall. After callose degradation microspores are released into the anther loculus and after a period of microspore maturation, they undergo mitosis to finally produce pollen grains. Mature pollen grain comprises a generative cell or two sperm cells, completely enclosed within cytoplasm of the vegetative cell. During the long period of pollen maturation, the vegetative cell accumulates storage compounds like carbohydrates and lipids, which will be used for pollen germination and early pollen tube elongation (Bednarska 1988; McCormick 1993; Rodríguez-García et al. 2003; Zienkiewicz et al. 2010, 2011, 2013). The entomophilous pollen grains accumulate relatively more lipids than anemophilous pollen grains, which accumulate starch as their main reserve (Baker and Baker 1979; Piffanelli et al. 1998). The presence of OBs in pollen grains has been reported in several species such as *Brassica napus*, *Tradescantia bracteata*, *Gossypium hirsutum*, *Lilium longiflorum*, *Arabidopis thaliana* or *Olea europaea* (Mepham and Lane 1970; Charzyńska et al. 1989; Wetzel and Jensen 1992; Van Aelst et al. 1993; Rodríguez-García et al. 2003; Jiang et al. 2007; Zienkiewicz et al. 2011). Pollen OBs are synthesized mainly in the vegetative cell of the pollen grain as it has been reported in *Olea europaea* (Rodríguez-García et al. 2003), *Brassica napus* (Charzyńska et al. 1989) or *Arabidopsis thaliana* (Owen and Makaroff 1995). However, OBs have been observed also in both pollen cells of lily (Nakamura and Miki-Hirosige 1985) and only in the generative cytoplasm in *Polystachia pubescens* (Schlag and Hesse 1992). The accumulation of lipid reserves takes place following the rapid lipid biosynthesis, soon after the vacuolation stage of the microspore (Fig. 4) (Evans et al. 1992; Piffanelli et al. 1997; Rodríguez-García et al. 2003; Zienkiewicz et al. 2011).

The increase of OB numbers during pollen development is positively correlated with high levels of OBs-associated proteins (Zienkiewicz et al. 2011). The expression of three genes encoding oleosins was found in the microspores of *Arabidopsis thaliana* (Kim et al. 2002). Oleosin mRNAs were detectable also in the olive developing microspore and pollen grain (Alché et al. 1999). In contrast, lily pollen oleosin is accumulated at further steps of pollen maturation but not at the pre-meiosis and microspore stages (Jiang et al. 2007). Possible function of these oleosins could be stabilization of OBs during pollen development and maturity. The level of olive pollen caleosin continuously increase after the asymmetric mitosis of microspore and during the subsequent steps of pollen maturation, and is positively correlated with increasing number of OBs inside developing pollen (Zienkiewicz et al. 2011).

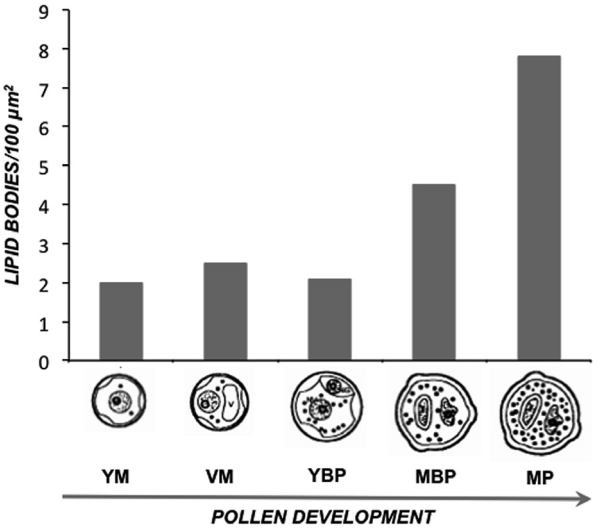

Figure 4. Oil body accumulation during olive pollen development. YM, young microspore; VM, vacuolated microspore; YBP, young bicellular pollen; MBP, mid bicellular pollen; MP, mature pollen (modified from Rodríguez-García et al. 2003).

OBs Mobilization during Pollen Germination

Fertilization in flowering plants relies on the growth and elongation of the pollen tube, which delivers the sperm cells to the female gametophyte in the ovule (Russell 1991). Rapid elongation of the pollen tube demands an energy production and biosynthetic capacity (Taylor and Hepler 1997). The mobilization of storage OBs resumes at a more rapid rate following germination and growth of the pollen tube (Piffanelli et al. 1998). It has been proposed that OBs serves as energy supply for the pollen tubes growth and as source for the rapid synthesis of membrane lipids after germination (Dorne et al. 1988; Zienkiewicz et al. 2013). In hydrated olive pollen, OBs polarize towards the exine and aperture and move to the emerging pollen tube, which is most likely caused by cytoplasmic streaming (Rodríguez-García et al. 2003). In the olive, OBs mobilization starts after pollen hydration and progress during the pollen tube growth (Zienkiewicz et al. 2010, 2013). During this period, the number of OBs decrease almost 20-fold in the pollen grain, whereas the opposite tendency is observed in the pollen tube, suggesting that oil bodies moved from the pollen grain towards the growing pollen tube as soon as the pollen grain begins to germinate (Fig. 5). After 12 h of *in vitro* germination the OBs were almost completely metabolized (Zienkiewicz et al. 2010). Moreover, sugar removal from the

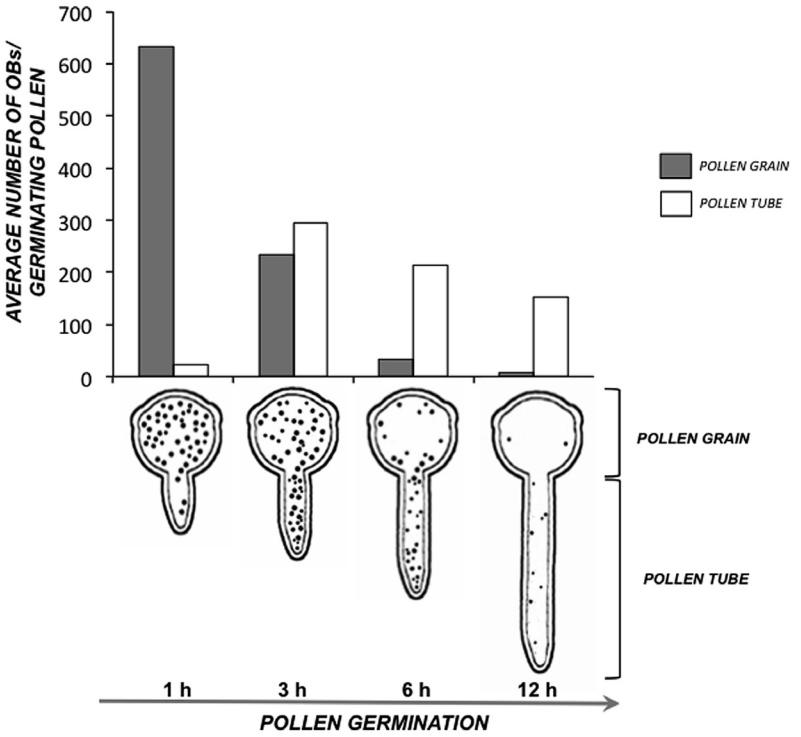

Figure 5. Schematic representation of changes in the number and localization of OBs within the olive pollen grain and pollen tube during *in vitro* germination.

germinating medium did not influence pollen tube growth rate, suggesting that OBs are sufficient as carbon supply for proper, early pollen tube growth (Zienkiewicz et al. 2013).

In mature pollen grain, OBs are frequently in close contact with the ER cisternae (Rodríguez-García and Fernández 1990), which persist during pollen germination and may facilitate mobilization of the OBs into membrane components. After lily pollen germination the OBs were individually surrounded by tubular membrane structures, encapsulated in the vacuoles (Jiang et al. 2007). These results suggested that degradation of OBs during pollen tube elongation might be carried out by vacuolar digestion. The apparent fusion of OBs with vacuoles has also been reported during seed germination in *Arabidopsis* (Poxleitner et al. 2006) and proposed as part of the TAG mobilization. Moreover, it was found that caleosin participates in OBs-vacuole interactions (Poxleitner et al. 2006). In the pollen tube of the olive, caleosin was localized in the intracellular membranes and in the tonoplast (Zienkiewicz et al. 2010). Therefore, it might

be possible that caleosin mediate OB-vacuole membrane fusion in pollen tube (Zienkiewicz et al. 2010). Caleosin was detected in olive pollen during the whole germination process and its level decreased coincidentally with the reduction in the number of OBs present in the pollen tube (Zienkiewicz et al. 2010).

In germinating seeds OBs breakdown occurs by the action of hydrolytic enzymes such as phospholipase A, lipoxygenase and lipase (Eastmond 2006; Rudolph et al. 2011). Lipid mobilization is initiated by the activation of TAG lipases, which hydrolyze TAGs and release glycerol and fatty acids (Graham 2008). The free fatty acids are then transported to the glyoxysome where their β-oxidation occurs. In an alternative pathway of OBs breakdown, lipoxygenase activity leads to oxygenation of storage TAGs to their hydroperoxy derivatives, which are subsequently cleaved by lipases (Feussner et al. 2001). However, before lipase and LOX get access to the TAGs it requires the proteolytic degradation of structural proteins of OBs and partial degradation of phospholipid monolayer of this organelle presumably by a patatin-like phospholipase (Matsui et al. 1999; Rudolph et al. 2011). Recently, we have demonstrated that phospholipase A, lipoxygenase and lipase are also likely involved in OBs mobilization during pollen tube growth (Zienkiewicz et al. 2013). The presence of phospholipase A, lipoxygenase and lipase activities associated to the surface of OBs was demonstrated during olive pollen germination. Interestingly, phospholipaseA activity on the OBs surface was detected in mature and germinated pollen, meanwhile lipase activity and lipoxygenase protein were associated with OBs boundaries after germination (Zienkiewicz et al. 2013). These results suggest that the mobilization of storage lipids by lipase and lipoxygenase during pollen germination is promoted by a phospholipase A (Zienkiewicz et al. 2013). Moreover, the capacity of olive pollen to germinate was strongly hampered by lipoxygenase and lipase inhibitors (Rejón et al. 2012; Zienkiewicz et al. 2013). This effect was usually accompanied by an accumulation of OBs at the germinative aperture. Taken together, all these data support the essential role of storage lipids in pollen tube growth and a strong functional relationship between OBs mobilization pathway and pollen performance.

In oilseeds, after germination free fatty acids derived from TAGs produces acetyl-CoA, which is converted to sucrose through the glyoxylate cycle and gluconeogenesis (Eastmond et al. 2000). Two enzymes unique to the glyoxylate cycle, malate synthase (MS) and isocitrate lyase (ICL) have been localized in seed gloxysomes. The presence of glyoxysome-like microbodies (Charzyńska et al. 1989) and expression of *MS* and *ICL* genes was also indicated in developing *Brassica napus* pollen (Zhang et al. 1994). However, expression of both genes is not activated during pollen

germination when OBs are mobilizated (Zhang et al. 1994). The presence of a functional glyoxylate cycle during pollen germination has yet to be confirmed.

Conclusions

Lipids provide the structural basis for cell membranes and fuels for metabolism in all living organisms. Recently, it has been found that lipids function also as mediators in many plant processes including signal transduction, cytoskeletal rearrangements and membrane trafficking. These processes are crucial for cell survival, growth and differentiation and for plant response to water, temperature, salinity and pathogens. It should be clearly stated, that understanding of physiological and molecular nature of pollen performance is extremely important because of its direct connexion with food production, industrial biotechnology and medicine. However, it can be seen that our knowledge of plant lipid biology during sexual plant reproduction is insufficient. Despite highly developed methods of molecular biology we still don't know which, how many and how proteins associated to lipid metabolism are involved in cellular processes regulated by lipids. In this context pollen grain still remains unexplored. Thus, further investigations should be extended in order to explore the pollen machinery directly connected to conversion of storage lipids into substrates used in lipid signalling, energy production and cellular membranes synthesis during pollen performance, with a special emphasis on pollen-pistil interaction.

References

Abell, B.M., M. Hahn, L.A. Holbrook and M.M. Moloney. 2004. Membrane topology and sequence requirements for oil body targeting of oleosin. Plant J. 37: 461–470.

Alché, J.D., A.J. Castro and M.I. Rodríguez-García. 1999. Expression of oleosin genes in the olive (*Olea europea* L.) anther. *In*: C. Clément, E. Pacini and J.C. Audran (eds.). Anther and Pollen. From Biology to Biotechnology. Springer, Berlin, pp. 91–99.

Baker, H.G. and I. Baker. 1979. Starch in angiosperm pollen grains and its evolutionary significance. Am. J. Bot. 66: 591–600.

Bednarska, E. 1988. Ultrastructural transformations in the cytoplasm of differentiating *Hyacinthus orientalis* L. pollen cells. Acta Bot. Pol. 57: 235–245.

Charzyńska, M., M. Murgia and M. Cresti. 1989. Ultrastructure of the vegetative cel of *Brassicanapus* pollen with particular reference to microbodies. Protoplasma 152: 22–28.

Chen, D.H., C.L. Chyan, P.L. Jiang, C.S. Chen and J.T.C. Tzen. 2012. The same oleosin isoforms are present in oil bodies of rice embryo and aleurone layer while caleosin exists only in those of the embryo. Plant Physiol. Biochem. 60: 18–24.

Chen, J.C.F., C.C.Y. Tsai and J.T.C. Tzen. 1999. Cloning and secondary structure analysis of caleosin, a unique calcium-binding protein in oil bodies of plant seeds. Plant Cell Physiol. 40: 1079–1086.

Dorne, A.-J., R. Kappler, U. Kristen and E. Heinz. 1988. Lipid metabolism during germination of tobaco pollen. Phytochemistry 27: 2027–2031.

Eastmond, P.J. 2006. *SUGAR-DEPENDENT* encodes a patatin domain triacylglycerol lipase that initiates storage oil breakdown in germinating *Arabidopsis* seeds. Plant Cell 18: 665–675.

Eastmond, P.J., V. Germain, P.R. Lange, J.H. Bryce, S.M. Smith and I.A. Graham. 2000. Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. PNAS 97: 5669–5674.

Evans, D.E., P.E. Taylor, M.B. Singh and R.B. Knox. 1992. The interrelationship between the accumulation of lipids, protein and the level of acyl carrier protein during the development of *Brassica napus* L. pollen. Planta 186: 343–354.

Feussner, I., H. Kuhn and C. Wasternack. 2001. Lipoxygenase-dependent degradation of storage lipids. Trends Plant Sci. 6: 268–273.

Frandsen, G.I., F. Müller-Uri, M. Nielsen, J. Mundy and K. Skriver. 1996. Novel plant Ca^{+2}-binding protein expressed in response to abcisic acid snd osmotic stress. J. Biol. Chem. 271: 343–348.

Goldberg, R.B., T.P. Beals and P.M. Sanders. 1993. Anther development: basic principles and practical application. Plant Cell 51: 1217–1229.

Graham, I.A. 2008. Seed storage oil mobilization. Annu. Rev. Plant Biol. 59: 115–142.

Hanano, A., M. Burcklen, M. Flenet, A. Ivancich, M. Louwagie, J. Garin and E. Blee. 2006. Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif. J. Biol. Chem. 281: 33140–33151.

Hsieh, K. and A.H.C. Huang. 2004. Endoplasmatic reticulum, oleosins and oils in seeds and tapetum cells. Plant Physiol. 136: 3427–3434.

Huang, A.H.C. 1994. Structure of plant seed oil bodies. Curr. Opin. Struct. Biol. 4: 493–498.

Huang, A.H.C. 1996. Oleosin and oil bodies in seeds and other organs. Plant Physiol. 110: 1055–1061.

Jiang, P.L., C.S. Wang, C.M. Hsu, G.Y. Yauh and J.T. Tzen. 2007. Stable oil bodies sheltered by a unique oleosin in lily pollen. Plant Cell Physiol. 48: 812–821.

Jiang, P.L., G.Y. Yauh, C.S. Wang and J.T. Tzen. 2008. A unique caleosin in oil bodies of lili pollen. Plant Cell Physiol. 49: 1390–1395.

Jiang, P.L., J.C. Chen, S.T. Chiu and J.T. Tzen. 2009. Stable oil bodies sheltered by a unique caleosin in cycad megagametophytes. Plant Physiol. Biochem. 47: 1009–1016.

Kim, H.U., K. Hsei, C. Ratnayake and A.H.C. Huang. 2002. A novel group of oleosins is present inside the pollen of *Arabidopsis*. J. Biol. Chem. 277: 22677–22684.

Kim, Y.Y., K.W. Jung, K.S. Yoo, J.U. Jeung and J.S. Shin. 2011. A stress-responsive caleosin-like protein, AtCLO4, acts a a negative regulator of ABA respones in *Arabidopsis*. Plant Cell Physiol. 52: 874–884

Leprince, O., A.C. van Aelst, H.W. Pritchards and D.J. Murphy. 1998. Oleosins prevent oil-body coalescence during seed imbibition as suggested by a low-temperature scanning electron microscope study of desiccation-tolerant and -sensitive oilseeds. Planta 204: 109–119.

Lin, L.J., S.S.K. Tai, C.C. Peng and J.T.C. Tzen. 2002. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. Plant Physiol. 128: 1200–1211.

Lin, L.J. and J.T.C. Tzen. 2004. Two distinct steroleosins are present in seed oil bodies. Plant Physiol. Biochem. 42: 601–608.

Liu, H., P. Hedley, L. Cardle, K.M. Wright, I. Hein, D. Marshall and R. Waugh. 2005. Characterisation and functional analysis of two barley caleosins expressed during barley caryopsis development. Planta 221: 513–522.

Matsui, K., K. Hijiya, Y. Tabuchi and T. Kajiwara. 1999. Cucumber cotyledon lipoxygenase during postgerminative growth. Its expression and action on lipid bodies. Plant Physiol. 119: 1279–1287.

Mayfield, J.A. and D. Preuss. 2000. Rapid initiation of *Arabidopsis* pollination requires the oleosin-domain protein GRP17. Nat. Cell Biol. 2: 128–130.

McCormick, S. 1993. Male gametophyte development. Plant Cell 5: 1265–1275.

Mepham, R.H. and G.R. Lane. 1970. Observation on the fine structures of developing microspores of *Tradescantia bracteata*. Protoplasma 70: 1–20.

Murphy, D.J. 1993. Structure, function and biogenesis of storage lipid bodies and oleosins in plants. Prog. Lipid Res. 32: 247–280.

Murphy, D.J. 2001. Biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog. Lipid Res. 40: 325–438.

Murphy, D.J. 2006. The extracellular pollen coat in members of the *Brassicaceae*: composition, biosynthesis and functions in pollination. Protoplasma 228: 31–39.

Murphy, D.J. 2012. The dynamic roles of intracellular lipid droplets: from archaea to mammals. Protoplasma 249: 541–585.

Naested, H., G.I. Frandsen, G.Y. Jauh, I. Hernandez-Pinzon, H.B. Nielsen, D.J. Murphy, J.C. Rogers and J. Mundy. 2000. Caleosins: Ca^{+2}–binding proteins associated with lipid bodies. J. Plant Mol. Biol. 44: 463–476.

Nakamura, S. and H. Miki-Hirosige. 1985. Fine-structural study on the formation of the generative cell wall and intine-3 layer in a growing pollen grain of *Lilium longiflorum*. Am. J. Bot. 72: 365–375.

Owen, H.A. and C.A. Makaroff. 1995. Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L.) Heynh. Ecotype Wassilewskija (Brassicaceae). Protoplasma 185: 7–21.

Piffanelli, P., J.H.E. Ross and D.J. Murphy. 1997. Intra and extracellular lipid composition and associated gene expression patterns during pollen development in *Brassica napus*. Plant J. 11: 549–652.

Piffanelli, P., J.H.E. Ross and D.J. Murphy. 1998. Biogenesis and function of the lipidic structures of pollen grains. Sex. Plant Reprod. 11: 65–80.

Poxleitner, M., S.W. Rogers, A.L. Samuels, J. Browse and J.C. Rogers. 2006. A role for caleosin in degradation of oil-body storage lipids during seed germination. Plant J. 47: 917–933.

Purktova, Z., P. Jolivet, M. Miquel and T. Chardot. 2008. Structure and function of seed lipid-body-associated proteins. CR Biol. 331: 746–754.

Rejón, J.D., A. Zienkiewicz, M.I. Rodríguez-García and A.J. Castro. 2012. Profiling and functional classification of esterases in olive (*Olea europaea*) pollen during germination. Ann. Bot. 110: 1035–1045.

Robert, L.S., J. Gerster, S. Allard, L. Cass and J. Simmonds. 1994. Molecular characterization of two Brassica napus genes related to oleosins, which are highly expressed in the tapetum. Plant J. 6: 927–933.

Roberts, M.R., R. Hodge and R. Scott. 1995. *Brassica napus* pollen oleosins possess a characteristic C-terminal domain. Planta 195: 469–470.

Rodríguez-García, M.I. and M.C. Fernández. 1990. Ultrastructural evidence of endoplasmatic reticulum changes during the maturation of the olive pollen grain (*Olea europaea* L., *Oleaceae*). Pl. Syst. Evol. 171: 221–231.

Rodríguez-García, M.I., M. M'rani-Alaoui and M.C. Fernández. 2003. Behavior of storage lipids during development and germination of olive (*Olea europaea* L.). pollen. Protoplasma 221: 237–244.

Ross, J.H.E. and D.J. Murphy. 1996. Characterization of anther-expressed genes encoding a major class of extracellular oleosin-like proteins in the pollen coat of Brassicaceae. Plant J. 9: 625–637.

Rudolph, M., A. Schlereth, M. Körner, K. Feussner, E. Berndt, M. Melzer, E. Hornung and I. Feussner. 2011. The lipoxygenase-dependent oxygenation of lipid body membranes is promoted by a patatin-type phospholipase in cucumber cotyledons. J. Exp. Bot. 62: 749–760.

Russell, S.D. 1991. Isolation and characterization of sperm cells in flowering plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 42: 189–204.

Schlag, M. and M. Hesse. 1992. The formation of the generative cell in *Polystachia pubescens* (Orchidaceae). Sex. Plant Reprod. 5: 131–137.

Scott, R.J., M. Spielman and H.G. Dickinson. 2004. Stamen structure and function 16: 46–60.

Shimada, T.L., T. Shimada, H. Takahashi, Y. Fukao and I. Hara-Nishimura. 2008. A novel role for oleosins in freezing tolerance of oilseeds in *Arabidopsis thaliana*. Plant J. 55: 798–809.

Siloto, R.M.P., K. Findlay, A. Lopez-Villalobos, E.C. Yeung, C.L. Nykiforouk and M.M. Moloney. 2006. The accumulation of oleosins determines the size of seed oil bodies in *Arabidopsis*. Plant Cell 18: 1961–1974.

Taylor, L.P. and P.K. Hepler. 1997. Pollen germination and tube growth. Ann. Rev. Plant Physiol. Plant Mol. Biol. 58: 461–491.

Ting, J.T.L., K. Lee, C. Ratnayake, K.A. Platt, R.A. Balsamo and A.H.C. Huang. 1996. Oleosin genes in maize kernels having diverse oil contents are constitutively expressed independent of oil contents: size and shape of intracellular oil bodies are determined by the oleosins/oils ratio. Planta 1: 158–165.

Ting, J.T.L., S.S. Wu, C. Ratnayake and A.H. Huang. 1998. Constituents of the tapetosomes and elaioplasts in Brassica campestris tapetum and their degradation and retention during microsporogenesis. Plant J. 16: 541–551.

Tzen, J.T.C. and A.H.C. Huang. 1992. Surface structure and properties of plant seed oil bodies. J. Cell Biol. 117: 327–335.

Tzen, J.T.C., Y. Cao, P. Laurent, C. Ratnayake and A.H.C. Huang. 1993. Lipids, proteins and structure of seed oil bodies from diverse species. Plant Physiol. 1001: 267–276.

Tzen, J.T.C., M.M. Wang, J.C.F. Chen, L.J. Lin and M.C.M. Chen. 2003. Seed oil body proteins: oleosin, caleosin and steroleosin. Curr. Topic Biochem. Res. 5: 133–139.

Tzen, J.T.C. 2012. Intergral proteins in plant oil bodies. ISRN Botany 2012: 16.

van Aelst, A.C., E.S. Person, J.L. Van Went and M. Cresti. 1993. Ultrastructural changes of *Arabidopsis thaliana* pollen during final maturation and rehydration. Zygote 1: 173–179.

Wetzel, C.L.R. and W.A. Jensen. 1992. Studies of pollen maturation in cotton: the storage reserve accumulation phase. Sex. Plant Reprod. 5: 117–127.

Wu, L.S.H., G.H.H. Hong, R.F. Hou and J.T.C. Tzen. 1999. Classification of the single oleosin isoform and characterization of seed oil bodies in gymnosperms. Plant Cell Physiol. 40: 326–334.

Yamamoto, Y., M. Nishimura, I. Hara-Nishimura and T. Noguchi. 2003. Behavior of vacuoles during microspore and pollen developmentin *Arabidopsis thaliana*. Plant Cell. Physiol. 44: 1192–1201.

Zhang, J.Z., D.L. Laudencia-Chingcuanco, L. Comai, L. Morris and J.J. Harada. 1994. Isocitrate lyase and malate synthase genes from *Brassica napus* L. are active in pollen. Plant Physiol. 104: 857–864.

Zhu, J., H. Chen, H. Li, J.F. Gao, H. Jiang, Ch. Wang, Y.F. Guan and A.N. Yang. 2008. Defective in *Tapetal Development* and *Function 1* is essential for anther development and tapetal function for microspore maturation in *Arabidopsis*. Plant J. 55: 266–277.

Zienkiewicz, A., K. Zienkiewicz, J.D. Rejón, M.I. Rodríguez-García and A.J. Castro. 2013. New insights into the early steps of oil body mobilization during pollen germination. J. Exp. Bot. 64: 293–302.

Zienkiewicz, K., A.J. Castro, J.D. Alché, A. Zienkiewicz, C. Suarez and M.I. Rodríguez-García. 2010. Identification and localization of a caleosin in olive (*Olea europaea* L.) pollen during *in vitro* germination. J. Exp. Bot. 61: 1537–1546.

Zienkiewicz, K., A. Zienkiewicz, M.I. Rodríguez-García and A.J. Castro. 2011. Characterization of a caleosin expressed during olive (*Olea europaea* L.) pollen ontogeny. BMC Plant Biol. 11: 12.

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Pollination Biology and Breeding System of European *Fritillaria meleagris* L. (Liliaceae)

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ABSTRACT

Fritillaria L. is a relatively large genus of the family Liliaceae, comprising 100–130 bulbiferous, mostly spring-flowering perennial plant species distributed in the Northern Hemisphere. Although most of *Fritillaria* species are of conservation concern, surprisingly little is known about the reproductive biology of these plants. For example, it would appear that, to date, the pollination biology of only two species has been studied extensively, and details of the breeding system are known only for a further small number of taxa. The present chapter explores the reproductive biology of the type species, *F. meleagris*, one of the most widely distributed representatives of the genus, red-listed or rare for all European countries of its range. Recent findings show that, contrary to earlier studies, this plant is not dichogamous nor obligatorily outcrossed. Fritillaria meleagris is a self-compatible plant, the P/O ratio indicates outcrossing species, and selfing (rarely occurring in natural populations) results in fully developed seeds. Throughout the species range its flowers are visited by almost 30 insect species, mostly social

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and solitary bees. The main reward for pollinators is pollen and nectar. The latter is produced by six perigonal nectaries and presented to pollinators throughout the flowering period of an individual flower. The maximum nectar secretion overlaps with that of maximum pollen presentation and stigmatic receptivity, and the nectar is resorbed at the final stages of flowering. The nectar composition is composed of sucrose, glucose and fructose in approximately equal quantities and this composition does not change significantly during subsequent stages of flowering. In the natural populations *F. meleagris* is not pollen limited. Although the largest recorded pollen loads are transferred by small pollen-collecting solitary bees, there are no significant differences in pollen deposition and removal among the key floral visitors. However, due to their abundance on flowers, seasonal and floral constancy and tolerance of bad weather conditions, the key pollinators of *F. meleagris* are bumblebees (mostly of the most common species *Bombus terrestris* and *B. lapidarius*). All available literature suggests that the current decline of *F. meleagris* seems not to be caused by the species' pollination or breeding systems but by the plant's habitat loss. However, *ex situ* experiments suggest that smaller populations may be prone to pollen-limitation and, in such cases, the plant species' dependence on generally rare pollinators and largely out-crossed breeding system may prompt local extinctions of the species.

Keywords: *Bombus*, *Fritillaria*, Fritillary, Liliaceae, pollination, red list, solitary bees

Introduction

Fritillaria L. (type species *F. meleagris* L.) is a genus of the family Liliaceae, comprising 100–130 species distributed in the Northern Hemisphere (Tamura 1998; Rønsted et al. 2005; Mabberley 2008), with a large representation in the Mediterranean region (Zaharof 1986; Teksen and Aytac 2011) and California (Rønsted et al. 2005). These species are bulbiferous, mostly spring-flowering perennials, with an erect flowering stem usually producing a single flower, but occasionally, a multi-flowered raceme. Although *Fritillaria* is a relatively large genus, and most of its species are of conservation concern, little is known about the reproductive biology of these plants. For example, it would appear that, to date, the pollination biology of only two species, namely, *F. imperialis* L. and *F. meleagris*, has been studied extensively, and details of the breeding system are known only for a further small number of taxa.

Flowers of *Fritillaria* species are usually nodding, actinomorphic and have a typical tulip-like, trimerous, campanulate perianth (Tamura 1998). The perianth parts are usually white, greenish, yellow or purplish to reddish and the sepals of many species (e.g., the widely distributed European *F. meleagris*) have a characteristic checkerboard pattern, hence

the name of the genus (Latin *fritillus*, a dice-box). The plants generally produce bisexual flowers, but some cases of andromonoecy, androdioecy or gender disphasy are also known (Knuth 1899; Shimizu et al. 1998; Mancuso and Peruzzi 2010; Peruzzi 2012; Peruzzi et al. 2012). Some species (e.g., *F. imperialis, F. meleagris,* and *F. koidzumiana* Ohwi) are considered to be dichogamous-protogynous (Knuth 1899; Hedström 1983; Burquez 1989; Kawano et al. 2008). This suggestion, however, may not necessarily reflect the true protogyny of the plants but may, in some cases, be based on the characteristically protruding, trifid stigma with its well-developed papilla, which is already present at the bud stage of the flower (Knuth 1899). This structure is mainly found in representatives of the subgenus *Fritillaria* (Rønsted et al. 2005), and is thought to indicate that stigma receptivity may precede the pollen presentation phase. Recently, Zych and Stpiczyńska (2012) have demonstrated experimentally that this is not true, at least for *F. meleagris*, and that stigmas in this species become receptive concomitantly with anthesis. This was also supported by pollen germination tests and the fact that flowers showed no receptivity prior to anther dehiscence.

Published data show that the breeding systems of *Fritillaria* species range from self-incompatible (Burquez 1989; Yashima et al. 1997) to self-compatible, demonstrating various degrees of out-crossing, including facultatively autogamous species (Hedström 1983; Kawano et al. 2008; Mancuso and Peruzzi 2010; Zhang et al. 2010; Zych and Stpiczyńska 2012). Knuth (1899) suspected that some *F. meleagris* plants with cylindrical and closed corollas, as found in German populations of the species, may even be cleistogamous, but this is not true of cultivated plants or for another European population studied by Zych and Stpiczyńska (2012). Cases of nearly-sterile species of hybrid origin have also been reported (U.S. Fish and Wildlife Service 2003).

Based on nectar characteristics, Rix and Rast (1975) concluded that most *Fritillaria* species are probably pollinated by bees and wasps. These authors, however, studied only European and Asiatic taxa. Indeed, floral visitors to *Fritillaria* flowers often include, as well as Hymenoptera (mostly various species of bees and wasps), other insects, such as Diptera, Lepidoptera and Coleoptera (Hedström 1983; Bernhardt 1999; Kawano et al. 2008; Zych and Stpiczyńska 2012; Zych et al. 2013), an even birds, as in the case of Asiatic *F. imperialis* and some North American species, such as *F. gentneri* Gilkey and *F. recurva* Benth. (Burquez 1989; Peters et al. 1995; Bernhardt 1999; U.S. Fish and Wildlife Service 2003). This, however, does not necessarily reflect the whole spectrum of pollinator vectors for this genus, since a detailed assessment of the effectiveness of animals as pollinators has so far been undertaken only for *F. imperialis* (Burquez 1989) and *F. meleagris* (Zych and Stpiczyńska 2012; Zych et al. 2013).

One of the most widely distributed representatives of the genus is *F. meleagris* (Fig. 1), the type species. Recently, the reproductive biology of this taxon has been extensively studied (Hedström 1983; Stpiczyńska et al. 2012; Zych and Stpiczyńska 2012; Zych et al. 2013). Since it possesses many of the characteristic features of the genus, it is considered a good reference species for comparison with other representatives of both genus and family.

Figure 1. Left: *Fritillaria meleagris* in flower with both purple- and white-flowered individuals. Photo by M. Zych. Right: *Fritillaria meleagris* L. (Liliaceae). Photograph by M. Zych, with kind permission from Springer Sciences and Business Media.

Color image of this figure appears in the color plate section at the end of the book.

Fritillaria meleagris L.

Fritillaria meleagris L. is distributed from Western to Central Europe, growing on wet, eutrophic meadows (usually flood-plains) and in open woodlands. In the Alps, it is found in alpine pastures (Rix 1968), and British and Scandinavian populations of the species are regarded to have anthropogenic origins (Zhang and Hytteborn 1985; Harvey 1996). The plant is red-listed or rare for all European countries of its range, and considered vulnerable (VU) for the whole of Central Europe (Schnittler and Günther 1999).

Fritillaria meleagris is a long-lived perennial (life span approx. 30 years; Horsthuis et al. 1994), that flowers in spring (April-May). During the flowering period (6–7 d; Zych and Stpiczyńska 2012), the plant grows to a height of approximately 15–60 cm and produces on the upper part of

the stem 4–5 narrow, linear leaves and usually a single, large, pendulous, broadly campanulate terminal flower, or very rarely 2–3 flowers. These measure approximately 20 mm in diameter, consist of six purplish-pink (rarely white or whitish) tepals, some 30–45 mm long, marked with a characteristic tessellate pattern resembling that of a chess-board, six stamens whose filaments measure 10–13 mm in length, and a single tricarpellary, superior ovary bearing a style terminating in a trifid stigma (Knuth 1899; Rix 1968; Piórecki 2001).

The ratio of white-flowering to purple-flowering individuals varies between populations. In the large, natural population found in Krówniki in SE Poland (>>1,000,000 flowering individuals) and studied by Zych and Stpiczyńska (2012), most flowers were purple (the authors encountered only 10–30 white individuals per annum), whereas in the smaller population at Stubno (approx. 15 km NE from Krówniki), only purple-flowered individuals were found. Richards (1997, p. 195) states that all British populations contain both white- and purple-flowered individuals. The Scandinavian populations described by Hedström (1983), however, contained 3.6–5.8% white-flowered plants, depending on the year. By contrast, the now extinct population at Sławno, near Koszalin, a coastal region of Poland, contained only approximately 5% purple-flowered plants. This population was, however, regarded to be anthropogenic in origin (Stecki et al. 1961). According to Hedström (1983), the white-coloured form reflects more UV light than does the purple one, but in the Swedish population (Uppsala region) studied by that author, flower visitors (mostly bumblebees and honeybees) did not distinguish between differently coloured flowers. We obtained similar results for an *ex situ* population created at the University of Warsaw Botanic Garden (described by Zych and Stpiczyńska 2012). The proportion of the white-flowered form in this population was quite high (approx. 0.25), and our observations, conducted over 3 d in 2011, during the peak flowering period, failed to demonstrate a preference by pollinators for either of the two colour forms (Spearman r = 0.033, $P = 0.94$), and deposited similar numbers of pollen grains on the stigmas of both purple and white flowers (ANOVA on log-transformed data $F_{1, 45} = 3.18$, $P = 0.08$; Fig. 2).

Breeding System

An individual flower of *F. meleagris* produces, on average, 148 ± 26 ovules (Zych et al. 2013), and has a P/O ratio (the ratio of pollen grains to ovules) of 1825 and an OCI index (outcrossing index) of 4 (corolla > 6 mm wide, flowers herkogamous), indicating, according to Cruden (1977), that this is a xenogamous (outcrossing) species. This was confirmed experimentally for a Swedish population by Hedström (1983), who showed that self-pollinated plants did not set fruit. A similar experiment was also performed by Zych

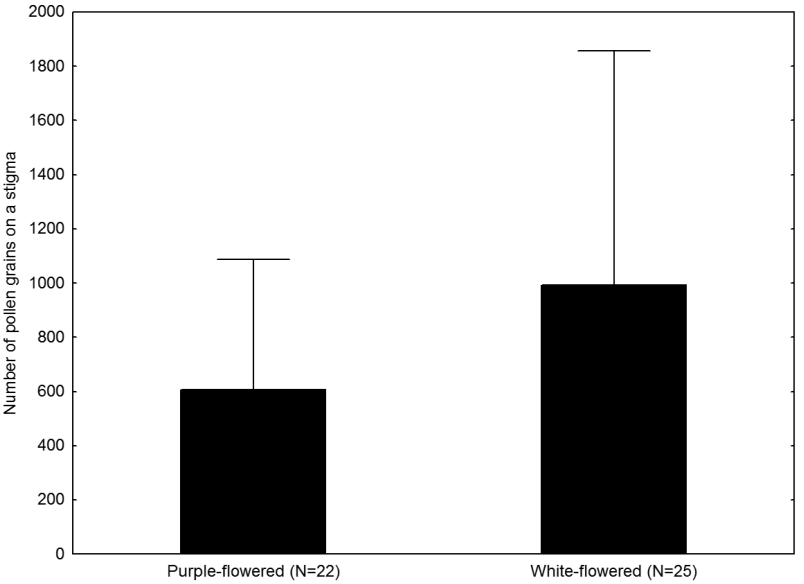

Figure 2. Stigmatic pollen loads (mean ± SD) of purple- and white-flowered forms of *Fritillaria meleagris* are not significantly different (ANOVA on log-transformed data $F_{1,45} = 3.18$, $P = 0.08$). Stigmas from randomly chosen plants were collected during the final stage of flowering (bud-like, wilting flowers) from an *ex situ* population described by Zych and Stpiczyńska (2012), and analyzed using the method of Zych et al. (2013).

and Stpiczyńska (2012) for the largest Polish population of this species, and also on commercially available, cultivated plants. These authors showed that although autonomous self-pollination, probably due to floral herkogamy, indeed resulted in very low seed production (on average 0–6 seeds in bagged flowers vs. 86–118 seeds in open pollinated, wild plants), induced self-pollination (self-pollen actively transferred to the stigma) yielded much higher seed set (on average 8–69 seeds per annum; Fig. 2), indicating some potential for selfing (estimated by these authors as 3–34% seed production per annum).

Nectaries and Nectar

In *F. meleagris*, the main reward offered to pollinators is nectar. It is produced by specialized secretory structures, the floral nectaries. These are of the perigonal type and are positioned adaxially on each of six perianth segments, in a groove located 2–4 mm above the base of the tepal (Knuth 1899; Hedström 1983). All the nectaries, regardless of whether they are located on the inner or outer tepals, are of similar size and, at anthesis, are equally accessible to potential pollinators (Stpiczyńska et al. 2012). The

nectar is presented on a relatively exposed, glabrous surface, unlike the subgenus *Rhinopetalum*, where the nectary surface is bordered by lobes or hairy ridges (Bakhshi Khaniki and Persson 1997) that may restrict nectar feeding by insects with relatively long proboscises. Moreover, the nodding flowers of *F. meleagris* also limit reward access to a relatively small group of visitors, predominantly large Hymenoptera (Zych and Stpiczyńska 2012).

Stpiczyńska et al. (2012) described in detail the floral nectar secretion in *F. meleagris.* These authors showed that nectar was presented to pollinators for approximately 5–6 d. The light green nectaries contrasted markedly with the white- and dark-purple-patterned tepals. SEM observations also revealed that the secretory surface was clearly different from that of the non-secretory region, since the cuticle of the former had distinct blisters that usually coincided with the position of the middle lamella between adjoining epidermal cells. Often, perforations were visible on these cuticular blisters, together with nectar residues. In terms of structure, the nectaries consisted of a single-layered epidermis lacking stomata and 3–4 layers of subepidermal, nectariferous parenchyma. The nectary was supplied by a single, main vascular bundle and several smaller vascular bundles that ended in the subepidermal secretory layer. These bundles contained xylem and phloem elements. The nectary secretory cells of *F. meleagris* shared many features with those of other plant species, in that they were small with large nuclei and contained small vacuoles and dense, intensely staining cytoplasm. TEM revealed abundant arrays of endoplasmic reticulum, dictyosomes and secretory vesicles. However, unlike the nectaries of the majority of investigated plant species, where amyloplasts or chloro-amyloplasts were present, at least during the pre-secretory stage, starch was not detected in the nectary cells of *F. meleagris*. In the absence of starch, sugars secreted in the nectar are probably delivered by the phloem sap.

Stpiczyńska et al. (2012) also reported the presence of transfer cells with prominent labyrinthine wall ingrowths in the nectariferous cells of *F. meleagris.* These outgrowths develop in the cells of many organs in plants, as well as in nectaries. They were also noted in fungi. Generally, cells with wall ingrowths are termed transfer cells, since wall ingrowths increase the surface area of the plasmalemma and thus improve transport capacity (for a review see Offler et al. 2002). In *F. meleagris*, cell wall ingrowths are particularly prominent on the tangential walls of epidermal and nectariferous parenchyma cells during the stage of maximum secretory activity. The presence of wall ingrowths frequently polarizes the direction of solute flow, and it is possible that cell wall protuberances in *F. meleagris* facilitate the transport of nectar within the nectary, as well as nectar secretion and nectar resorption during the final stage of anthesis, since these structures persist unchanged in both nectariferous epidermal cells and subepidermal parenchyma (Stpiczyńska et al. 2012). These authors

also showed that nectaries commence secretory activity, and small droplets of nectar appear on the surface of the nectary, just as the floral buds are opening (Stpiczyńska et al. 2012). The availability of secreted nectar increases concomitantly with anthesis, and the whole nectary groove becomes filled with nectar. Nectar remains on the nectary surface until the final stage of anthesis, but then disappears. This coincides with the tepals losing their turgor, and the flower reassuming a bud-like form. The period of maximum nectar secretion overlaps with that of maximum pollen presentation and stigmatic receptivity. Accumulation of rewards (nectar and pollen) at a given time may enhance the attractiveness of the flower to pollinators, and allow maximum benefit to be gained from just a single visit. This is particularly important since the frequency of pollinator visits to the flowers of *F. meleagris* is relatively low (0.2 visits per flower per h; Zych and Stpiczyńska 2012).

Stpiczyńska et al. (2012) also demonstrated that a single tepalar whorl, on average, secreted 5.4 ± 6.6 mg of nectar, whereas a single flower secreted 10.9 ± 13.0 mg of nectar. These authors also observed differences in nectar production from year to year. When perianth whorls were considered separately, the inner three tepals (inner whorl) produced approximately 20% more nectar than the outer whorl. In *F. meleagris*, the nectar concentration varied between 3–75%, and had an average sugar concentration that exceeded 50% (means calculated across years and floral stages). However, both nectar production and concentration depended on the floral stage being sampled (Fig. 3), with the highest scores, on average, being obtained for flowers displaying full anthesis (21.7 ± 16.8 mg; 70.5% mass and concentration, respectively) and the lowest obtained towards the end of anthesis (1.3 ± 2.69 mg; 16.9% mass and concentration, respectively). Intermediate results were obtained for flowers at the commencement of anthesis (9.8 ± 5.81 mg, 44% mass and concentration, respectively). A significant decline in the mass of nectar and nectar concentration during the final stage of anthesis indicated that nectar resorption occurs in the flowers of *F. meleagris*. Nectar resorption is a long-known phenomenon, but has rarely been addressed in studies based on nectar secretion (Nepi and Stpiczyńska 2008), and has generally been demonstrated (as in *Fritillaria*) only for the final stage of anthesis, as a post-pollination phenomenon, or following the completion of sexual stages in dichogamous flowers. It was generally considered to be a resource-recovery strategy resulting at least partially in the recycling of metabolites invested in nectar production. Moreover, it was thought that the modulation of two contrasting processes (nectar secretion and nectar resorption) resulted in homeostasis and maintained nectar composition within a narrow range appropriate for pollinators (Nepi et al. 2007). Reclamation of nectar components in Fritillary is facilitated by the presence of micro-channels or pores in the cuticle of the secretory epidermis (Stpiczyńska et al. 2012). Moreover, the efficiency

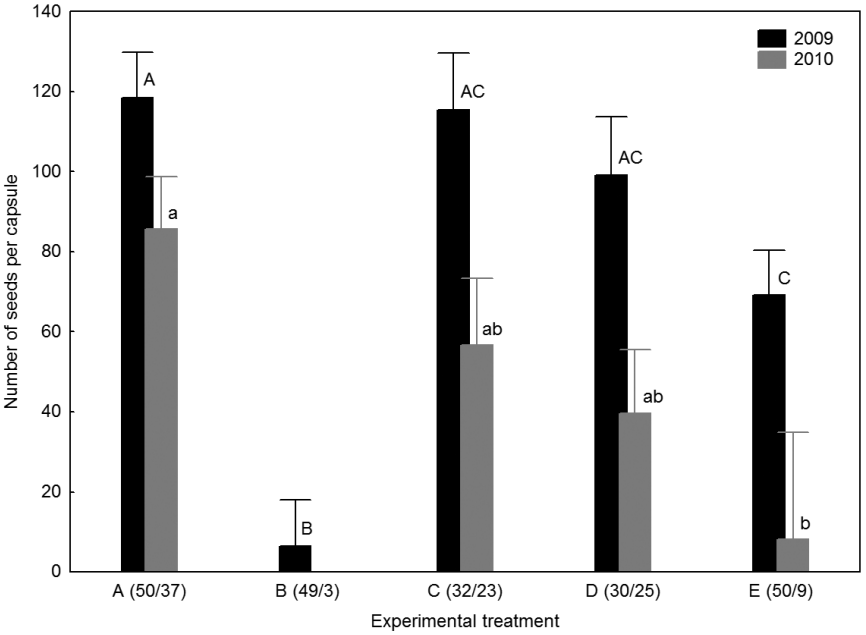

Figure 3. Average seed set (± 0.95 confidence interval) in *Fritillaria meleagris* plants from a natural population at Krówniki (SE Poland) subjected to five experimental pollination treatments in 2009 (black bars) and 2010 (grey bars): (A) open pollination (control), (B) autonomous self-pollination, (C) spontaneous cross-pollination (emasculated flowers), (D) supplemental pollination (emasculated flowers), (E) induced self-pollination. The numbers in brackets next to the treatment symbol indicate the number of experimental plants in 2009/2010, respectively. Means with different letters differ at $P < 0.01$ (Kruskal–Wallis test for multiple comparisons for a particular year). Owing to the very small sample size for B in 2010, this treatment was excluded from the Kruskal–Wallis test. Data based on Zych and Stpiczyńska (2012).

of nectar resorption (and also secretion) may be further improved in this species by the persistence of wall ingrowths in nectariferous epidermal and parenchymatous cells, even during the final stages of anthesis, when nectar resorption occurs.

The nectar of *F. meleagris* is composed of sucrose, glucose and fructose (Rix and Rast 1975; Stpiczyńska et al. 2012). Detailed analysis, provided by the latter authors, showed that these sugars appear in approximately equal quantities, with the amount of fructose slightly exceeding that of glucose and sucrose in the nectar profile for all stages investigated (33:28:39; sucrose:glucose:fructose ratio expressed as the relative percentage of total sugars; means calculated across all flowering stages). No other sugars were detected in the nectar. Furthermore, nectar composition did not change significantly during subsequent stages of flowering. As shown by these

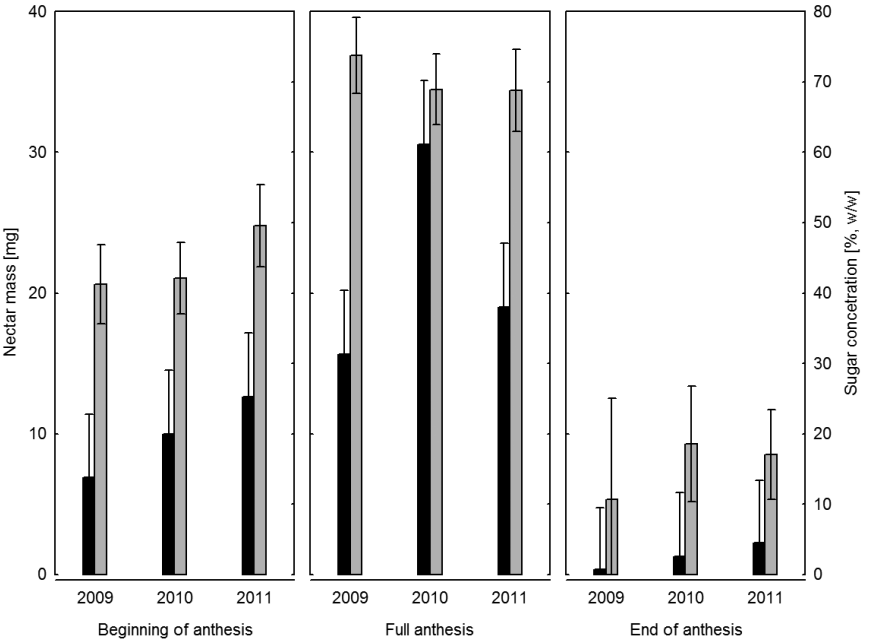

Figure 4. Nectar characteristics for *Fritillaria meleagris* during a 3-year study of a natural population from SE Poland; data based on Stpiczyńska et al. (2012). Left Y scale = mean nectar production per flower (mg)—black bars; and right Y scale = mean nectar sugar concentration (%, w/w) —grey bars. Error bars indicate 0.95 confidence interval.

authors, all nectar sugar constituents in *Fritillaria* nectar were resorbed to a similar degree, since the proportion of individual sugars at the final stage of anthesis remained almost unchanged.

Floral Scents

An overview of *F. meleagris* floral odours was given by Hedström (1983), who analysed floral scent by means of GC/MS and found it to contain terpene, ketone and alcohol components. Methyl vinyloketone, α-pinene, β-pinene, myrcene, linalool oxide, decanal, linalool, α-farnesene, and possibly 4-methyl-2-pentanol, were identified for the scent profile, as well as other compounds.

Floral Visitors and Pollinators

Ever since the study by Knuth (1899), *F. meleagris* has been regarded a bumblebee-pollinated species. However, published records show that throughout its geographical range, flowers of this species are visited by

almost 30 insect taxa, mostly Hymenopterans (Table 1). The most exhaustive data relating to insect visits to flowers of *F. meleagris* were recorded by Hedström (1983) and Zych and Stpiczyńska (2012). The latter authors made observations based on four flowering seasons for the largest Polish population of this species. In both cases, the most frequent visitors were bumblebees (*Bombus* spp.), and some years, these represented 100% of floral visitors to the Polish population (Zych and Stpiczyńska 2012). Visits by non-bee insects were reported to be extremely rare. This is in accordance with the observations of Knuth (1899), who, for a German population of *F. meleagris*, reported that flowers were visited only by *B. terrestris* (his observations, however, lasted for just one day). In contrast to other insects, e.g., honeybees (*Apis mellifera*) or solitary bees that appear on Fritillary flowers only during sunny and warm days, bumblebees visited flowers even during adverse weather conditions (Hedström 1983; Zych and Stpiczyńska 2012).

Regarding the quality component of pollination (Herrera 1987; Olsen 1997), some floral visitors, especially flies and beetles, proved to be ineffective pollinators, because they did not come into contact with the sexual parts of the flower or carried very little or no pollen (Hedström 1983; Zych and Stpiczyńska 2012). As shown by Zych et al. (2013), bumblebees and other large-bodied solitary bees (e.g., *Anthophora plumipes*) visit flowers of *F. meleagris* for nectar, whereas small solitary bees (e.g., *Andrena* spp.) visit for pollen. The large bees usually cling to sepals and, during their passage into the flower, they receive pollen on their thorax and wings, which is unlikely to be deposited in the same flower on exit, since these insects tend only to touch the outer part of the stigma, which is not receptive (Knuth 1899; Hedström 1983).

Table 1. Insect Visitors to Flowers of *Fritillaria meleagris* L. based on data by Knuth (1899), Hedström (1983), Zych and Stpiczyńska (2012) and Zych et al. (2013) from Germany, Poland and Sweden.

Taxonomic order	Families and species
Hymenoptera	Andrenidae: *Andrena bicolor* Fabr., A. *helvola* (L.), A. *lapponica* Zett., *A. mitis* Schmiede., *Evylaeus* (*Lasioglossum*) *calceatus* (Scop.), *Andrena* sp., Apidae: *Anthophora plumipes* (Pall.), *Apis mellifera* L., *Bombus hortorum* (L.), *B. hypnorum* (L.), *B. lapidarius* (L.), *B. lucorum* (L.), *B. muscorum* (L.), *B. pascuorum* (L.), *B. ruderarius* (Müller), *B. sylvarum* (L.), *B. terrestris* (L.), *Psithyrus bohemicus* (Seidl.), Megachilidae: *Osmia bicolor* (Schr.)
Diptera	Calliphoridae: *Cynomya mortuorum* (L.), Chironomidae: *Chironomus* Meig., Empididae: *Rhamphomyia* Meig., Stratiomyidae: *Odontomyia argentata* (Fabr.), Syrphidae: *Helophilus trivittatus* (Fabr.)
Coleoptera	Curculionidae: *Phyllobius calcaratus* (Scop.)
Ephemeroptera	Leptophlebiidae: *Leptophlebia marginata* (L.)

Small *Andrena* bees usually wander over the androecium and leave flowers with pollen grains that completely cover all their body surfaces. Their visits are generally longer than those of both bumblebees and honeybees (Zych and Stpiczyńska 2012; Zych et al. 2013). This behaviour is probably caused by problems encountered by some pollinators while foraging on pendulous flowers (Makino and Thomson 2012) and this orientation of the flower may, in turn, provide an effective strategy for reducing the number of visits to a flower by inferior pollinators (Thomson 2003). Makino and Thomson (2012) demonstrated that generally, bumblebees prefer upwardly-facing flowers, as these are easier to handle and probably reflect the innate preferences of insects. Nevertheless, under field conditions, these insects may concentrate their visits on pendulous flowers, especially if the latter, as is probably the case for *F. meleagris*, offer a particularly attractive food source. This probably also explains the relatively larger average *Fritillaria* body pollen loads found by Zych and Stpiczyńska (2012) on small solitary bees, suggesting that, at least in terms of quality, they are perhaps superior pollinators. However, the above authors also showed that these insects simultaneously carry significantly more heterogenous pollen loads that contains a large proportion (>80%) of non-*Fritillaria* pollen grains, indicating that they readily switch to other floral resources, whereas bumblebees and honeybees appear to be more faithful floral visitors (>90% of Fritillary pollen in an average body pollen load). However, body pollen load is just one criterion used to estimate the effectiveness of a pollinator, and is not necessarily the best (Adler and Irwin 2006; Zych et al. 2013). In order to determine more precisely the effectiveness of an insect pollinator, direct methods are preferable, e.g., pollen deposition on a stigma by a given pollinator species, seed set following visits by insects or the exclusion of particular pollen vectors (Johnson and Steiner 2000; Pellmyr 2002; Willmer 2011). The results of such a survey were presented by Zych et al. (2013) for an *ex situ* population of *F. meleagris* located at the University of Warsaw Botanic Garden (Warsaw, Poland). In a small garden compartment, these authors established a regular 10 × 5 grid of test tubes containing water, where they provided virgin flowers, each at the stage of pollen presentation. As the experiment was located at the very centre of the city, a small beehive and a commercially available colony of *Bombus terrestris* were placed in the vicinity of experimental plants in order to saturate the local pollinator community. Following a single visit by a bee to an experimental flower, the flower was collected and its stigma and anthers removed for further examination of stigmatic pollen load (pollen deposition) and pollen removal, respectively. These authors showed that flowers of this *ex situ* population, like those growing under natural field conditions, were serviced by overwintered, wild bumblebee queens and solitary bees. However, they did not observe any visits by individuals from the introduced honeybee or bumblebee colonies.

Pollen deposition did not differ significantly between the three functional groups that visited the flowers (*Bombus* spp., *Anthophora plumipes* males and *Andrena* spp.), and a single visit resulted, on average, in the deposition of 5715 ± 5954 *Fritillaria* pollen grains per stigma (mean ± SD)—sufficient to fertilize all ovules. At the same time, pollinators removed from each flower 18–37% of available pollen grains, but this varied greatly according to taxon, and consequently, the authors were not able to detect significant differences between visitor guilds. The estimated pollinator efficiency of pollen transfer was 5.8–7.6%, and approximately 1.3–2.2% of pollen produced by an individual Fritillary flower reached a conspecific stigma during a single visit. Given the dominance of *Bombus* visitors in all published records of *F. meleagris* pollination (Knuth 1899; Hedström 1983; Zych and Stpiczyńska 2012; Zych et al. 2013), the above results confirm that they are the most effective pollinators of this species throughout its geographical range in terms of both quality and quantity.

Concluding Remarks

Fritillaria meleagris is a threatened plant species throughout its geographical range (Schnittler and Günther 1999), and its populations are generally in decline (Zhang and Hytteborn 1985; Horsthuis et al. 1994; Iljanić et al. 1998; Čačko 2005; Cheffings and Farrell 2005; Piórecki 2005; Tomović et al. 2007; Andrienko and Tchorney 2009). The plant is zoogamous and mostly outcrossing, and is pollinated by bumblebees, insects that generally show a reduction both in population size and species diversity (Kosior et al. 2007; Goulson et al. 2008), indicating that pollination and subsequent seed production is a "demographically sensitive life history stage" (*sensu* Schemske et al. 1994). However, the pollination biology and breeding system characteristics of the species do not explain its decline, at least in Poland (Zych and Stpiczyńska 2012; Zych et al. 2013). All reported cases (Knuth 1899, Hedström 1983; Zych and Stpiczyńska 2012; Zych et al. 2013) show that Fritillary flowers are serviced mainly by *B. lapidarius*, *B. ruderarius* and *B. terrestris*, bumblebee species that are generally common (Pawlikowski 1996; Goulson 2003), and in some cases have even extended their range (MacDonald 2001; Dafni et al. 2010). Zych et al. (2013) also showed that this plant can be successfully pollinated by other floral visitors, even when present in relatively small populations. These authors, however, reported that a small population may be less attractive to honeybees or bumblebee workers, suggesting that there may be a "threshold" population size below which it no longer remains an attractive food source for certain pollinators. This well-known phenomenon of insect foraging, namely, floral constancy (for a general reference see, e.g., Goulson 2003; Willmer 2011), is generally correlated with the quality of food plant resources, as perceived

by pollinators. When floral resources explored by pollinators become scarce, pollinators switch to other food sources, even when the original plant offers a larger reward per-flower. There is currently no data available on this phenomenon for *F. meleagris*, and breeding system experiments showed no pollen limitation for the large, natural population studied by Zych and Stpiczyńska (2012). Nevertheless, in the smaller *ex situ* population created by these authors at the botanic garden, seed production by pollen-supplemented flowers was greater than in control, open pollinated plants. Although these differences were not statistically significant, and could, as suggested by the authors, be caused by the close genetic proximity of experimental plants, it is possible that they also indicate a decline in plant fertility associated with the reduction in size of the population (the so-called Allee effect; Stephens et al. 1999). If a population reaches a critical density or size, a severe pollen limitation may occur resulting in diminished seed production. Such an effect has been observed for many plant species growing in a range of different habitats (e.g., Lamont et al. 1993; Brys et al. 2004; Cheptou and Avendaño 2006; Elam et al. 2007; Dauber et al. 2010), and is most likely to occur in small populations of Fritillary. Such a situation is probable if habitat fragmentation continues, and the present decline in this species coincides with the Europe-wide loss of traditionally used, extensive wet meadows (Grootjans et al. 1996) and habitat destruction, both the result of the intensification of, or changes to agricultural practices (Piórecki 2005). Smaller populations, which are usually more prone to herbivore attacks (see, e.g., Kolb 2008), can become unattractive to pollinators and suffer from a shortage of pollination events which, in turn, leads to local extinctions. Thus, any attempt to conserve this endangered plant should consider both the conservation of its habitat and its biology, in particular its pollination and breeding strategies.

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References

Adler, L.S. and R.E. Irwin. 2006. Comparison of pollen transfer dynamics by multiple floral visitors: experiments with pollen and fluorescent dye. Ann. Bot. 97: 141–150.

Andrienko, T.L. and I.I. Tchorney. 2009. *Fritillaria meleagris* L. *In:* J.P. Diduch (ed.). Ukrainian Red Book. Plants., Globalconsulting, Kiev, pp. 137.

Bakhshi Khaniki, G. and K. Persson. 1997. Nectary morphology in South West Asian *Fritillaria* (Liliaceae). Nord. J. Bot. 17: 579–611.

Bernhardt, P. 1999. The rose's kiss. A natural history of flowers, Island Press, Washington, D.C.

Brys, R., H. Jacquemyn, P. Endels, F. Van Rossum, M. Hermy, L. Triest, L. De Bruyn and G.D.E. Blust. 2004. Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. J. Ecol. 92: 5–14.

Burquez, A. 1989. Blue tits, *Parus caeruleus*, as pollinators of the Crown Imperial, *Fritillaria imperialis*, in Britain. Oikos 55: 335–340.

Čačko, L. 2005. *Fritillaria meleagris* L. *In:* D. Průša, P. Eliáš jr., D. Dítě, L. Čačko, P. Krása, Z. Podešva, L. Kovář, M. Průšová, L. Hoskovec and L. Adamec (eds.). Chránené rastliny Českej a Slovenskej republiky, Computer Press, Brno, pp. 133.

Cheffings, C. and L. Farrell (eds.). 2005. The vascular plant red data list for Great Britain. http://www.jncc.gov.uk/page-3354, accessed on 6 Feb 2013.

Cheptou, P.-O. and V.L.G. Avendaño. 2006. Pollination processes and the Allee effect in highly fragmented populations: consequences for the mating system in urban environments. New Phytol. 172: 774–783.

Cruden, R.W. 1977. Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. Evolution 31: 32–46.

Dafni, A., P. Kevan, C.L. Gross and K. Goka. 2010. *Bombus terrestris*, pollinator, invasive and pest: An assessment of problems associated with its widespread introductions for commercial purposes. Appl. Entomol. Zoolog. 45: 101–113.

Dauber, J., J.C. Biesmeijer, D. Gabriel, W.E. Kunin, E. Lamborn, B. Meyer, A. Nielsen, S.G. Potts, S.P.M. Roberts, V. Sober, J. Settele, I. Steffan-Dewenter, J.C. Stout, T. Teder, T. Tscheulin, D. Vivarelli and T. Petanidou. 2010. Effects of patch size and density on flower visitation and seed set of wild plants: a pan-European approach. J. Ecol. 98: 188–196.

Elam, D.R., C.E. Ridley, K. Goodell and N.C. Ellstrand. 2007. Population size and relatedness affect fitness of a self-incompatible invasive plant. Proc. Nat. Acad. Sci. USA 104: 549–552.

Goulson, D. 2003. Bumblebees. Their behaviour and ecology, Oxford University Press, Oxford.

Goulson, D., G.C. Lye and B. Darvill. 2008. Decline and conservation of bumble bees. Ann. Rev. Entomol. 53: 191–208.

Grootjans, A.P., L.F.M. Fresco, C.C. de Leeuw and P.C. Schipper. 1996. Degeneration of species-rich *Calthion palustris* hay meadows; some considerations on the community concept. J. Veget. Sci. 7: 185–194.

Harvey, J.H. 1996. Fritillary and Martagon: wild or garden? Garden Hist. 24: 30–38.

Hedström, I. 1983. Kungsängsliljans pollinationsekologi. Svensk Bot. Tidskr. 77: 313–325.

Herrera, C.M. 1987. Components of pollinator quality—comparative analysis of a diverse insect assemblage. Oikos 50: 79–90.

Horsthuis, M.A.P., A. Corporaal, J.H.J. Schaminee and V. Westhoff. 1994. Ecology, distribution and syntaxonomy of *Fritillaria meleagris* L. in the Netherlands and surrounding countries. Phytocoenologia 24: 627–647.

Iljanić, L., Z. Stančić, J. Topić and N. Šegulia. 1998. Distribution and phytosociological relationships of snake's-head (*Fritillaria meleagris* L.) in Croatia. Acta Bot. Croatica 57: 65–88.

Johnson, S.D. and K.E. Steiner. 2000. Generalization versus specialization in plant pollination systems. Trends Ecol. Evol. 15: 140–143.

Kawano, S., J. Masuda and K. Hayashi. 2008. Life-history monographs of Japanese plants. 10: *Fritillaria koidzumiana* Ohwi (Liliaceae). Plant Species Biol. 23: 51–57.

Knuth, P. 1899. Handbuch der Blütenbiologie, II Band, 2. Teil: Lobeliaceae bis Gnetaceae., Verlag von Wilhelm Engelmann, Leipzig.

Kolb, A. 2008. Habitat fragmentation reduces plant fitness by disturbing pollination and modifying response to herbivory. Biol. Conserv. 141: 2540–2549.

Kosior, A., W. Celary, P. Olejniczak, J. Fijał, W. Król, W. Solarz and P. Płonka. 2007. The decline of the bumble bees and cuckoo bees (Hymenoptera: Apidae: Bombini) of Western and Central Europe. Oryx 41: 79–88.

Lamont, B.B., P.G.L. Klinkhamer and E.T.F. Witkowski. 1993. Population fragmentation may reduce fertility to zero in *Banksia goodii*—a demonstration of the Allee effect. Oecologia 94: 446–450.

Mabberley, D.J. 2008. Mabberley's plant-book. A portable dictionary of plants, their classification and uses, 3rd ed., Cambridge University Press, Cambridge.

MacDonald, M.A. 2001. The colonisation of Northern Scotland by *Bombus terrestris* (L.) and *B. lapidarius* (L.) (Hym., Apidae), and the possible role of climate change. Entomol. Monthly Mag. 137 1–13.

Makino, T.T. and J.D. Thomson. 2012. Innate or learned preferences for upward-facing flowers? Implications for the costs of pendent flowers from experiments on captive bumble bees. J. Pol. Ecol. 9(11): 79–84.

Mancuso, E. and L. Peruzzi. 2010. Male individuals in cultivated *Fritillaria persica* L. (Liliaceae): real androdioecy or gender disphasy? Turk. J. Bot. 34: 435–440.

Nepi, M. and M. Stpiczyńska. 2008. The complexity of nectar: secretion and resorption dynamically regulate nectar features. Naturwissenshaften 95: 177–184.

Nepi, M., M. Guarnieri, D. Artese, L. Cresti, M. Stpiczyńska and E. Pacini. 2007. Dynamics of nectar production and reabsorption in *Cucurbita pepo*. *In:* 9-th international pollination symposium on plant-pollinator relationships—diversity in action, Ames Iowa (USA), pp. 34–35.

Offler, C.E., D.W. McCurdy, J.W. Patrick and M.J. Talbot. 2002. Transfer cells: cells specialized for a special purpose. Ann. Rev. Plant Biol. 54: 431–454.

Olsen, K.M. 1997. Pollination effectiveness and pollinator importance in a population of *Heterotheca subaxillaris* (Asteraceae). Oecologia 109: 114–121.

Pawlikowski, T. 1996. Keys for the identification of Polish Insects, XXIV 68h, Bees—Apidae. Subfamiliy: Apinae. Polskie Towarzystwo Entomologiczne, Toruń.

Pellmyr, O. 2002. Pollination by animals. *In:* C.M. Herrera and O. Pellmyr (eds.). Plant-Animal Interactions. An Evolutionary Approach, Blackwell, Oxford, pp. 157–184.

Peruzzi, L. 2012. Male flowers in Liliaceae are more frequent than previously thought. Bocconea 24: 301–304.

Peruzzi, L., E. Mancuso and D. Gargano. 2012. Males are cheaper, or the extreme consequence of size/age-dependent sex allocation: sexist gender diphasy in *Fritillaria montana* (Liliaceae). Bot. J. Linn. Soc. 168: 323–333.

Peters, W.S., M. Pirl, G. Gottsberger and D.S. Peters. 1995. Pollination of the Crown Imperial *Fritillaria imperialis* by Great Tits *Parus major*. J. Ornithol. 136: 207–212.

Piórecki, J. 2001. *Fritillaria meleagris* L. *In:* R. Kaźmierczakowa and K. Zarzycki (eds.). Polska czerwona księga roślin, Instytut Botaniki im. W. Szafera i Instytut Ochrony Przyrody PAN, Kraków, pp. 416–418.

Piórecki, J. 2005. Szachownica kostkowana *Fritillaria meleagris* L., warunki występowania, zagrożenia i perspektywy ochrony. Chrońmy Przyr. Ojcz. 61: 15–29.

Richards, A.J. 1997. Plant breeding systems. Bios Scientific, Oxford.

Rix, E.M. 1968. *Fritillaria*. *In:* T.G. Tutin (ed.). Flora Europaea, Vol. 5, Cambridge University Press, Cambridge, pp. 31–34.

Rix, E.M. and D. Rast. 1975. Nectar sugars and subgeneric classification in *Fritillaria*. Biochem. Syst. Ecol. 2: 207–209.

Rønsted, N., S. Law, H. Thornton, M.F. Fay and M.W. Chase. 2005. Molecular phylogenetic evidence for the monophyly of *Fritillaria* and *Lilium* (Liliaceae; Liliales) and the infrageneric classification of *Fritillaria*. Mol. Phylogen. Evol. 35: 509–527.

Schemske, D.W., B.C. Husband, M.H. Ruckelshaus, C. Goodwillie, I.M. Parker and J.G. Bishop. 1994. Evaluating approaches to the conservation of rare and endangered plants. Ecology 75: 584–606.

Schnittler, M. and K.F. Günther. 1999. Central European vascular plants requiring priority conservation measures—an analysis from national Red Lists and distribution maps. Biodivers. Conserv. 8: 891–925.

Shimizu, T., Y. Hatanaka, H. Zentoh, T. Yashima, E. Kinoshita, Y. Watano and T. Shimizu. 1998. The role of sexual and clonal reproduction in maintaining population in *Fritillaria camtschatcensis* (L.) Ker-Gawl. (Liliaceae). Ecol. Res. 13: 27–39.

Stecki, K., A. Mamulski and A. Biernacki. 1961. Łąki z szachownicą kostkowatą w południowowschodniej Polsce. Chrońmy Przyr. Ojcz. 17: 13–24.

Stephens, P.A., W.J. Sutherland and R.P. Freckleton. 1999. What is the Allee effect? Oikos 87: 185–190.

Stpiczyńska, M., M. Nepi and M. Zych. 2012. Secretion and composition of nectar and the structure of perigonal nectaries in *Fritillaria meleagris* L. (Liliaceae). Plant Syst. Evol. 298: 997–1013.

Tamura, M.N. 1998. Liliaceae. *In:* K. Kubitzki (ed.). The families and genera of vascular plants. III. Flowering plants. Monocotyledons, Lilianae (except Orchidaceae). Springer, Berlin, pp. 343–353.

Teksen, M. and Z. Aytac. 2011. The revision of the genus *Fritillaria* L. (Liliaceae) in the Mediterranean region (Turkey). Turk. J. Bot. 35: 447–478.

Thomson, J.D. 2003. When is it mutualism? Am. Nat. 162: S1–S9.

Tomović, G., S. Vukojičić, M. Niketić, B. Zlatković and V. Stevanović. 2007. *Fritillaria* (Liliaceae) in Serbia: distribution, habitats and some taxonomic notes. Phytol. Balcan. 13: 359–370.

U.S. Fish and Wildlife Service. 2003. Recovery plan for *Fritillaria gentneri* (Gentner's Fritillary). U.S. Fish and Wildlife Service, Portland, Oregon.

Willmer, P. 2011. Pollination and floral ecology. Princeton University Press, Princeton and Oxford.

Yashima, T., E. Kinoshita and S. Shimizu. 1997. Flowering phenology and self-incompatibility in *Fritillaria camtschatcensis* (L.) Ker-Gawl. J. Phytogeogr. Taxon. 45: 129–133.

Zaharof, E. 1986. Variation and taxonomy of *Fritillaria graeca* (Liliaceae) in Greece. Plant Syst. Evol. 154: 41–61.

Zhang, L. and H. Hytteborn. 1985. Effect of ground water regime on development and distribution of *Fritillaria meleagris*. Holarc. Ecol. 8: 237–244.

Zhang, X., D. Cui, X. Zong, R. Ren, J. Wei, L. Situ and Y. Zhang. 2010. Pollination biology and breeding system of *Fritillaria ussuriensis* Maxim. Acta Bot. Boreal. Occ. Sin. 30: 1404–1408.

Zych, M. and M. Stpiczyńska. 2012. Neither protogynous nor obligatory out-crossed: pollination biology and breeding system of the European Red List *Fritillaria meleagris* L. (Liliaceae). Plant Biol. 14: 285–294.

Zych, M., J. Goldstein, K. Roguz and M. Stpiczyńska. 2013. The most effective pollinator revisited: pollen dynamics in a spring-flowering herb. Arthropod Plant Interact. 7: 503–516.

9

Genetics and Reproductive Biology of Cultivated Potato (*Solanum tuberosum* L.): Implications in Breeding

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ABSTRACT

This article gives an overview of potato genetics and reproductive biology and their implication in conventional potato breeding. The commonly cultivated potato (*Solanum tuberosum* ssp. *tuberosum* L.) is an autotetraploid (2n=4x=48, 4EBN) with tetrasomic inheritance. Both interlocus (epistasis) and intralocus (heterozygosity) interactions occur and the more they are, the greater the heterosis. Favourable traits are fixed in F_1 generation due to clonal propagation. Potato exhibits inbreeding depression characteristic to cross pollinated species yet most of the seeds obtained from open-pollinated fruits are the result of self fertilization; flowering and fruiting are mainly affected by

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List of abbreviations after the text.

genotype, day length, and temperature. Continued self-pollination results in inbreeding depression; this results in reduction of germination percentage, plant vigour, flowering, male fertility, and open-pollinated fruit set. Hybrids are generally more vigorous than open-pollinated seeds; the open-pollinated seeds are generally selfs. The cultivated potato has many wild relatives; which provide genetic diversity as well as genes for valuable production and quality traits. However, introgression of useful genes from these wild relatives is difficult due to fertilization barriers. These barriers include incompatibility between pollen and pistil, male sterility resulting from interactions between nuclear and cytoplasmic genes, and differences in endosperm balance number (EBN), or effective ploidy. These are overcome through use of fertility restorer gene, sexual polyploidization and somatic polyploidization through protoplast fusion. The principal method of potato breeding is the conventional hybridisation followed by recurrent selection in the clonal generations. The choice of parents is determined by the breeding objective; crossability and unrelatedness should also be considered. The simplest method for predicting the value of cross combinations is to evaluate progenies at seedling stage. Conventional potato breeding takes long (about 10 years) before a cultivar is released, mostly due to the slow multiplication rate of the crop. This time can be reduced through use of marker assisted selection in identifying parents with desirable traits and selecting superior clones genotypically at seedlings stage.

Keywords: Early generations selection, Potato breeding, Reproductive biology

Introduction

World wide, the cultivated potato of commerce is *Solanum tuberosum* ssp. *tuberosum* L. The Andean mountains of Peru and Bolivia is the primary center of genetic variability of tuberous cultivated potatoes, where it has been cultivated for over 2,400 years (Acquaah 2007). More than 200 potato varieties were developed by the Aymara Indians on the Titicaca plateau, 10 to 20 degrees south and 3,000 to 4,600 meters above sea level (Sleper and Poehlman 2006). The secondary center of diversity of cultivated potatoes is in southern South America, particularly in Chile (Bukasov 1966). From here, the potato was introduced to Europe between 1565 and 1580, by the Spaniards, from where it was introduced into Germany in the 1620's and then into France after the seven year war, and thereafter, to the rest of Europe (Hijmans 2001; Acquaah 2007). It was introduced to Virginia, USA in 1621 (Sleper and Poehlman 2006; Acquaah 2007). In Africa, it was brought later by the colonialists. Potato is a crop of major economic importance worldwide (Tsegaw 2005; FAO 2008). On a global scale, potato is the third

most cultivated food crop after wheat and rice (FAO 2008). Potato is the most important among root and tuber crops, with an annual production of approximately 330 million tonnes grown on about 19.7 million hectares (FAO 2010); it is followed by cassava, sweet potato, and yam (FAO 2004, 2008). Potato is grown in more than 150 countries worldwide from latitudes 65ºN to 50ºS, and from sea level to 4,000 metres above sea level (Acquaah 2007). The world average potato production is 17 t ha^{-1}, while direct consumption as human food is 31.3 kg per capita (kg yr^{-1}) (FAO 1995, 2004). Potatoes can be grown wherever it is neither too hot (ideal average daily temperature below 21ºC) nor too cold (above 5ºC), and there is adequate water from rain or irrigation. On regional basis, Asia and Europe are the major potato producing regions, accounting for more than 80% of world production, while Africa produces the least, accounting for about 5% (FAO 2008).

There are approximately 200 wild *Solanum* species distributed from the south-western United States to Chile and central Argentina, and are concentrated in Peru and Bolivia (Spooner and Hijmans 2001). The geographical distributions of many species overlap (Spooner and Hijmans 2001). These species are able to maintain their genetic uniqueness in the absence of geographical separation due to internal reproductive barriers (Dvøra' 1983; Singh et al. 1989). These barriers include incompatibility between pollen and pistil, male sterility resulting from interactions between nuclear and cytoplasmic genes, and differences in endosperm balance number (EBN), or effective ploidy (Camadro et al. 2004). The wild *Solanum* relatives provide genetic diversity as well as genes for valuable production and quality traits. In order to utilize the wealth of genetic resources in potato, breeders and geneticists must first understand the causes of interspecific hybridization failure. They can then devise strategies to overcome these barriers.

This chapter gives an overview of genetics and reproductive biology of *Solanum tuberosum* L. and their implication in potato breeding. Commonly cultivated potato herewith will be referring to *Solanum tuberosum* ssp. *tuberosum* L.

Potato Genetics

The genus *Solanum* contains over 2000 species, of which only 150 are tuber bearing (Plaisted 1980; Sleper and Poehlman 2006). The common potato, *Solanum tuberosum* L. belongs to the tuber-bearing section *Petota:* this section is subdivided into 21 series containing 228 wild and 7 cultivated species (Hawkes 1994). *Solanum tuberosum* L. belongs to series *Tuberosa;* this series contains 14 wild and 7 cultivated species (Matsubayashi 1991). The seven cultivated species are *Solanum ajanhuiri, S. chaucha, S. curtilobum, S. juzepczukii, S. phureja, S. stenotomum* and *S. tuberosum* (Hawkes 1990).

All species of the section *Petota* have the same basic chromosome number (x = 12) and they constitute a polyploid series ranging from diploids (2n=2x = 24) to hexaploids (2n=6x=72) (Douches and Jastrzebski 1993; Carputo et al. 2003; Carputo and Barone 2005). Five genomes (A, B, C, D and P) are recognized in the tuber-bearing species of section *Petota* (Plaisted 1980; Matsubayashi 1991). All diploid tuber-bearing species comprise one major genomic group A; no diploid species have ever been identified with B, C, D and P genomes (Matsubayashi 1991). Two major mechanisms have been proposed to explain the origin of polyploidy: chromosome doubling of the somatic cells and formation of unreduced gametes (sexual polyploidization) (Gavrilenko 2007). Almost all polyploids in nature appear to have originated through sexual polyploidization (Harlan and Wit 1975). This is particularly true for the species of section *Petota*, many of which often form both 2*n* pollen and 2*n* eggs (Watanabe and Peloquin 1991). The 2*n* gametes provide opportunities for gene flow between species with different ploidy levels and/or different endosperm balance number (EBN) (Den Nijs and Peloquin 1977). Thus, in addition to causing polyploidization, the ability to form 2*n* gametes also facilitated interspecific hybridization which has played an important role in the evolution of wild and cultivated potatoes and in the formation of polyploidy complexes in the section *Petota* (Gavrilenko 2007). The section *Petota* contains both alloploids and autoploids; the seven cultivated species are autoploids (Matsubayashi 1991; Gavrilenko 2007). *Solanum tuberosum* L. is an autotetraploid with AAAA genome and displays tetrasomic inheritance ratios (Bradshaw and Mackay 1994). Generally, strict allotetraploids and allohexaploids species are sexually fertile, show regular meiosis, are self-compatible and display disomic inheritance (Hawkes 1990; Douches and Jastrzebski 1993). Allotriploid, allopentaploid and autotetraploid species have irregular meiosis, are sterile or have very low levels of fertility (Gavrilenko 2007). Nearly all of the diploid species are outbreeders, with a single S-locus, multiallelic, gametophytic self-incompatibility system (Dodds 1965; Hawkes 1990). One hundred and eighty one tuber-bearing species of *Solanum* have known ploidy levels: 76% are diploids, 3% triploids (2n=3x=36), 12% tetraploids (2n=4x=48), 2% pentaploids (2n=5x=60) and 7% hexaploids (2n=6x=72) (Hawkes 1990; Spooner et al. 2004). The three commonly cultivated diploid species are *Solanum stenotomum, Solanum phureja,* and *Solanum ajanhuiri* (Sleper and Poehlman 2006). *Solanum stenotomum* is adapted to high altitude areas in southern Peru and Bolivia. *Solanum phureja* is widely used in bridge-crossing, and as a source of resistance to bacterial wilt (Fock et al. 2000, 2001). It is adapted to lower altitudes in the frost-free Andean valleys of Venezuela, Colombia, Ecuador and northern Peru (Hawkes 1956). It lacks tuber dormancy and can be replanted immediately in areas where continuous cropping is possible (Plaisted 1980). There are two cultivated

triploid species, *Solanum chaucha,* and *Solanum juzepczukii,* one tetraploid species *S. tuberosum*, and one pentaploid species *Solanum curtilobum* (Hawkes 1990). A common wild allohexaploid, *Solanum demissum*, is the source of the major R genes that confer resistance to late blight of potatoes (Carputo et al. 2003; Sleper and Poehlman 2006).

The commonly grown potato, *Solanum tuberosum* L., is an autotetraploid ($2n=4x=48$, 4EBN) species that displays tetrasomic inheritance (Bradshaw and Mackay 1994; Sleper and Poehlman 2006). The species has a monophyletic origin, which means that it developed out of one wild plant, and hence it has a narrow genetic diversity. There are two major subspecies of *Solanum tuberosum*; *andigena* or Andean, and *tuberosum* or Chilean (Raker and Spooner 2002). The Andean potato is adapted to the short-day conditions prevalent in the equatorial and tropical regions where it originated (Raker and Spooner 2002). It is indigenous to the Andean region from Venezuela to northern Chile and Argentina (Hawkes 1990). The Chilean potato is adapted to the long-day conditions prevalent in the higher latitude region of southern Chile, especially Chiloé Island and Chonos Archipelago, where it is thought to have originated (Hawkes 1990; Hijmans 2001). The genetic relationship between subspecies *tuberosum* and *andigenum* is unresolved (Raker and Spooner 2002). The transition from subspecies *andigena* to subspecies *tuberosum* apparently resulted from transporting material from the short-day environment of the Peruvian/Bolivian Andes to the long days conditions. This transportation accompanied by adaptation is believed by Hawkes (1990) to have occurred twice: the first event would have taken place in Chile where original subspecies *andigena* material, brought here by migrating Indian tribes from the Andes, underwent adaptation to long-days and cool climatic conditions. The second time this development took place was in Europe after the Spaniards introduced potato there. Grun (1990), suggested that *tuberosum* was distinct from *andigenum* based on cytoplasmic sterility factors, geographical isolation, and ecological differences. *Solanum tuberosum ssp. tuberosum* contains at least seven different cytoplasmic sterility factors (Sps, Sm^s, In^s, TA^s, ASF^s, VSAs and Fm^s) that conditions sterilities in presence of dominant chromosomal genes (Sp, SM, In, TA, ASF, VSA and Fm) that occur in ssp. *andigena* (Grun et al. 1977). The cytoplasmic sterility sensitivity factors that are typical of ssp. *tuberosum* have not been found to occur in ssp. *andigena*. In addition, the two subspecies are well separated geographically in their native areas with ssp. *andigena* at high altitudes in the northern and central Andes Mountains and ssp. *tuberosum* at sea level in southern Chile. There are also well developed internal isolation barriers separating the two subspecies because the hybrids ssp. *tuberosum* x ssp. *andigena* are usually male sterile and often female sterile (Grun et al. 1977; Grun 1979). Hawkes (1990) distinguished the two subspecies on the grounds that subspecies *tuberosum* has fewer stems, more horizontal foliage,

less-dissected leaves, wider leaflets, and thicker pedicels than *andigenum*. In addition, subspecies *andigenum* has five chloroplast genotypes (A, C, S, T, and W) while subspecies *tuberosum* has only three (A, T, and W) (Hosaka and Hanneman 1988).

In *Solanum tuberosum* there can be four different alleles at a locus (Ross 1986). The tetraploid nature of cultivated potato can be exploited by the breeder to improve desirable characteristics. Because of the potato's autotetraploid nature, intralocus interactions (heterozygosity) and interlocus interactions (epistasis) occur, and are important when selecting breeding procedures to improve certain traits. Heterozygosity in potato is attributed to self-incompatibility and has most likely been enhanced by millions of years of asexual propagation by tubers. Selection based on maximum heterozygosity, rather than additive genetic variance, is critical in potato breeding, especially for quantitative traits. It is assumed that increased heterozygosity leads to increased heterosis (Bradshaw and Mackay 1994; Sleper and Poehlman 2006). Heterosis in potato is when the progeny surpasses the value of the best parent or the parental mean. The exploitation of heterosis is by far the most important goal in potato breeding. The inheritance of heterosis is by minor genes or by the side effects of the major genes. Their action can proceed in an additive (general combing ability) or in a non-additive manner (specific combing ability): in most case both operate (Ross 1986). Heterosis in potato is based mainly on non-addititve interactions of genes and it comprises intralocus (over dominance) as well as interlocus (epistasis) interaction between genes and alleles (Ross 1986). Asexually propagated species such as potatoes have evolved taking advantage of non-additive or epistatic gene action (Sleper and Poehlman 2006). The level of heterozygosity in potatoes is influenced by how different the four alleles are within a locus; the more diverse they are, the higher the heterozygosity and the greater the number of interlocus (epistatic) interactions and hence the greater the heterosis (Ross 1986; Bradshaw and Mackay 1994; Sleper and Poehlman 2006). To see how increased heterozygosity can lead to more epistatic interactions, it is necessary to identify the allelic conditions possible in an autotetraploid (Caligari 1992; Sleper and Poehlman 2006). Five tetrasomic conditions are possible at an individual locus in an autotetraploid (Table 1).

It is hypothesized that the tetraallelic condition provides the maximum heterosis because more interlocus interactions are possible for this tetrasomic condition than for the other configurations (Ross 1986; Sleper and Poehlman 2006). For example, in the tetrallelic condition, the six first-order interactions are: a_1a_2, a_1a_3, a_1a_4, a_2a_3, a_2a_4, a_3a_4. The four second-order interactions are: $a_1a_2a_3$, $a_1a_2a_4$, $a_1a_3a_4$, $a_2a_3a_4$. The one third-order interaction is $a_1a_2a_3a_4$.

There are a total of 11 different interactions possible for the tetraallelic condition. This is in contrast to the monoallelic condition, which has no

Table 1. The number of first, second and third-order possible interactions and their sums for the five different tetrasomic conditions in an autotetraploid

Number of heteroallelic interaction					
Tetrasomic Condition	**First order**	**Second order**	**Third order**	**Total**	**Portion of Haploids(2x) conserving one first-order Interaction**
$a_1a_2a_3a_4$	6	4	1	11	All
$a_1a_1a_2a_3$	3	1	0	4	5/6
$a_1a_1a_2a_2$	1	0	0	1	2/3
$a_1a_1a_1a_2$	1	0	0	1	1/2
$a_1a_1a_1a_1$	0	0	0	0	none

Adapted from Sleper and Poehlman (2006).
The $a_1a_1a_1a_1$ is a monoallelic locus where all alleles are identical.
$a_1a_1a_1a_2$ is an unbalanced diallelic locus where two different allele are present in unequal frequency.
$a_1a_1a_2a_2$ is a balanced diallelic locus where two different alleles occur with equal frequency.
$a_1a_1a_2a_3$ is a triallelic locus where three different alleles are present.
$a_1a_2a_3a_4$ is a tetraallelic locus where four different alleles are present.

interactions. The highest level of heterosis will occur as the frequency of tetraallelic loci increase. The greatest number of interlocus interactions will also occur as the frequency of tetraallelic loci increase. In breeding potatoes for higher tuber yields, inter- and intralocus interactions have been shown to be important; procedures that maximize the frequency of tetraallelic loci should be considered in breeding potato for increased yields (Ross 1986; Bradshaw and Mackay 1994; Sleper and Poehlman 2006). Therefore, the segregation of heterotic seedlings in a population is likely to be greatest when three conditions are fulfilled: 1) the parents possess as low a coefficient of inbreeding as possible, 2) as many loci as possible have different alleles and, 3) the parents belong to different gene pools which improves the chances of allelic diversity, i.e., wide hybridisation (should be as unrelated as possible) (Ross 1986). In potatoes, heterosis is of direct relevance for improving traits under consideration as it gets fixed in F_1 generation (Upadhya and Cabello 2000) due to vegetative propagation of the crop. Because potato is a highly heterozygous crop, an increase in heterozygosity results in heterosis. Distantly related genotypes are more complementary and they produce heterotic progenies (Ross 1986).

Reproductive Biology in Cultivated Potato

It is difficult to classify the cultivated *Solanum tuberosum* L. as to the extent of natural cross pollination. It exhibits inbreeding depression characteristic to cross pollinated species yet most of the seeds obtained from open-pollinated fruits are the result of self fertilization (Plaisted 1980). Potato is predominantly

self-pollinated, although some cross pollination is often accomplished by bumblebees (Caligari 1992; Acquaah 2007). Wind pollination plays a minor role in nature. Outcrossing is enforced in cultivated (and most wild) diploid species by a single S-locus, multiallelic, gametophytic self-incompatibility system (Dodds 1965). While self-incompatibility does not operate in *S. tuberosum*, 40% (range 21–74%) natural cross-pollination was estimated to occur in ssp. *andigena* in the Andes (Brown 1993) and 20% (range 14–30%) in an artificially constructed *andigena* population (Glendining 1976).

Flowering

Potato has a terminal inflorescence consisting of 1 to 30 (but usually 7 to 15) flowers, depending on cultivar (Plaisted 1980; Acquaah 2007). The flower is 3–4 cm in diameter and contains five sepals and petals, and a bilobed stigma (Acquaah 2007). The five petals give the flower a star shape (Plaisted 1980; Caligari 1992). The petals vary in size with the cultivar, and the colour varies from white to a complex range of blue, red, and purple (Sleper and Poehlman 2006; Acquaah 2007). The petals are united and tubular. The stamens are attached to the corolla tube and bear erect anthers (Almekinders and Struik 1996). The anthers are bright yellow except for those produced on male sterile plants, which are light yellow or yellow-green in colour; the stigma protrudes above a cluster of large, bright yellow anthers (Sleper and Poehlman 2006).

Flowers open starting with those nearest the base of the inflorescence proceeding upwards at a rate of about 2–3 flowers per day (Acquaah 2007). At the peak bloom, there are usually 5–10 open flowers (Caligari 1992; Acquaah 2007). Flowers stay open for only 2–4 days, while the receptivity of the stigma and duration of pollen production is about two days (Sleper and Poehlman 2006). Flowers open mostly in the early morning, although a few may continue to open throughout the day (Sleper and Poehlman 2006). Genotype, day length, light intensity and temperature are the main factors that determine flowering and fruiting in potato (Turner and Ewing 1988; Sleper and Poehlman 2006). Flowering in potato is best when long days (around 16 hours), abundant moisture, and cool temperatures prevail (Almekinders and Struik 1996; Sleper and Poehlman 2006). Photoperiod of 12–14 hours and night temperatures of 15–20°C has been shown to favour flower production and berry setting in potato (Almekinders 1992; Gopal 2006). Short days during flowering may lead to abscission of the floral bud, giving the impression that the cultivars do not flower well (Almekinders and Struik 1996). Therefore, in tropics and sub-tropics, conditions conducive for flowering and fruiting are available only at high altitudes (>1500 m above sea level) (Gopal 1994). Turner and Ewing (1988) found that reducing light intensity completely suppressed flower development; all floral buds

aborted at an early stage of development. A wide genetic variability has been observed for days to flowering, duration of flowering, flowering intensity, and berry setting (Gopal 2006). Gopal (1994) conducted a survey of the flowering behaviour, male sterility, and berry setting in 676 accessions of tetraploid *Solanum tuberosum* from 25 countries. He conducted his studies outdoors at Kufri in India (32°N, 70°E, 2500 masl) during summer (12.1–14.1 hours of day length). He found that flowering intensity ranged from the dropping of floral buds just after initiation to profuse blooming. Majority of the accessions (58.3%) bloomed profusely, but 20.4% did not bloom at all. Time to flowering ranged from 6 to 15 weeks and duration of flowering from 1 to 10 weeks. Pollen stainability (as an indicator of fertility) ranged from 0 to 90%, with 23% of the accessions being completely male sterile. Berry setting ranged from zero to more than five berries per plant, with none in 31.8% of the flowering accessions. Therefore, only 54.3% of the accessions were found to be fertile in all respects and could be used as male and female parents. He also found that premature bud abscission was the major cause of sterility. In addition to genotype, temperature, and photoperiod, other factors such as inflorescence position (Almekinders and Wiersema 1991), plant/stem density (Almekinders 1991), competition between flower and tuber (Thijn 1954), precipitation and date of planting (Jauhnl 1954) and soil nutrient level (Bamberg and Hanneman 1988) are also known to influence production of flowers and fruits in potato. Increasing stem density has been shown to decrease the number of flowers per plant, berry set and seed production from every inflorenscence (Mok 1985; Almekinders 1991; Almekinders and Wiersema 1991). Increasing plant density was shown to increase the proportion of primary flowers in the total number of flowers per plant and reduced the proportion of flowers on lateral stems (Almekinders 1991). However, increasing plant density was shown to increase the number of flowers and seed weight per m^2 (Mok 1985). Nitrogen enhances the export of cytokinins from the roots to the shoots resulting in delayed senescence of the plants. Berries therefore have a longer time to mature on the mother plants and a better chance for high quality seed production (Van Staden et al. 1982). Studies have shown that periodic supplemental nitrogen applications to the soil during seed development at rates higher than recommended for tuber production enhance flowering and delay plant maturity thereby prolonging the berry development period (CIP 1985). Flower production was increased by more than three times when supplemental N rates up to a total of 240 $kgha^{-1}$ were applied at weekly intervals. In addition, significant increases in 100-seed weight were obtained with supplemental application of N (Pallais 1986). In a subsequent experiment, the highest 100-tps weight was found in plants receiving N at 600 kg ha^{-1}. Therefore N rates greater than those required for tuber production enhance quality TPS production.

Some techniques that are used by breeders to enhance flowering and seed set include shading of glasshouses to reduce temperature below 22ºC, girdling or constriction of the stem, and grafting of young potato shoots onto tomato or other compatible *Solananceous* plants (Caligari 1992; Sleper and Poehlman 2006). However, the last method gives weaker growth (Gopal 1994). Another method includes growing the seed tuber on a brick and the brick is covered with sand and peat. The roots grow over the brick and when they have penetrated the soil on which the brick is lying, the covering sand is washed away. The tips of the stolons, which would otherwise produce new tubers, are then removed. This promotes vigorous stem growth and enhances flowering (Gopal 1994). Other methods which have been reported include foliar spray with GA_3 (El-Gizawy et al. 2006).

Pollination in Cultivated Potato

Controlled pollination may be done in the field or in the greenhouse under controlled conditions. Crosses made in the field are liable to suffer losses from wind, rain, heat and drought and most breeders therefore prefer to work in the greenhouse. The best time for crossing is early morning when temperatures are not high (Acquaah 2007). Prior to crossing, flower buds that are mature and plump with the petals ready to separate are selected for emasculation (Acquaah 2007). If pollinations are done in the field, it is important to emasculate just prior to crossing as the wind can break off the stigma before pollination occurs if they are emasculated too far ahead of pollination. Normally, the unopened flowers of the female parent must be emasculated one to two days before crossing to avoid self contamination. For emasculation, choose the bud that has developed the petal colour but is unopened. Pull back the petals carefully to expose the immature anthers; pull-off all the anthers carefully with a blunt scalpel or tweezers. To facilitate emasculation of the selected buds, and to prevent contamination of the emasculated flowers by the open flowers, the remaining buds and open flowers in the inflorescence should be removed (Sleper and Poehlman 2006; Acquaah 2007). Removing the extra flowers increases the chance that pollination will be successful and reduces competition for photoassimilates (Almekinders and Struik 1996). In the male plant, look for a newly opened flower and pull off the anthers. Slit open the anthers using a blunt scalpel to collect pollen; dab the pollen onto stigma of the emasculated female flower and label it; pollinate again the following day (Plaisted 1980).

Flowers with plump bright yellow anthers and brown tips are most apt to be good sources of pollen. Pollen is most abundant in the morning (Plaisted 1980). Potato pollen is robust if it kept cool and dry. It can be kept for a month if store at 2.5ºC or a year at –24ºC (Blomquist and Lauer 1962). Pollen viability is prolonged if stored under dry conditions such as

in a dessicator containing silica gel (Howard 1958). Pollen stored at room temperature and humidity losses viability after one day.

Alternatively, open flowers are collected from the male plant and laid out to dry overnight (Almekinders and Struik 1996). In the following morning, the pollen is collected from them by shaking into gelatine capsules or small tubes (Sleper and Poehlman 2006). To pollinate, the stigma is dipped into the pollen and then the pollination tag is attached. The emasculated flowers do not need covering to avert contamination. Germination of the pollen is completed after 30 minutes, and the ovary is fertilized within 12 hours (Bradshaw and Mackay 1994). The berries appear within a few weeks and to prevent losses, they are bagged with nylon netting of large mesh. Potato fruits (berries) contain about 50 to 500 seeds with an average of 200 seeds (CIP 1984).

Pollinations can also be done on flowers attached to stems that have been cut and placed in jars of water with an anti-bacterial agent to reduce contamination (Peloquin and Hougas 1959; Wolfgang et al. 2009).

Infertility Problems in Cultivated Potato

Biological seed production in potatoes is low due to: failure of plants to flower, dropping of buds and flowers either before or after pollination, low pollen production, failure to produce viable pollen, male sterility, and self-incompatibility (Sleper and Poehlman 2006). Because open pollinated berries in potato result predominantly from self pollination, formation of open pollinated berries indicates that the genotype bearing them is both male and female fertile, self compatible, and that after fertilization, flowers do not drop, but develop into fruits. Non-formation of berries, on the other hand, can be due to any one or more of the causes listed above. Berry setting is the ultimate test of fertility if these berries carry seed in them, which is generally the case (Gopal 1994). Hence, from a practical point of view, genetic blockage at any stage from floral bud initiation to seed set should be considered as sterility.

Male sterility

In male-sterile plants, flowers do not produce functional anthers or viable pollen but the ovaries function normally. Male sterility may be controlled by the action of the nuclear genes (genetic male sterility) or by the cytoplasm (cytoplasmic male sterility) (Sleper and Poehlman 2006). Male sterility due to deleterious nuclear genes is a very common and serious constraint in potato breeding (Gopal 2006). The deleterious recessive alleles can accumulate in tetraploid potato cultivars because they are more easily masked than in diploids. The failure to produce pollen may be an inherent characteristic

with sterility being dominant over fertility. Presence of a tetrasomic gene, which is lethal when present in a homozygous condition, or partly lethal when present in a heterozygous condition has also been reported (Sleper and Poehlman 2006). Though both pollen and ovule sterility can occur, pollen sterility ranging from partial to complete absence of pollen grains is very common in potato (Pushkarnath and Dwivedi 1961). Almost one third of the potato cultivars derived from *Solanum tuberosum* ssp. *tuberosum* do not form berries (Ross 1986). Male sterility in potatoes is probably controlled by more than one gene, with partial dominance or by nuclear-cytoplasmic interactions (Howard 1978) and depends partially on environmental factors. In cultivated *S. tuberosum* potato seven types of cytoplasmic factors exists each of which condition, in combination with a specific genotype, a specific type of sterility (Grun 1970). These cytoplasmic factors are: indehiscence (Ins), sporads (Sp^s), shrivelled microspores (SM^s), anther style fusion (Af^s), thin anthers (TA^s), females sterility (Fm^s), and deformed flower (df^s) (Gopal 2006). Variations, depending on the stage at which development is blocked have also been observed within a particular type of sterility. A block can occur at any place in the development process and depending on the particular gene-plasmon interaction involved, different kinds of blockage may occur (Grun 1970, 1990). The prevalence of sterility to such a large extent limits the use of many germplasm clones as parents, but at the same time this is advantageous because males sterile clones do not require emasculation when used as females in controlled pollinations (Gopal 2006). Because the marketable product in potato is not seed, there is no selection pressure for high fertility in breeding programs. In fact, fruit development may partition resources away from tuber yield, so breeders may inadvertently select against high fertility (Jansky and Thompson 1990). Levels of cytoplasmic genetic male sterility are frequently variable, presumably due to genetic and environmental influences (Hanneman Jr. and Peloquin 1981). Breeders can, therefore, overcome this type of sterility by either carrying out reciprocal crosses or selecting parents that do not contain sensitive cytoplasm or dominant nuclear sterility genes (Iwanaga et al. 1991; Tucci et al. 1996). Fertility restorer genes have been found for the latter (Jansky 2009). For example, there is a dominant gene (*Rf*) that restores fertility to plants that contain the dominant male sterility gene (*Ms*) in the presence of sensitive cytoplasm (Iwanaga et al. 1991).

Incompatibility

Crossability in a broad sense can be defined as any natural or artificial fusion of two genetically different cells leading to hybrid progeny. Incompatibility is a form of infertility caused by the failure of plants with normal pollen and ovules to set seed due to some physiological hindrance that prevents

fertilization. Incompatibility may be caused by failure of pollen tube either to penetrate the stigma or to grow normally the full length of the style so that fertilization may occur (Sleper and Poehlman 2006). Incompatibility restricts self-fertilization and inbreeding and fosters cross-fertilization and outbreeding. Incompatibility can be bilateral (in both directions) or unilateral (in only one direction); both self- and cross- incompatibility governed by a series of allelomorphs are widely prevalent in potato. Self- and cross incompatibility are closely interrelated: self-compatible species (SC) will cross with other self-compatible species; self-incompatible (SI) species will cross with other self-incompatible species; self-compatible species will cross as females with self-incompatible species, but self-incompatible species as females will not cross with self-compatible species (Hanneman Jr. 1999). This SC x SI and SI x SC reciprocal differences rule was termed unilateral incompatibility (Lewis and Crowe 1958). Unilateral incompatibility is a phenomenon in which SC species can be crossed as a female, but not as a male, to SI species (Abdalla and Hermsen 1972). The cultivated diploids are obligate outbreeders due to self incompatibility governed by the S locus system (Simmonds 1997; Hosaka and Hanneman 1998). This incompatibility system does not operate in tetraploids. If effectively pollinated, the sequence of decreasing seed fertility (i.e., seed production per plant) goes: diploids>*andigena*>*tuberosum*. The least fertile class is certainly the 4x *Solanum tuberosum* subspecies *tuberosum.* Experience supports the assertion as to the superior fertility of andigena over tuberosum group. There is a widespread view that the cross *tuberosum* x *andigena* gives progeny superior to the reciprocal *andigena x tuberosum*. However, the matter is still unresolved (Simmonds 1997). In general the tetraploids bear fewer seeds per berry but larger than the diploids. Seed size in any potato has a large maternal element in its determination even though seed numbers per berry are bi-parentally controlled (Simmonds 1995). While self-incompatibility does not operate in tetraploid *Solanum tuberosum*, 40% (21 to 74%) natural cross pollination was estimated to occur in subsp. *andigena* in the Andes (Brown 1993) and 20% (14 to 30%) in an artificially constructed *andigena* population (Glendining 1976). The cultivated *Solanum tuberosum* is a tetraploid in which recombination among all the four homologs is possible (Bradshaw and Mackay 1994). Such species do not exclusively self-fertilize in their natural habitats (Brown 1993) and they maintain high levels of heterozygosity across generations (Hosaka and Hanneman 1998).

Endosperm balance number hypothesis

In angiosperms, double fertilization results in the production of an embryo and endosperm, both of which are critical for the development of viable seed. Successful development of embryos and seeds requires proper endosperm

development (Sleper and Poehlman 2006). The endosperm (3n) is formed as a result of fertilization of the polar nuclei or central nucleus (2*n*) by a male nucleus (*n*). The embryo (2*n*) results from the fertilization of an egg (n) by a male nucleus (*n*). The endosperm balance number (EBN) hypothesis states that normal endosperm development occurs when the ratio of maternal to paternal EBN contribution to their progeny is 2:1 (Johnston et al. 1980). Any deviation from this ratio (2 EBN maternal: 1 EBN paternal) will result in no seed set. Intraspecific intraploidy crosses in potato typically produce viable seeds containing well-developed endosperm. Conversely, in most interploidy crosses, unviable seeds are produced due to endosperm failure (Brink and Cooper 1947). However, endosperm may also fail to develop adequately in some intraploidy, interspecific crosses, while some interploidy crosses succeed. A 2:1 maternal: paternal ratio of endosperm balance factors, rather than genomes, is necessary for normal endosperm development in potato (Johnston et al. 1980). The EBNs are independent of ploidy levels but have been described as the "effective ploidy" of the parent (Hanneman Jr. 1999). The nature of these endosperm balance factors has yet to be elucidated although genetic models have been proposed (Ehlenfeldt and Hanneman Jr. 1988a; Camadro and Masuelli 1995). *Solanum* species have been assigned endosperm balance numbers (EBN) based on their ability to hybridize with each other (Hanneman Jr 1994). Barring other crossing barriers, viable seeds will be produced from crosses between plants with matching EBN values. This will produce a 2:1 maternal: paternal ratio of endosperm balance factors after fertilization of the central cell to produce endosperm. The most common ploidy, EBN combinations in potato are 6x (4EBN), 4x (4EBN), 4x (2EBN), 2x (2EBN) and 2x (1EBN) (Hawkes and Jackson 1992). Breeders use EBN values to determine whether interspecific crosses will succeed. The EBN concept also allows them to design strategies to access wild germplasm by manipulating EBN (Johnston et al. 1980). Endosperm balance number can be increased through somatic doubling (Ross et al. 1967; Sonnino et al. 1988) or the production of 2*n* gametes. Endosperm balance number can be reduced through anther culture or parthenogenesis (Veilleux 2005). Furthermore, embryo rescue can be used to secure a hybrid where embryo abortion is due to a defective endosperm (Jansky 2006). As the largest compatibility group is EBN = 2, it is now common for potato breeders to secure tetraploid hybrids from 4*x* (*S. tuberosum*) × 2*x* (2*x S. tuberosum* × wild species) crosses in which an unbalanced endosperm prevents the development of triploid embryos.

Another problem in pollinating potato is poor nicking (i.e., unsynchronized flowering of the parents). This can be prevented by planting both the male and female parents in a greenhouse. The luxuriant growth of plants in the greenhouse ensures a long period of pollen production, which can be stored under appropriate conditions for later pollination. Pollen can

be stored dessicated in the refrigerator for 1 to 2 weeks and in the freezer for 6 months to one year (Sleper and Poehlman 2006).

Overcoming Crossability Problems

Sexual Polyploidization (Production of 2n gametes)

Specific genes have been identified in wild and cultivated species that produce unreduced male gametes by at least three different mechanisms (Mok and Peloquin 1975b). The three distinct meiotic mutations during microsporogenesis are parallel spindles (*ps*) (including also fused and tripolar spindles), premature cytokinesis-1 (*pc*-1), and premature cytokinesis-2 (*pc*-2), all inherited as simple Mendelian recessives (Mok and Peloquin 1975a). When these mutations are present at the end of meiosis, dyads with two 2*n* microspores are formed. The *ps* is the most common mutation leading to 2*n* pollen production in the potato (Watanabe and Peloquin 1991, 1993). The *ps* gene causes the spindles of the two second meiotic metaphases, which normally are perpendicular to one another in the same cell, to be parallel. The chromosomes of the two second meiotic metaphase plates, under the influence of *ps* gene go to two instead of four poles resulting in restitution of the 2*n* chromosome number. Although this restitution occurs in the second meiotic division, it is referred to as first division restitution (FDR) because it brings back together in one nucleus most of the genes, proximal to the kinetochore, that were separated in the first meiotic division. All loci from the centromere to the first crossover that are heterozygous in the parent will be heterozygous in the gametes, and half the heterozygous parental loci beyond the first crossover will be heterozygous in the gametes (due to small chromosome size, there is normally only one cross-over per chromosome arm). The result of FDR is formation of unreduced and highly heterozygous male gametophytes that grow vigorously down the style (Simon and Peloquin 1976). The heterozygosity of the male unreduced gametes in turn results in vigour of the offspring plants (Mok and Peloquin 1975a). Several mutations leading to 2*n* egg formation have also been found (Werner and Peloquin 1991). Meiotic mutations affecting megasporogenesis and resulting in formation of unreduced eggs is most commonly the omission of the second meiotic division, which is genetically similar to SDR (Werner and Peloquin 1987). The recessive gene (*os*) controls the formation of unreduced gametes by this mechanism. While the genetic consequence of 2*n* pollen formation in potato is typically FDR, that of 2*n* egg formation is second division restitution (Werner and Peloquin 1990). The combined presence of *os* gene producing unreduced eggs and *ps* genes producing unreduced male gametes has resulted in production of 4*x* following 2*x* x 2*x* crosses. Formation of unreduced gametes by FDR and SDR allows

the transfer of large portions of intralocus (heterozygous) and interlocus (epistasis) interactions from the 2*x* parent to the resulting 4*x* progeny. This is in contrast with normal meiosis in 2*x* parents which would transfer little or no intralocus and interlocus interactions. The genetic consequences of FDR 2*n* gametes are very different from those of SDR 2n gametes. In an FDR 2*n* gamete, all loci from the centromere to the first crossover on each chromosome have the same genetic constitution as the parent of that gamete. That is, all dominance (intralocus) interactions up to the first crossover are maintained in the gametes. Even in the chromosomal region beyond the first crossover, half of the loci that were heterozygous in the parent will remain so in 2*n* gametes. Since potato chromosomes are small, there is typically only one crossover per chromosome (Yeh et al. 1964; Carputo et al. 2003). Consequently, FDR 2*n* gametes provide a unique and powerful method of transmitting blocks of advantageous dominance (intralocus) and epistatic (interlocus) interactions to polyploid offspring even following meiosis, which usually breaks up such interactions. In contrast, SDR 2*n* gametes contain non-sister chromatids from the centromere to the first crossover. It has been estimated that FDR can transfer 80% of the heterozygosity and a significant portion of epistasis from parent to progeny. The SDR is less efficient and transfers less than 40% of the heterozygosity of the 2*x* female parent to the 4*x* progeny. Both FDR and SDR allow breeders to transfer desirable linkage groups and gene interactions intact from parent to offspring without having them broken up through the normal meiosis (Sleper and Poehlman 2006). This is important because the potato is clonally propagated; once heterosis if fixed in F1, it is not broken up again.

Formation of unreduced gametes by either male or female parent is called unilateral sexual polyploidization (USP); simultaneous occurrence of unreduced gametes in the male and female parent is bilateral sexual polyploidization (BSP) (Sleper and Poehlman 2006). Unilateral sexual polyploidization offers a modified form of conventional breeding that can maximize the effects of heterosis. Exceptionally high tuber yields have been observed in tetraploid (2n = 4x = 48) progenies obtained from 4x X 2x matings in potatoes (Hanneman Jr and Peloquin 1967, 1968; Mok and Peloquin 1975a). The progeny of 4x X 2x crosses are typically vigorous and relatively uniform for high tuber yield, which may at first seem surprising, considering the heterozygosity of the parents. The heterotic response is most commonly observed when the tetraploid is used as the female parent and the diploid parent produces 2*n* pollen by FDR (Jansky 2006). In addition, families from 4x X 2x (FDR 2n pollen) crosses outyield 4x X 2x (SDR 2n pollen) and 4x X 4x families by about 50% (Mok and Peloquin 1975b). Because intralocus and interlocus interactions contribute to high yield in potato, this significant increase in yield by 4x X 2x (FDR 2*n* pollen) hybridization is most likely due to the increase in transmission of

heterozygosity and epistasis by 2n FDR gametes (Mendiburu and Peloquin 1977). However, high tuber yield must be accompanied by acceptable tuber quality. Previous reports showed that a large number of haploid (2x) X diploid *S. chacoense* hybrids used in USP schemes produced tetraploid offspring with good tuber appearance and size, along with high yield (Schroeder and Peloquin 1983). Bilateral sexual polyploidization provides an alternative sexual polyploidization strategy. In this scheme, both parents are diploid and produce 2*n* gametes. The potential advantage of bilateral sexual polyploidization is that highly heterotic offspring can be produced by crossing diverse diploid parents. The disadvantage is that both parents must produce 2*n* gametes. In addition, since the meiotic mutations that produce 2*n* gametes exhibit variable expressivity (*n* and 2*n* gametes are produced by the same plant), both diploid and tetraploid offspring will be produced. Tetraploid progeny from bilateral sexual polyploidization are highly heterotic and typically outyield their diploid full-sibs (Mendiburu and Peloquin 1977) and even tetraploid commercial cultivars (Werner and Peloquin 1991c). The yield gains from bilateral sexual polyploidization are typically higher than those from unilateral sexual polyploidization, presumably due to the contributions of heterozygosity from both parents (Werner and Peloquin 1991c).

Endosperm balance number can be reduced through anther culture or parthenogenesis. The production of haploids through anther culture is possible, but can be difficult because it requires the presence of genes for androgenic competence, which are not always found in potato cultivars (Sonnino et al. 1989). In contrast, it is relatively easy to produce parthenogenetically derived haploids from Tuberosum Group tetraploids by crossing them to selected pollinators (Hougas et al. 1958). These 2x (2EBN) haploids cross readily to 2x (2EBN) wild species, often producing hybrids with good yield, adaptation and fertility (Yerk and Peloquin 1986; Hermundstad and Peloquin 1986). Seed set when haploids are crossed to wild species is similar to that when cultivars are intercrossed (Budin and Gavrilenko 1994). The production of haploids is dependent on a pollination mechanism which permits fertilization of the central cell but not the egg, allowing the egg to develop into a plant through parthenogenesis (Hougas et al. 1958). Haploids (2n=2x=24) of the common potato (2n=4x=48) have been obtained in large numbers from a wide range of parental clones (Hougas et al. 1964). They have been successfully hybridized with cultivated and wild, 24-chromosome, tuber-bearing *Solanum* species from Mexico and South America.

Polyploidization Through Somatic (protplast) fusion

Diploid hybrids can be somatically doubled through chemical means such as colchicine (Ross et al. 1967) or through tissue culture (Sonnino et al. 1988) to bring them to teraploid level. However, tetraploids produced by this method do not exhibit a yield increase because new interlocus and intralocus interactions are not created (Rowe 1967; Maris 1990; Tai and Jong 1997). Somatic doubling can produce only one type of heterozygote (duplex-AAaa) and a maximum of two alleles per locus. A common somatic fusion strategy fuses protoplasts of tetraploid cultivars with those of sexually incompatible diploid wild species. The resulting hexaploid hybrids are often fertile and crossable with the tetraploid cultivars (Carputo et al. 1997). The pentaploid offspring are also fertile and tetraploid clones are recovered after a few backcrosses. Alternatively, diploid cultivated potato clones can be fused with diploid wild species to produce tetraploid hybrids (Rokka et al. 1994; Carputo et al. 1997). However, hexaploid somatic hybrids from 4x + 2x fusions are typically more successful in crosses to cultivars than are tetraploid hybrids from 2x + 2x fusions (Helgeson et al. 1988). Most somatic fusions have been carried out to capture disease resistance genes, but somatic fusion hybrids with improved salt tolerance have also been developed (Bidani et al. 2007). While chromosomes from both parents are typically found in somatic fusion hybrids, the genetic contributions of some wild species are lost more rapidly than others in backcross generations (Naess et al. 2001). Somatic fusion has allowed the production of fertile hexaploid hybrids between tetraploid *S. tuberosum* (EBN = 4) and diploid EBN = 1 species, such as the non-tuber-bearing species *S. brevidens* that has tuber soft rot and early blight resistances (Tek et al. 2004) and *S. bulbocastanum* that has a major gene for broad spectrum resistance to late blight (Naess et al. 2000).

Conventional Potato Breeding

Conventional potato breeding involves initial crossing of parents possessing complementary traits followed by selection in the subsequent clonal generations (Sleper and Poehlman 2006; Bradshaw and Bonierbale 2010). Because the crop is generally vegetatively propagated, favourable traits are fixed in the F_1 generation. The clones are highly heterozygous and exploit heterosis. However, progenies produced by selfing of clones reveal strong inbreeding depression (Arndt and Peloquin 1990). For effective potato breeding, there is need to understand the crop's reproductive biology as well as the breeding procedures.

Selection of Parents and Prediction of Cross Outcome

Making crosses between pairs of parents with complementary features has traditionally been, and still is the main route for the development of new cultivars (Caligari 1992; Sleper and Poehlman 2006; Acquaah 2007). The parents are usually chosen on the basis of their phenotypes (Caligari 1992; Bradshaw and Mackay 1994). The aim is to generate genetic variation on which to practice phenotypic selection over a number of vegetative generations for clones with as many desirable characteristics as possible for release as new cultivars (Caligari 1992; Sleper and Poehlman 2006; Acquaah 2007). The choice of parents depends largely on the aims and objectives of the breeder (Caligari 1992).

An important criterion for the choice of parents is their crossability and unrelatedness (Wolfgang et al. 2009). Often the parents are chosen due to their performance *per se*. Theoretically, this cannot be secure in clonal crops like potatoes; clonal varieties are highly heterozygous hybrids and polyploids, so that segregation in crossings is almost unpredictable (Wolfgang et al. 2009). Suggestions that have been made for better assessment of parents is the offspring performance from test crosses; the other suggestion is to work on reduced polyploidy level, which has been especially proposed for breeding tetraploid potatoes (Ross 1986). In general, parents should have a good combing ability and good performance over all traits. In potatoes, it has been observed that SCA is nearly as large as GCA, and in some cases SCA has been observed that is clearly larger than GCA (Tai 1976; Killick 1977). In situations where not much is known about the performance of a cross, the number of cross combinations should be increased to the maximum of the breeder's capacity and the number of genotypes per cross should be kept small (Wolfgang et al. 2009). This is based on selection theory which shows that "if the breeder has no prior knowledge on the cross, the breeder has to make as many crosses as possible"; this also minimizes the risk of raising genotypes with poor performance (Wricke and Weber 1986). Potatoes are highly heterozygous so that dominance and epistatic effects contribute considerably to clone performance. Therefore, it should be assumed not much is known about the value of a cross combination until it has been made and the progeny tested.

Crossing success can be predicted based on endosperm balance number (EBN), or effective ploidy, of the parents if they are known. Once the cross is successful, the simplest method for predicting the value of cross combinations is to evaluate progenies at seedling stage (Neele and Louwes 1989; Neele et al. 1991). If a close relation between seedling performance and performance in subsequent field generations exists, as found by Brown and Caligari (1989) for tuber yield and plant appearance and by Neele and

Louwes (1989) for crisp quality and dry-matter content, progeny selection could be carried out at the seedling stage. Progeny tests offer the means to replace phenotypic recurrent selection with a much more efficient, multi-trait, genotypic recurrent selection programme, in which the generation cycle time can be reduced by several years, because parents with good GCA can be recognised shortly after each round of hybridization. Then their progeny can be used for subsequent crossing cycles and selection, whilst cultivars are being produced from resowings of the best progenies (Bradshaw and Mackay 1994). Progeny means is therefore a reliable approach in identifying superior cross combinations (Brown and Caligari 1989; Gopal 1997).

For highly heritable traits, the midparent value is a good predictor of the mean performance of the offspring, and a few carefully chosen crosses can be made (Bradshaw et al. 2000). However, with an only moderately heritable trait such as yield, offspring mean is less predictable, and more crosses need to be made to ensure that they include the best possible cross for the trait (Bradshaw 2007). For such a trait breeders still have rely on phenotypic data and the concepts of quantitative genetics to determine crossing strategies (Bradshaw 2007).

Mid parent values for yield and quality traits can complement the results of the seedling progeny tests (Maris 1989). The mid-parent value is the predictor that is generally used in potato breeding programmes because the method is quick, cheap, and easy. No time is lost with the production of hybrid seed and seedlings, and the data are available from experiments already performed with the clones of interest. If additional information is required, the cost of trials is not likely to interfere with a large number of entries to be tested (Neele et al. 1991). Neele et al. (1991) found the seed potato harvest prediction by the mid-parent value to be very good for most characters, with many correlation coefficients between progeny mean and mid-parent value exceeding r = 0.8. Moderate correlation coefficients were noted for foliage weight, number of stems, tuber shape and number of tubers. However, at ware potato harvest, the correlation coefficients were lower; tuber yield in particular was poorly predicted by the mid-parent value. Maris (1989) obtained moderate to good correlation coefficients (r=0.51 to r=0.85) between the mid-parent value and the actual progeny performance for various agronomically important characters. For yield and number of tubers, however, the correlations were moderate (r=0.51 and r=0.59 respectively). Brown and Caligari (1989) were not able to accurately predict the progeny performance by the mid-parent value. This suggests that a prediction based on the mean of the parental values might have limited value and might not result in progenies with the best prospects.

Implications of Genetics in Potato Breeding

The 2*n* gametes represent unique tools for genetic studies of potatoes. This is important given that the tetrasomic inheritance of the tetraploid potato makes such studies very difficult. The 2*n* gametes have been used to determine gene-centromere map distances, such as those of isozymes and the yellow tuber flesh color loci (Douches and Quiros 1987), and those of genes conferring resistance to viruses and nematodes (Wagenvoort and Zimnoch-Guzowska 1992). They have also been employed to infer the physical location of QTLs controlling total tuber yield (Buso et al. 1999). A very important feature of 2*n* gametes is that they make the potato the best organism in which to manipulate all sets of chromosomes for breeding purposes. They allow breeders to broaden the genetic diversity, introducing both new genes for the improvement of traits of interest and allelic diversity to maximize heterozygosity in tetraploid varieties. In the potato, as in other polysomic polyploids, the genetic variance for several important polygenic traits (e.g., tuber yield), is almost entirely non-additive, depending on intralocus (heterozygosity) and inter-locus (epistasis) interactions (Mendoza and Heynes 1974). Thus, breeding for polygenic traits should be oriented towards maximizing heterozygosity and maintaining valuable epistatic combinations. The significance of 2*n* gametes in these crossing schemes lies not only in the possibility of returning to the 4*x* level, but also in their ability to transmit non-additive genetic effects (heterozygosity and epistasis) from the 2*x* parent to the 4*x* offspring. This aspect is important given that *n* gametes from diploids are only capable of transmitting additive effects, whereas *n* gametes from tetraploids can only transmit a certain number of first-order intralocus interactions; epistasis is not transmitted due to the disruptive effects of meiosis. Due to the high level of intra- and inter-locus interactions transmitted by FDR gametes and on the wide natural occurrence of 2*n* pollen, much emphasis has been given to the 4*x* x 2*x*—FDR breeding approach to produce superior potato genotypes. Besides the advantages of the allelic interactions transmitted, FDR 2*n* gametes are highly uniform, and thus unilateral sexual polyploidization is expected to produce vigorous and fairly homogeneous progeny in crosses with tetraploids (Ortiz 1997). The availability of so many species with a 24-chromosome complement, the ease with which most of these species can be crossed with *S. tuberosum* haploids, and the widespread occurrence of meiotic mutations leading to 2*n* gametes, strongly favour sexual polyploidization crossing-schemes for germplasm introgression from species which are crossable with *S. tuberosum* haploids.

Dihaploids obtained from autoteraploid potato could exhibit disomic inheritance and a smaller population is required to generate a particular genotype than with the tetraploids. Such dihaploids can be more easily bred

for recessive traits than their polyploid parents. The selected germplasm can then return to the original polyploidy level by somatic polyplodization using colchicines or sexual polyplodization using 2*n* gametes (Sleper and Poehlman 2006). Dihaploids may also be used to transfer genes from polyploidy to a related diploid species and vice versa.

Somatic fusion can be applied in combining disease resistance genes from two sexually incompatible parents. When protoplasts of diploids carrying a major gene for PVX resistance (Hines and Marx 2001) were fused with those of diploids carrying a major gene for PVY resistance (*Ry*), most of the hybrids expressed both genes (Thach and Wenzel 1993). Similarly, when clones carrying genes for resistance to different pathotypes of potato cyst nematode were fused, some of the hybrids were resistant to both pathotypes (Rasmussen et al. 1996). Foliar and tuber late blight resistance have also been combined in somatic fusion hybrids (Rasmussen et al. 1998). The fusion of diploid *S. verrucosum* protoplasts with those of cultivated potato clones combined PLRV resistance from *S. verrucosum* with adaptation and tuber yield from the cultivated potato donor (Carrasco et al. 2000). Resistance to bacterial wilt in the tetraploid potato, has frequently been sought from the diploid *S. phureja* which produces unreduced gametes; another diploid, *S. stenotomum* has been used at experimental level through somatic fusion (Fock et al. 2000, 2001). The potential use of somatic hybridization has been demonstrated by the successful introduction of traits of resistance to viruses (Gibson et al. 1988; Valkonen and Rokka 1998), to extreme climatic conditions such as frost (Preiszner et al. 1991), to fungi (Mattheij et al. 1992) and to insects (Serraf et al. 1991) into the cultivated potato. Resistance against bacterial wilt has successfully been transferred from *S. commersonii* (Laferriere et al. 1999) and *S. phureja* (Fock et al. 2000) into potato through somatic hybridization.

Marker Assisted Selection in Potato Breeding

In potato, molecular markers have been used for construction of genetic linkage maps (Bonierbale et al. 1988; Gebhardt et al. 1991; Bonierbale et al. 1994), trait tagging (Gebhardt et al. 1993; Bryan et al. 2002), fingerprinting analysis (Milbourne et al. 1997), phylogeny studies (Raker and Spooner 2002), and characterization of accessions from germplasm banks (Ghislain et al. 2006). Powell et al. (1991) have suggested using genetic distance based on molecular markers to select diverse parents capable of producing high-performing progeny. Molecular markers have been identified for resistance against late blight (Colton et al. 2006), nematodes and viruses in potatoes (Gebhardt et al. 2006).

There are three major types of genetic markers: (Harris 1976) (1) morphological (also 'classical', 'phenotypic' or 'visible') markers which

themselves are phenotypic traits or characters; (2) biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA sequence (Winter and Kahl 1995). The major disadvantages of phenotypic and biochemical markers are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter and Kahl 1995). In addition, biochemical markers are expensive. Despite these limitations, these markers have been extremely useful to plant breeders (Weeden et al. 1994; Eagles et al. 2001). Molecular markers are the most widely used mainly due to their abundance. They are also environmentally neutral and independent, and therefore more robust and unbiased compared to phenotypic descriptors. They arise from different classes of DNA mutations such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Paterson 1996). The most widely used molecular markers in potatoes are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR) or microsatellites (Collard et al. 2005). Single nucleotide polymorphisms (SNP) are the latest markers (Hamilton et al. 2011).

Conclusions and Recommendations

Potato is easy to breed because it is clonally propagated; once variation is released through crossing, there is no problem with stabilizing (fixing) any desirable combination that arises, as any clone can be multiplied unchanged by asexual reproduction. However, generating TPS is a problem due to various infertility systems operating in potatoes; these can be overcome through use of 2*n* gametes and somatic fusion. Conventional potato breeding takes long,about 10 years before a cultivar is released, mostly due to the slow multiplication rate of the crop. This time can be reduced by use of progeny tests to discard whole families before starting the within family selection; use of modern methods of rapid multiplication may shorten the time even further.

A big impact on the efficiency and rate of progress would be the identification of superior clones genotypically as seedlings in the glasshouse. This will require molecular marker assisted selection or preferably direct recognition of the desired allele at a genetic locus. Molecular makers have been used extensively in potatoes in genetic studies; they could be used in identifying parents with desired traits and hence shorten the breeding cycle.

Abbreviations

EBN	Endosperm balance number
GCA	General combining ability
SCA	specific combining ability

References

Abdalla, M.M.F. and J.G.T. Hermsen. 1972. Unilateral incompatibility: Hypotheses, debate, and its implications for plant breeding. Euphytica 21: 32–47.

Acquaah, G. 2007. Principles of plant genetics and breeding. Blackwell Publishing Ltd., 350 Main Street, Malden, MA, USA.

Almekinders, C.J.M. 1991. Flowering and true seed production in potato (*Solanum tuberosum* L.) 2. Effects of stem density and pruning of lateral stems. Potato Research 34: 379–388.

Almekinders, C.J.M. 1992. The effect of photoperiod on flowering and TPS production in the warm tropics. Potato Research 35: 433–442.

Almekinders, C.J.M. and S.G. Wiersema. 1991. Flowering and true seed production in potato (*Solanum tuberosum* L.) 1. Effects of inflorescence position, nitrogen treatment and harvest date of berries. Potato Research 34: 365–377.

Almekinders, C.J.M. and P.C. Struik. 1996. Shoot development and flowering in potato (*Solanum tuberosum* L.). Potato Research 39: 581–607.

Arndt, G.C. and S.J. Peloquin. 1990. The identification and evaluation of hybrid plants among open pollinated true seed families. American Potato Journal 67: 293–304.

Bamberg, J.B. and R.E. Hanneman. 1988. Enhanced production of botanical seed of tuber bearing *Solanum* species via supplemental fertilizer applications. American Potato Journal 65: 470.

Bidani, A., O. Nouri-Ellouz, L. Lakhoua, D. Sihachakr, C. Cheniclet, A. Mahjoub, N. Drira and B.R. Gargouri. 2007. Interspecific potato somatic hybrids between *Solanum berthaultii* and *Solanum tuberosum* L. showed recombinant plastome and improved tolerance to salinity. Plant, Cell, Tissue, and Organ Culture 91: 179–189.

Blomquist, A.W. and F.L. Lauer. 1962. A simplified technique for handling and storing potato pollen. American Potato Journal 39: 340–343.

Bonierbale, M.W., R.L. Plaisted and S.D. Tanksley. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120: 1095–1103.

Bonierbale, M.W., R.L. Plaisted, O. Pineda and S.D. Tanksley. 1994. QTL analysis of trichome-mediated insect resistance in potato. Theoretical and Applied Genetics 87: 973–987.

Bradshaw, J.E. 2007. Breeding potato as a major staple crop. *In*: M.S. Kang and P.M. Priyadarshan (eds.). Breeding major food staples. Blackwell Publishing professional, Ames, Iowa, USA. edited. pp. 277–332

Bradshaw, J.E. and G.R. Mackay. 1994. Breeding strategies for clonally propagated potatoes. *In*: J.E. Bradshaw and G.R. Mackay (eds.). Potato genetics. CAB International, Wallingford, UK, pp. 467–497.

Bradshaw, J.E. and M. Bonierbale. 2010. Potatoes. *In*: J.E. Bradshaw (ed.). Root and tuber crops, Handbook of plant breeding 7. Springer Science +Business Media, New York. edited. pp. 1–52.

Bradshaw, J.E., D. Todd and R.N. Wilson. 2000. Use of tuber progeny tests for genetical studies as part of a potato (*Solanum tuberosum* subsp. *tuberosum*) breeding programme. Theoretical and Applied Genetics 100: 772–781.

Brink, R.A. and D.C. Cooper. 1947. The endosperm in seed development. Botanical Review 13: 423–541.

Brown, C.R. 1993. Outcrossing rate in cultivated autotetraploid potato. American Potato Journal 70: 725–734.

Brown, J. and P.D.S. Caligari. 1989. Cross prediction in a potato breeding programme by evaluation of parental material. Theoretical and Applied Genetics 77: 246–252.

Bryan, G.J., K. McLean, J.E. Bradshaw, W.S. De Jong, M. Phillips, L. Castelli and R. Waugh. 2002. Mapping QTLs for resistance to the cyst nematode *Globodera pallida* derived from the wild potato species *Solanum verneii*. Theoretical and Applied Genetics 105: 68–77.

Budin, K.Z. and T.A. Gavrilenko. 1994. Genetic bases of remote hybridization in potato. Russian Journal of Genetics 30: 1188–1196.

Bukasov, S.M. 1966. Die Kulturarten der Kartoffeln und ihre wildwachsenden Vorfahren. Z. Pflanzenzucht 55: 139–164.

Buso, J.A., L.S. Boiteux, G.C.C. Tai and S.J. Peloquin. 1999. Chromo- some regions between centromeres and proximal crossovers are the physical size of major effect loci for yield in potato: Genetic analysis employing meiotic mutants. Proceedings of the National Academy of Science USA 96: 1773–1778.

Caligari, P.D.S. 1992. Breeding new varieties. *In*: P. Harris (ed.). The potato crop. 2nd ed., Chapman and Hall, London, pp. 334–372.

Camadro, E.L. and R.W. Masuelli. 1995. A genetic model for the Endosperm Balance Number (EBN) in the wild potato S. acaule Bitt. and two related diploid species. Sexual Plant Reproduction 8: 283–288.

Camadro, E.L., D. Carputo and S.J. Peloquin. 2004. Substitutes for genome differentiation in tuber-bearing *Solanum*: interspecific pollen- pistil incompatibility, nuclear-cytoplasmic male sterility, and endosperm. Theoretical and Applied Genetics 109: 1369–1376.

Carputo, D. and A. Barone. 2005. Ploidy level manipulations in potato through sexual hybridisation. Annals of Applied Biology 146: 71–79.

Carputo, D., L. Frusciante and S.J. Peloquin. 2003. The role of 2n gametes and endosperm balance number in the origin and evolution of polyploids in the tuber-bearing *Solanums*. Genetics 163: 287–294.

Carputo, D.C.T., M. Speggiorin, A. Zoina and L. Frusciante. 1997. Resistance to blackleg and tuber soft rot in sexual and somatic interspecific hybrids with different genetic background. American Potato Journal 74: 161–172.

Carrasco, A.R.D.G.J.I., A. Rico and E. Ritter. 2000. Transfer of PLRV resistance from *Solanum verrucosum* Schlechdt to potato (*S. tuberosum* L.) by protoplast electrofusion. Potato Research 43: 31–42.

CIP. 1984. Potato for the developing world. A collaborative experience. Centro Internacional de la Papa, Lima, Peru.

CIP. 1985. Annual Report 1984. Centro Internacional de la Papa, Lima, Peru.

Collard, B.C.Y., M.Z.Z. Jahufer, J.B. Brouwer and E.C.K. Pang. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142: 169–196.

Colton, L.M., H.I. Groza, S.M. Wielgus and Jiang. 2006. Marker-Assisted Selection for the broad-spectrum potato late blight resistance conferred by gene RB derived from a wild potato species. Crop Science 46: 589–594.

Den Nijs, T.P.M. and S.J. Peloquin. 1977. The role of endosperm in hybridization. American Potato Journal 54: 488–489 (abstr).

Dodds, K.S. 1965. The history and relationships of cultivated potatoes. *In*: J.B. Hutchinson (ed.). Essays in crop plant evolution. Cambridge University Press, Cambridge, England, pp. 123–141.

Douches, D.S. and C.F. Quiros. 1987. Use of 4x–2x crosses to determine gene-centromere map distances of isozyme loci in Solanum species. Genome 29: 519–527.

Douches, D.S. and K. Jastrzebski. 1993. Potato, *Solanum tuberosum* L. *In*: G. Kalloo and B.O. Bergh (eds.). Genetic improvement of vegetable crops., Pergamon Press, Oxford, U.K., pp. 605–644.

Dvøra' k.J. 1983. Evidence for genetic suppression of heterogenetic chromosome pairing in polyploid species of *Solanum*, sect. *Petota*. Canadian Journal of Genetics and Cytology 25: 530–539.

Eagles, H., H. Bariana, F. Ogbonnaya, G. Rebetzke, G. Hollamby, R. Henry, P. Henschke and M. Carter. 2001. Implementation of markers in Australian wheat breeding. Australian Journal of Agricultural Research 52: 1349–1356.

Ehlenfeldt, M.K. and R.E. Hanneman Jr. 1988a. Genetic control of Endosperm Balance Number (EBN): Three additive loci in a threshold-like system. Theoretical and Applied Genetics 75: 825–832.

El-Gizawy, A.M., A.A. El-Yazied, A.A. Tawfik and A.A. El-Kaddour. 2006. Effect of Gibberellic acid (GA_3) on enhancing flowering and fruit setting in selected potato cultivars. Annals of Agricultural Science, Ain Shams University, Cairo 51: 173–189.

FAO. 1995. Potato in the 1990s. Situation and prospects of the world potato economy. A joint study by the Basic Foodstuffs Service, FAO Commodities and Trade Division, and Post Harvest Management Marketing Program. Food and Agriculture Organisation of the United Nations, Rome.

FAO. 2004. Production statistics [Online]. Available at http://www.apps.fao.org (Accessed 10 March 2010). Food and Agriculture Organisation of the United Nations, Rome.

FAO. 2008. International year of the potato [Online]. Available at http://www.potato2008.org (Accessed 10 March 2010). Food and Agriculture Organisation of the United Nations, Rome.

FAO. 2010. Strengthening potato value chains: Technical and policy options for developing coutries. Food and Agriculture Organisation of the United Nations, Rome.

Fock, I., C. Collonnier, A. Purwito, J. Luisetti, V. Souvannavong, F. Vedel, A. Servaes, A. Ambroise, H. Kodja, G. Ducreux and D. Sihachakr. 2000. Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja*. Plant Science 160: 165–176.

Fock, I., C. Collonnier, J. Luisetti, A. Purwito, V. Souvannavong, F. Vedel, A. Servaes, A. Ambroise, H. Kodja, G. Ducreux and D. Sihachakr. 2001. Use of *Solanum stenotomum* for introduction of resistance to bacterial wilt in somatic hybrids of potato. Plant Physiology and Biochemistry 39: 899–908.

Gavrilenko, T. 2007. Potato Cytogenetics. *In*: Dick Vreugdenhil, J.E. Bradshaw, C. Gebhardt, F. Govers, D.K.L. Mackerron, M.M. Taylor and H.A. Ross (eds.). Potato Biology and Biotechnology. Advances and Perspectives. Elsevier B.V. pp. 203–216.

Gebhardt, C., D. Mugnieri, E. Ritter, F. Salamini and E. Bonnel. 1993. Identification of RFLP markers closely linked to the H1 gene conferring resistance to *Globodera rostochiensis* in potato. Theoretical and Applied Genetics 85: 541–544.

Gebhardt, C., D. Bellin, H. Henselewski, W. Lehmann, J. Schwarzfischer and J.P.T. Valkonen. 2006. Marker-assisted combination of major genes for pathogen resistance in potato. Theoretical and Applied Genetics 112: 1458–1464.

Gebhardt, C., E. Ritter, A. Barone, T. Debener, B. Walkemeier, U. Schachtschabel, H. Kaufmann, R.D. Thompson, M.W. Bonierbale, M.W. Ganal, S.D. Tanksley and F. Salamini. 1991. RFLP maps of potato and their alignment with the homologous tomato genome. Theoretical and Applied Genetics 83: 49–57.

Ghislain, M., D. Andrade, F. Rodríguez, R.J. Hijmans and D.M. Spooner. 2006. Genetic analysis of the cultivated potato *Solanum tuberosum* L. Phureja Group using RAPDs and nuclear SSRs. Theoretical Applied Genetic 113: 1515–1527.

Ghislain, M., D. Andrade, F. Rodríguez, R.J. Hijmans and D.M. Spooner. 2006. Genetic analysis of the cultivated potato *Solanum tuberosum* L. Phureja Group using RAPDs and nuclear SSRs. Theoretical Applied Genetic 113: 1515–1527.

Gibson, R.W., M.G.K. Jones and N. Fish. 1988. Resistance to potato leaf roll virus and potato virus Y in somatic hybrids between dihaploid *Solanum tuberosum* and *S. brevidens*. Theoretical and Applied Genetics 76: 113–117.

Glendining, D.R. 1976. Neo-Tuberosum: New potato breeding material. 4. The breeding system of Neo-Tuberosum, and the structure and composition of the the Neo-Tuberosum genepool. Potato Research 19: 27–36.
Gopal, J. 1994. Flowering behaviour, male sterility, and berry setting in tetraploid *Solanum tuberosum* germplasm. Euphytica 72: 133–142.
Gopal, J. 1997. Progeny selection for agronomic characters in early generations of a potato breeding programme. Theoretical and Applied Genetics 95: 307–311.
Gopal, J. 2006. Considerations for successful breeding. *In*: J. Gopal and S.M.P. Khurana (eds.). Handbook of potato production, improvement, and postharvest management. Food Products Press, New York. edited. pp. 77–108.
Grun, P. 1970. Cytoplasmic sterilities that separate the cultivated potato from its putative diploid ancestors. Evolution 24: 750–758.
Grun, P. 1979. Evolution of the cultivated potato: a cytoplasmic analysis. *In*: J.G. Hawkes, R.N. Lester and A.D. Skelding (eds.). The biology and taxonomy of the Solanaceae. Academic Press, London, pp. 655–665.
Grun, P. 1990. The evolution of cultivated potatoes. Economic Botany 44: 39–55.
Grun, P., C. Ochoa and D. Capage. 1977. Evolution of cytoplasmic factors in tetraploid cultivated potatoes (*Solanaceae*). American Journal of Botany 64: 412–420.
Hamilton, J.P., C.N. Hansey, B.R. Whitty, K. Stoffel, A.N. Massa, A.V. Deynze, W.S.D. Jong, D.S. Douches and C.R. Buell. 2011. Single nucleotide polymorphism discovery in elite north american potato germplasm. BMC Genomics 12: 302. http://www.biomedcentral.com/1471-2164/12/302.
Hanneman Jr, R.E. 1994. Assignment of Endosperm Balance Numbers to the tuber-bearing Solanums and their close non-tuber-bearing relatives. Euphytica 74: 19–25.
Hanneman Jr., R.E. 1999. The reproductive biology of the potato and its implication for breeding. Potato Research 42: 283–312.
Hanneman Jr., R.E. and S.J. Peloquin. 1967. Crossability of 24 chromosome potato hybrids with 48-chromosome cultivars. European Potato Journal 10: 62–73.
Hanneman Jr., R.E. and S.J. Peloquin. 1968. Ploidy levels of progeny from diploid-tetraploid crosses in the potato. American Potato Journal 45: 255–261.
Hanneman Jr., R.E. and S.J. Peloquin. 1981. Genetic-cytoplasmic male sterility in progeny of 4x-2x crosses in cultivated potatoes. Theoretical and Applied Genetics 59: 53–55.
Harlan, J.R. and J.M. Wit. 1975. On O Winge and a prayer: the origins of polyploidy. Botanical Review 41: 361–390.
Harris, O.C. 1976. Bacterial wilt in Kenya with particular reference to potatoes. *In*: L. Sequeira and A. Kelman (eds.). Proceedings of the first international planning conference and workshop on the ecology and control of bacterial wilt caused by *Pseudomonas solanacearum*. 18–24 January 1976. Raleigh, North Carolina., Springer-Verlag, Berlin, pp. 84–88.
Hawkes, J.G. 1956. Taxonomic studies on the tuber-bearing Solanums. I. *Solanum tuberosum* and the tetraploid species complex. Proceedings of the Linnean Society of London 166: 97–144.
Hawkes, J.G. 1990. The potato: Evolution, biodiversity, and genetic resources. Belhaven Press, London.
Hawkes, J.G. 1994. Origins of cultivated potatoes and species relationships. *In*: J.E. Bradshaw and G.R. Mackay (eds.). Potato genetics. CAB International, Wallingford, UK, pp. 3–42.
Hawkes, J.G. and M.T. Jackson. 1992. Taxonomic and evolutionary implications of the endosperm balance number hypothesis in potatoes. Theoretical and Applied Genetics 84: 180–185.
Helgeson, J.P., G.T. Haberlach, J. Pohlman and S. Austin. 1988. Somatic fusions of *Solanum* species. Plant Cell Tissue and Organ Culture 12: 185–187.
Hermundstad, S. and S.J. Peloquin. 1986. Tuber yield and tuber traits of haploid-wild species F1 hybrids. Potato Research 29: 289–297.

Hijmans, R. 2001. Global distribution of the potato crop. American Journal of Potato Research 78: 403–412.

Hines, P.J. and J. Marx. 2001. The endless race between plant and pathogen. Science 292: 2269–2289.

Hosaka, K. and R.E. Hanneman. 1988. Origin of chloroplast DNA diversity in andean potatoes. Theoretical and Applied Genetics 76: 333–340.

Hosaka, K. and R.E. Hanneman. 1998. Genetics of self-incompatibility in a self-incompatible wild diploid potato species *Solanum chacoense*.I. Detection of an S locus inhibitor (Sli) gene. Euphytica 99: 191–197.

Hougas, R.W., S.J. Peloquin and R.W. Ross. 1958. Haploids of the common potato. Journal of Heredity 49: 103–107.

Hougas, R.W., S.J. Peloquin and A.C. Gabert. 1964. Effect of seed-parent and pollinator on frequency of haploids in *Solanum tuberosum*. Crop Science 4: 593–597.

Howard, H.W. 1958. The storage of potato pollen. American Potato Journal 35: 676–678.

Howard, H.W. 1978. The production of new varieties. *In*: P.M. Harris (ed.). The potato crop. Chapman and Hall, London, pp. 607–644.

Iwanaga, M., R. Ortiz, M.S. Cipar and S.J. Peloquin. 1991. A restorer gene for genetic-cytoplasmic male sterility in cultivated potatoes. American Potato Journal 68: 19–28.

Jansky, S. 2006. Overcoming hybridization barriers in potato. Plant Breeding 125: 1–12.

Jansky, S. 2009. Breeding, genetics and cultivar development. *In*: J. Singh and L. Kaur (eds.). Advances in potato chemistry and technology. Academic Press, Burlington, VT, pp. 27–62.

Jansky, S.H. and D.M. Thompson. 1990. The effect of flower removal on potato tuber yield. Canadian Journal of Plant Science 70: 1223–1225.

Jauhnl, G. 1954. Zur Kenntnis des Bluuhverhaltens der Kartoffel. Verouff. Bundesanst. alp. Landw Admont 9: 40–74.

Johnston, S.A., T.P.M. den Nijs, S.J. Peloquin and R.E. Hanneman Jr. 1980. The significance of genic balance to endosperm development in interspecific crosses. Theoretical and Applied Genetics 57: 5–9.

Killick, R.J. 1977. Genetic analysis of several traits in potatoes by means of a diallel cross. Annals of Applied Biology 86: 279–289.

Laferriere, L.T., J.P. Helgeson and C. Allen. 1999. Fertile *Solanum tuberosum* + *Solanum commersonii* somatic hybrids as sources of resistance to bacterial wilt caused by *Ralstonia solanacearum*. Theoretical and Applied Genetics 98: 1272–1278.

Lewis, D. and L.K. Crowe. 1958. Unilateral interspecific incompatibility in flowering plants. Heredity 12: 233–256.

Maris, B. 1989. Analysis of an incomplete diallel cross among three ssp. *tuberosum* varieties and seven long-day adapted ssp. *andigena* clones of the potato (*Solanum tuberosum* L.). Euphytica 41: 163–182.

Maris, B. 1990. Comparison of diploid and tetraploid potato families derived from *Solanum phureja* x dihaploid *S. tuberosum* hybrids and their vegetatively doubled counterparts. Euphytica 46: 15–33.

Matsubayashi, M. 1991. Phylogenetic relationships in the potato and its related species. *In*: T. Tsuchiya and P.K. Gupta (eds.). Chromosome engineering in plants: genetics, breeding and evolution, part B. Elsevier, Amsterdam, pp. 93–118.

Mattheij, W.M., R. Eijlander, J.R.A.d. Koning and K.M. Louwes. 1992. Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophtora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens. Theoretical and Applied Genetics 83: 459–466.

Mendiburu, A.O. and S.J. Peloquin. 1977. Bilateral sexual polyploidization in potatoes. Euphytica 26: 573–583.

Mendoza, H.A. and F.L. Heynes. 1974. Genetic basis of heterosis for yield in the autotetraploid potato. Theoretical and Applied Genetics 45: 21–25.

Milbourne, D., R. Meyer, J.E. Bradshaw, E. Baird, N. Bonar, J. Provan, W. Powell and R. Waugh. 1997. Comparison of PCR based marker systems for the analysis of genetic relationships in cultivated potato. Molecular Breeding 3: 127–136.

Mok, D.W.S. and S.J. Peloquin. 1975a. Breeding value of 2n pollen (diplandroids) in tetraploid x diploid crosses in potatoes. Theoretical and Applied Genetics 46: 307–314.

Mok, D.W.S. and S.J. Peloquin. 1975b. Three mechanisms of 2n pollen formation in diploid potatoes. Canadian Journal of Genetics and Cytology 17: 217–225.

Mok, G. 1985. TPS research in Korea. *In*: Innovative methods for propagating potatoes. CIP, Lima, Peru, pp. 215–219.

Naess, S.K., J.M. Bradeen and S.M. Wielgus. 2000. Resistance to late blight in *Solanum bulbocastanum* is mapped to chromosome 8. Theoretical and Applied Genetics 101: 697–704.

Naess, S.K.B., J.M. Wielgus, S.M. Haberlach, G.T. McGrath, J.M. Bradeen and J.P. Helgeson. 2001. Analysis of the introgression of *Solanum bulbocastanum* DNA into potato breeding lines. Molecular Genetics and Genomics 265: 694–704.

Neele, A.E.F. and K.M. Louwes. 1989. Early selection for chip quality and dry matter content in potato seedling populations in greenhouse or screenhouse. Potato Research 32: 293–300.

Neele, A.E.F., H.J. Nab and K.K. Louwes. 1991. Identification of superior parents in apotato breeding programme. Theoretical and Applied Genetics 82: 264–272.

Ortiz, R. 1997. Breeding for potato production from true seed. Plant Breed Abstr 67: 1355–1360.

Pallais, N. 1986. Techniques to improve the sowing value of true potato seed. Hort Science 21: 826 (Abstr).

Paterson, A.H. 1996. Making genetic maps. *In*: A.H. Paterson (ed.). Genome Mapping in Plants. R.G. Landes Company, San Diego, California. Academic Press, Austin, Texas, pp. 23–39.

Peloquin, J.S. and R.W. Hougas. 1959. Decapitation and genetic markers as related to haplidy in in *Solanum tuberosum*. European Potato Journal 2: 176–183.

Plaisted, R.L. 1980. Potato. *In*: W.R. Fehr and H.H. Hadley (eds.). Hybridization of crop plants. The American Society of Agronomy, Inc, Madison, WI, pp. 483–494.

Preiszner, J., A. Feher, O. Veisz, J. Sutka and D. Dudits. 1991. Characterization of morphological variation and cold resistance in interspecific somatic hybrids between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil. Euphytica 57: 37–49.

Pushkarnath and R.S. Dwivedi. 1961. Potato floral biology series. II. Pollen and pollination. Indian Potato Journal 3: 76–85.

Raker, C.N. and D.M. Spooner. 2002. Chilean tetraploid cultivated potato *Solanum tuberosum* is distinct from Andean populations: Microsatellite data. Crop Science 42: 1451–1458.

Rasmussen, J.O., J.P. Nepeper and O.S. Rasmussen. 1996. Analysis of somatic hybrids between two sterile dihaploid *Solanum tuberosum* L. breeding lines. Restoration of fertility and complementation of *G. pallida* Pa2 and Pa3 resistance. Theoretical and Applied Genetics 92: 403–410.

Rasmussen, J.O., J.P. Nepeper, H.G. Kirk, K. Tolstrup and O.S. Rasumessen. 1998. Combination of resistance to potato late blight in foliage and tubers by intraspecific dihaploid protoplast fusion. Euphytica 102: 363–370.

Rokka, V.M., Y.S. Xu, J. Kankila, A. Kuusela, S. Pulli and E. Pehu. 1994. Identification of somatic hybrids of dihaploid *Solanum tuberosum* lines and *S. brevidens* by species specific RAPD patterns and assessment of disease resistance of the hybrids. Euphytica 80: 207–217.

Ross, H. (Ed.).1986. Potato breeding: Problems and perspectives., Verlag Paul Parey, Berlin and Hamburg.

Ross, R.W., J.A. Dionne and R.W. Hougas. 1967. Doubling the chromosome number of selected *Solanum* genotypes. European Potato Journal 10: 37–52.

Rowe, P.R. 1967. Performance of diploid and vegetatively doubled clones of *Phureja*-haploid *Tuberosum* hybrids. American Potato Journal 44: 195–203.

Schroeder, S.H. and S.J. Peloquin. 1983. Seed set in 4x-2x crosses as related to 2n pollen frequency. American Potato Journal 60: 527–536.

Serraf, I., D. Sihachakr, G. Ducreux, S.C. Brown, M. Allot, N. Barghi and L. Rossignol. 1991. Interspecific somatic hybridization in potato by protoplast electrofusion. Plant Science 76: 115–126.

Simmonds, N.W. 1995. Potatoes. *In*: J. Smartt and N.W. Simmonds (eds.). Evolution of crop plants. 2nd ed. Longman, London. edited. pp. 466–471.

Simmonds, N.W. 1997. A review of potato propagation by means of seed, as distinct from clonal propagation by tubers. Potato Research 40: 191–214.

Simon, P.W. and S.J. Peloquin. 1976. Pollen vigour as a function of mode of 2n gamete formation in potatoes. Journal of Heredity 67: 204–208.

Singh, A.K., F. Salamini and H. Uhrig. 1989. Chromosome pairing in 14 F1 hybrids among 11 diploid potato species. Journal of Genetics and Breeding 43: 1–5.

Sleper, D.A. and J.M. Poehlman. 2006. Breeding field crops, 5th ed., Blackwell Publishing Professional. 2121 State Avenue, Ames, Iowa.

Sonnino, A., M. Iwanaga and A. Henostroza. 1988. Chromosome number doubling of 2x potato lines with diverse genetic background through tissue culture. Potato Research 31: 627–631.

Sonnino, A., S. Tanaka, M. Iwanaga and L. Schilde-Rentscher. 1989. Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Reproduction 8: 105–107.

Spooner, D. and R.J. Hijmans. 2001. Potato systematics and germplasm collecting, 1989–2000. American Potato Journal 78: 237–268.

Spooner, D.M., R.G. Van den Berg, A. Rodriquez, J. Bamberg, R.J. Hijmans and S.I. Lara-Cabrera. 2004. Wild potatoes (*Solanum* sect. *Petota; Solanaceae*) of North and Central America. Systematic Botany Monograph 68: 13.

Tai, G.C.C. 1976. Estimation of general and specific combing abilities in potato. Canadian Journal of Genetics and Cytology 18: 463–470.

Tai, G.C.C. and H.D. Jong. 1997. A comparison of performance of tetraploid progenies produced by diploid and their vegetatively doubled (tetraploid) couterpart parents. Theoretical and Applied Genetics 94: 303–308.

Tek, A.L., W.R. Stevensen and J.P. Helgeson. 2004. Transfer of tuber soft rot and early blight resistances from *Solanum brevidens* into cultivated potato. Theoretical and Applied Genetics 109: 249–254.

Thach, N.Q.F.U. and G. Wenzel. 1993. Somatic fusion for combining virus resistances in *Solanum tuberosum* L. Theoretical and Applied Genetics 85: 863–867.

Thijn, G.A. 1954. Observations on flower induction with potatoes. Euphytica 3: 28–34.

Tsegaw, T. 2005. Response of potato to paclobutrazol and manipulation of reproductive growth under tropical conditions. Ph.D. Thesis., University of Pretoria, South Africa.

Tucci, M., D. Carputo, G. Bile and L. Frusciante. 1996. Male fertility and freezing tolerance of hybrids involving *Solanum tuberosum* haploids and diploid Solanum species. Potato Research 39: 345–353.

Turner, A.D. and E.E. Ewing. 1988. Effects of photoperiod, night temperature, and irradiance on flower production in the potato. Potato Research 31*:* 257–268.

Upadhya, M.D. and R. Cabello. 2000. Influence of seed size and density on the performance of direct seedling transplants from hybrid true potato seed. CIP Programme Report 1999–2000. International Potato Center, Lima, Peru.

Valkonen, J.P.T. and V.M. Rokka. 1998. Combination and expression of two virus resistance mechanisms in interspecific somatic hybrids of potato. Plant Science 131: 85–94.

Van Staden, J., J.E. Davey and N.A.C. Brown. 1982. Cytokinins in seed development and germination. *In*: A.A. Khan (ed.). The physiology and biochemistry of seed development, dormancy and germination. Elsevier, New York, pp. 185–200.

Veilleux, R.E. 2005. Cell and tissue culture of potato (*Solanaceae*). *In*: M.K. Razdan and A.K. Mattoo (eds.). Genetic improvement of Solanaceous crops, Volume I: Potato. Science Publishers Inc, Enfield, NH, pp. 185–208.

Wagenvoort, M. and E. Zimnoch-Guzowska. 1992. Gene-centromere mapping in potato by half-tetrad analysis: Map distances of H1, Rx, and Ry and their possible use for ascertaining the mode of 2n pollen formation. Genome 35: 1–7.

Watanabe, K. and S.J. Peloquin. 1991. The occurrence and frequency of 2n pollen in 2x, 4x, and 6x wild, tuber-bearing *Solanum* species from Mexico, and Central and South America. Theoretical and Applied Genetics 82: 621–626.

Watanabe, K. and S.J. Peloquin. 1993. Cytological basis of 2n pollen formation in a wide range of 2x, 4x, and 6x taxa from tuberbearing *Solanum* species. Genome 36: 8–13.

Weeden, N., G. Timmerman and J. Lu. 1994. Identifying and mapping genes of economic significance. Euphytica 73: 191–198.

Werner, J.E. and S.J. Peloquin. 1987. Frequency and mechanisms of 2n egg formation in haploid tuberosum-wild species F1 hybrids. American Potato Journal 64: 641–654.

Werner, J.E. and S.J. Peloquin. 1990. Inheritance and two mechanisms of 2n egg formation in 2x potatoes. Journal of Heredity 81: 371–374.

Werner, J.E. and S.J. Peloquin. 1991. Yield and tuber characteristics of 4x progeny from 2x × 2x crosses. Potato Research 34: 261–267.

Werner, J.E. and S.J. Peloquin. 1991c. Yield and tuber characteristics of 4x progeny from 2x x 2x crosses. Potato Research 34: 261–267.

Winter, P. and G. Kahl. 1995. Molecular marker technologies for plant improvement. World Journal of Microbiology and Biotechnology 11: 438–448.

Wolfgang, J., R. Mwanga, M. Andrade and J. Espinoza. 2009. Selection methods. Part 5: Breeding clonally propagated crops. *In*: S. Ceccarelli, E.P. Guimaraes and E. Weltzien (eds.). Plant breeding and farmer participation, FAO, Rome, pp. 275–322.

Wricke, G. and W.E. Weber. 1986. Qunatitative geentics and selection in plant breeding. de Gruyter, Berlin.

Yeh, B.P., S.J. Peloquin and R.W. Hougas. 1964. Meiosis in *Solanum tuberosum* haploids and haploid-haploid F1hybrids. Canadian Journal of Genetics and Cytology 6: 393–402.

Yerk, G.L. and S.J. Peloquin. 1986. Evaluation of tuber traits of 10, 2x (2EBN) wild species through hybrid x wild species hybrids. American Potato Journal 66: 731–739.

10

Reproductive Biological Characteristics of *Dendrobium* Species

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ABSTRACT

Dendrobium, as the second largest genera of Orchidaceae, is wildly distributed all over the world and has higher ornamental and medicinal values. Currently, research about the reproductive biological characteristics of the *Dendrobium* species are still limited. Therefore, only vegetative propagation (tissue culture) and sexual reproduction (flowering character, visitors, pollination and seed set) are reviewed in this article. Finally, the subjects of further research including flower morphology, pseudopollen, anther cap and lower fruiting rate are put forward.

Keywords: *Dendrobium*, vegetative propagation, sexual propagation, pseudopollen, pollinators

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Introduction

Dendrobium, which has about 1000–1400 original species with higher ornamental and medicinal values (Kuehnle 2006; Wang et al. 2007; He et al. 2008), is the second largest genera after *Bulbophyllum* of Orchidaceae. It is extensively distributed between Asia north of the equator and the Oceania regions (Ji et al. 1999; Wang et al. 2007). Seed and vegetative propagation are the major modes of reproduction of the *Dendrobium* species under natural conditions. However, *Dendrobium* species often have lower seed setting, and mycorrhizal symbiosis limits seed germination in seed propagation, resulting in the poor ability of natural reproduction (He et al. 2008; Li et al. 2009b). *Dendrobium* orchids are popular flowering potted and cut flowers around the world due to their flowering floriferousness, wide range in flower color, size, and shape, year-round availability, and lengthy post-harvest life (Kuehnle 2006). The earliest record about *Dendrobium* species can be traced back to "Shen Nong's Classic of Materia Medica" (also named as Shennong's Herbal), a classic work on plants and their utilization, written in the Han Dynasty (206 B.C.–220 A.D.) in China (Wang et al. 2007). Previous studies have focused on the many chemical ingredients, such as alkaloid, polysaccharide, sesquiterpenoids, phenanthrenes and bibenzyls in the *Dendrobium* species (Zhang et al. 2003; Ma and Wang 2008; Li et al. 2010), which could be used to effectively treat eosinophilic gastroenteritis, cataract, arthritis, thromboangiitis obliterans and chronic pharyngitis (Wei 2005). Chemical ingredients contained in *Dendrobium* species are effective in promoting digestion, extending life span, regulating the immune system, dilating blood vessels, lowering or normalizing blood pressure, and treating against tumor (Cheng and Guo 2001; Zhang 2003; Ma and Wang 2008; Zou and Liu 2010; Ng et al. 2012). Although lots of knowledge has been displayed in tissue culture and rapid propagation of *Dendrobium* species, reproductive biological characteristics of the plant are still limited. In this present chapter, reproductive biological characteristics of *Dendrobium* species including vegetative propagation and sexual reproduction are reviewed. Besides, a perspective on unknown reproductive biological characteristics of *Dendrobium* species will be put forward at the end of this chapter.

Vegetative Propagation

Characteristics of *Dendrobium* Species

Dendrobium species is either epiphytic or lithophytic plant without or with few lateral branches in cylindrical or flat-triangular prism stems (Ji et al. 1999). The base of the pseudobulb has ability for tillering, which is determined by environmental conditions, ages of tuft, plant nutritional

status and other factors. Generally, tiller growth increases with age of tuft when nutritional status and other conditions are not limited (He et al. 2009). In *Dendrobium* plants, pseudobulbs of different ages possess diverse functions varying among species (Table 1) (Tang et al. 2007; He et al. 2009). *Dendrobium officinale* has different characteristics of stems as shown in Table 2, compared with *D. nobile*. There are two reserve buds on the base of pseudobulb in *D. nobile*, from which the current buds will sprout. Usually, a pseudobulb and a bud together form tufted stems for growth and development. Buds sprout from the beginning of mid-February each year, and their detailed growth characteristics are shown in Table 3.

Table 1. The characteristics and functions of pseudobulbs of *D. nobile.*

Ages	Characteristics	Functions
2	green, withered and yellow leaves after summer, occasional falling leaves	sprouting buds and vegetative propagation
3	yellow-green, with or without damaged leaves	sexual reproduction (flowering and fruiting)
≥4	yellow-green, with abscission mark of peduncle and without leaves	supporting the whole plant and high bud breeding

Data from (Tang et al. 2007)

Table 2. The growth characteristics of stems of *D. officinale.*

Ages	Characteristics
1	producing fibrous root, Growth peak: spring, dormancy: autumn, without fallen leaves, evergreen life-form plant
2	accumulating nutrients and fertilizing flowers, Generally no longer elongation growth, falling leaves after the second growth period
3	flowing and fruiting, with or without leaves in blooming stem, without new leaves after falling leaves, naked stem
≥4	losing the tillering ability, becoming withered and then dying gradually

Data from (He et al. 2009)

Propagation Modes

After reaching the age limitation, high buds and aerial roots often grow in nodes of the *Dendrobium* species, which produce a scaly bud on the base of stems. Therefore, the vegetative propagation of *Dendrobium* occurs primarily through high bud breeding, cuttings, layering, division propagations and tissue culture, in which high bud breeding and division propagation are more commonly used (Wang et al. 2007; He et al. 2008; Li and Miao 2009).

Table 3. The growth characteristics of buds of *D. nobile.*

Time	Growth characteristics			
	Average ht (cm)	**No of leaves**	**New roots growth**	**New leaves growth**
mid-February	minority buds			
mid-March	majority buds			
late-March	1.5			
mid-April	3.5			
late-April	5.0	2	partial	
late-May	17	4–6	all	
late-June	21.8	5–7	all	
late-August	35.5	7–10	all	
late-September	39.5	10–13	all	Arc-shaped top, one acrophyll
late-October	40.8	10–13	all	Arc-shaped top, one acrophyll and height growth cessation

Data from (Tang et al. 2007)

Cutting Propagation

It has low cost and high yield, and can preserve the advantageous traits of the female parent. However, the buds have a different nutritional status, which easily lead to diverse seedling growth. Cuttings can be divided into different modes, such as one-year-old stem segment cuttings, rootless high bud cuttings and new bud cutting, according to the different shoot use for cuttings; Late mid-February to late March is the best time for cutting propagation (Wang et al. 2007).

Layering Propagation

Pseudobulbs are cut away from the female parent and laid onto the water grass in a 2–3 cm depth rectangular plastic box. The stems can be cut off after germination and rooting. Each stem can propagate 4–5 seedlings (Wang et al. 2007).

High Bud Breeding

It also can keep the advantageous traits of the female parent and its operation is very simple. However, very few numbers of high buds restrain propagation (Wang et al. 2007). Under field conditions, *D. loddigesii* produce new seedlings by means of high bud breeding (He et al. 2009). *D. officinale* propagate mainly by lateral buds, thus leading to a lower reproductive rate (Jin 2009).

Division Propagation

It also can keep the advantageous traits of the female parent and seedlings can be in blooming in current year. This method is very simple and survival rate is very high. But it is difficulty in large-scale cultivation due to the lower propagation and the easily damaged roots (Wang et al. 2007).

Tissue Culture

Tissue culture can also keep the advantageous traits of the female parent and grow regularly and fast. However, it needs higher technical skills, cost and longer time. The common culture mediums include MS, B5, N6, 1/2MS, KC (Knudsoc), W (White) and VW (Vacin and Went). NAA and BA are commonly used as the hormones of culture medium. Moreover, addendums such as potato, banana juice and casein are often used in culture medium (Wang et al. 2007). At present, explants of tissue culture contain seeds, roots, pseudobulb segments, leaves, protocorm and artificial seeds (Song et al. 2004; Wang et al. 2006; Li et al. 2010).

As mentioned before, seed germinations of *Dendrobium* species are difficult under natural conditions due to lack of mycosymbiosis, whereas in a synthetic medium, comprising of inorganic salts, sugars and agar without mycorrhizae it germinates successfully (Wang et al. 2007). Callus induced by seed and protocorm cultured by embryo have stronger differentiation potential and higher quality, respectively (Li et al. 2010). Seed culture is generally carried out before capsule dehiscing because of the easily separated powdered-seed, sowing and sterilization (only for capsule epidermis) at this period. The capsule after removal of persistent perianth and carpopodium is scrubbed with 70% alcohol for a minute or two, soaked in supernatant of 10% calcium hypochlorite or 2% chlorinated acetic acid for 20 min, and then washed 3 times with aquae sterilisata. Afterwards, we can use four methods for inoculation in a super clean bench: (1) cutting the peel and sowing it in culture medium surface; (2) sowing with inoculating needle after the capsule placed in 0.1% mercuric chloride for disinfection for 3 min; (3) seeds are transferred into 3% to 6% hydrogen peroxide, shaken for 20–30 min, sowed with a sterile pipette, and then evenly distributing them in the medium; (4) cutting at 1 cm of both the front and the back ends of the capsule, taking out the seed block, immersing in sterile water, shaking and then sowing seeds as droplets (Wang et al. 2007).

In order to improve the viability and production, stem sections with strong reproductive capacity should be selected for *Dendrobium* cultivation (Li and Xiao 1995). The stem sections as appropriate explants are washed with tap water and detergent on the super clean bench, scrubbed and sterilized for 15 s with 70% alcohol, put them into 0.1% mercuric chloride

(including 2 drops of Tween-60), and then sterilized for 20 min. Afterwards, they are rinsed with sterile water for 5 s, gently shaken and washed 6 times, then placed in the corresponding medium (Wang et al. 2007).

Cultivation conditions, such as hormones, pH value of cultivation medium and addendum, play different roles in *Dendrobium* cultivation. Coconut milk can promote the differentiation of buds as well as proliferation of shoots and protocorm. Moreover, banana juice can improve rooting (Jiang et al. 2005; Liao et al. 2006). Decreased basic element contents of cultivation medium are beneficial for the growth of protocorm, but do not benefit protocorm differentiation. In addition, NAA, IAA, IBA and GA3 can induce the growth and differentiation of protocorm, whereas acidic cultivation medium act against morphological variation of protocorm (Hou and Guo 2005). Cultivation mediums of protocorm, shoots and rooting added 1 mg/L $AgNO_3$ can improve the proliferation of protocorm, the differentiation of buds, and the growth of seedlings, and thus increase the survival rate of transplant (Li et al. 2007).

In the process of tissue culture, the selections of the types of medium, addendum, hormones and culture conditions mainly are based on *Dendrobium* species and the stage of culture.

Cultivation condition for spring *Dendrobium*: (1) seed culture: N6 culture medium (Wang et al. 2007; Zhou and Liu 2010); (2) stem induction: medium contains MS + 2.0 mg/L 6BA + 0.1 mg/L NAA, 3.0% sucrose and 0.21% crystal agar, temperature (24 ± 2°C), light 14 h/d, 500–2000 lux; (3) root induction: medium is the same as above except 0.1 mg/L NAA is substituted 0.05 mg/L for 0.1mg/L NAA (Zou and Liu 2010).

Cultivation condition for autumn *Dendrobium*: (1) shoot tip or vegetative buds culture: medium contains MS + 5 mg/L BA + 1 g/L activated carbon (pH 5.2–5.5), light 8–12 h/d, 1500–2000 lux, at 26°C; (2) vegetative buds or protocorm subculture: medium contains MS + 1 mg/L BA + 0.5 g/L activated carbon, light 8–12 h/d, 1500–2000 lux, at 26 ± 2°C; (3) monoclonal cultures: medium contains 1/2 MS + 0.05 mg/L BA + 0.5% activated carbon + 0.5 mg/L NAA + 5% banana juice (pH 5.2–5.5), light 12 h/d, 4000–5000 lux, at 26 ± 2°C (Zou and Liu 2010).

Cultivation condition for *D. Candidum*: (1) protocorm proliferation: medium contains 1/2 MS + 0.2 mg/L KT + 1.0 mg/L NAA + 0.5% activated carbon; (2) protocorm differentiation: medium contains 1/2 MS + 0.5 mg/L NAA + 0.5% activated carbon; (3) rooting: medium contains 1/2 MS+ 3.0 mg/L NAA + 0.5% activated carbon (Jiang et al. 2003); (4) test-tube seedling: medium contains B5 or 1/2 MS, 10% banana extract, 2 mg/L NAA and 2% sucrose (Liu and Zhang 1998).

Cultivation condition for *D. nobile*: the basic conditions of seed germination and seedling development is light intensity of 1000–1500 lux and photoperiod of 10–12 h at 18–27°C (Song et al. 2004).

Furthermore, the type and concentration of disinfectant and disinfection time also have effects on non-symbiotic germination of *Dendrobium* seed (Song et al. 2004).

Sexual Reproduction

Flowering Character and Phenology

Dendrobium species has one or more flowers with normal structure (3 sepals, 3 petals and 1 gynostemium) at the top of inflorescence axes. Compared to the sepal and petal, the labellum structure is more complex and obviously differs in different species. For example, *D. fimbriatum*: sunken labellum, edged with fringed beard, covering with pubescence; *D. loddigesii*: labellum with pubescence, outward circle is white, edge destined hairy red tassels; *D. hercoglossum*: labellum epidermis with tall and slender paper structure; *D. jiajiangense*: labellum with pseudopollen (He 2008; Wang et al. 2009; Pang et al. 2012). *Dendrobium* species have various colors including red, yellow, white, green, pink and purple. Thus, the flowers can be subdivided according to labellum colors (Wang et al. 2007; Li et al. 2009a). Many D*endrobium* species have fairly strong, pleasant scents. Of the 140 species evaluated, 40% produced scents ranging from floral to fruity to herbaceous (Kaiser 1993). However, most of them have no scents, such as *D. devonianum* (Lian and Li 2003). Furthermore, partial D*endrobium* species have a small amount of nectar, such as *D. fimbriatum* and *D. setifolium* (Inoue et al. 1995; Wang et al. 2009). Flowers of *Dendrobium* species have significant interspecific differences. Flowers characteristics of some D*endrobium* species are presented as Table 4.

Table 4. Flower characteristics of *Dendrobium.*

Species	Color (core of flower)	Flower diameter (cm)	No of raceme	Flowering order	Scents
D. hercoglossum	pink	2.5	2–3	scattering	
D. henryi	yellow	1.5–2.5	1–2	scattering	
D. hancockii	yellow	4.0–6.0	1–2	scattering	
D. primulinum	pink	4.0–6.0	1–2	scattering	
D. wardianum	white (Yellow)	7.0–8.5	1–3	assembling	Yes
D. chrysotoxum	yellow	3.0–4.5	12–20	assembling	Yes
D. crystallinum	pink (Yellow)	5.0–5.5	1–3	assembling	
D. crepidatum	rose (yellow)	3.0–4.0	1–4	assembling	
D. capiilipes	yellow	3.0	2–5	assembling	
D. gratiosissimum	pink (yellow)	2.0–3.0	1–2	assembling	
D. longicornu	white	5.0	1–3	relative assembling	
D. aphyllum	pink	4.0–5.0	1–3	relative assembling	
D. thyrsiflorum	white (yellow)	3.0–4.5	20–33	relative assembling	
D. devonianum	white (yellow)	4.0–5.0	1–2	relative assembling	

Data from (Li et al. 2009a)

During cultivation, *Dendrobium* can be divided into spring *Dendrobium* and autumn *Dendrobium*, according to the flowering period. Spring *Dendrobium*, mainly as a potted flower plant, generally is a deciduous species and flower from internodes in spring. Autumn *Dendrobium*, mainly as cut-flower, generally, is an evergreen species and about 20 flowers bloom from the stem tip (Zou and Liu 2010). The flowering period of *Dendrobium* is affected by environmental factors such as temperature and humidity (Table 5). Furthermore, pollination also impact flowering (Lian and Li 2003; Jin 2009; Wang et al. 2009). In some species, the flower begins to wilt once the pollinia is removed or pollinate to stigma. Under natural conditions, an unfertilized flower of *D. Jiajiangense* could generally open for about 10 d but fertilized flowers would wither in about 4 d (Pang et al. 2012).

Table 5. Comparison of flowering period of *Dendrobium in situ* and greenhouse.

Species	Flowering period (mon)		Ahead of time in greenhouse (mon)	Flowering of duration (mon)
	Situ	Greenhouse		
D. aphyllum	3–4	2–3	1	1–3
D. primulinum	3–4	2–3	1	1–3
D. wardianum	3–5	2–3	1	1–3
D. capiilipes	3–5	1–3	2	1–3
D. chrysotoxum	3–5	1–8	2	1–3
D.crepidatum	4–5	2–3	2	
D. gratiosissimum	4–5	2–3	2	
D. thyrsiflorum	4–5	2–4	2	
D. hercoglossum	5–6	2–3	3	
D. crystallinum	5–7	2–5	2	
D. hancockii	5–6	1–3	4	
D. devonianum	4–5	4–5	0	
D. henryi	6–9	7–9	–1	
D. longicornu	9–11	1–4	–5	
D. nobile	3–11			

Data from (Li et al. 2009a)

Pollen and Stigma Biology

Gynostemium of *Dendrobium* is formed by androecium and pistil. Androecium, on top of the gynostemium, is made up by anther cap and pollinia. Pistil locate at the lower part of the gynostemium. Waxy pollen, 4 pollinia (2:1) is formed by 4 single pollinia, the anther cell is covered by anther cap. The anther cap fallen off by external force (Lian and Li 2003; Pan et al. 2010).

Obvious differences in pollen vary within *Dendrobium* species. Previous studies have found that the order of pollen size is *D. officinale* >

D. huoshanense > *D. moniliforme*. Pollinia surface has less and more sculpture in *D. officinale* and *D. huoshanense* respectively, while the surface of *D. moniliforme* is smooth (Wang and Wang 1989).

There are differences in pollen vitality among species or during different flowering periods. Throughout the whole flowering period, pollen always has vitality in some species, such as *D. speciosum*, whose pollen vitality are greater than 95% (Slater and Calder 1988). The others have significant differences during different flowering periods. For example, pollen vitalities of *D. crepidatum*, *D. chrysotoxum*, *D. moniliforme* and *D. nobile* are as below: bud stage (11.5%–32.0%), flowering (55.5%–84.0%), full flowering period (31.0%–71.5%) and flower withering period (0–9.5%) (Pan et al. 2009). For *D. candidum*, the proportion of pollen with vitality in the bud stage is 29.4%, reaches the maximum 70.6% in the first day of flowers and then decreases. After the flowers have been open for a week, the proportion of pollen with vitality is 31.9% and decrease to 21.8% 12 d later (Zhu et al. 2011). In *D. hercoglossum*, pollen reaches the maturing stage within 4 d of flowering and drop rapidly on 12–14 d. Pollen within 2 d of flowering or 2 d impending withering of the flower is not suitable for pollination. The optimal period for pollen collection is 5–12 d after flowering (Li et al. 2009b). In *D. nobile*, pollen has the strongest germination and seed rate after the first day of flowering. Pollinia are still available after 9 d of flowering (Wang et al. 2006).

Stigma receptivity also differs among some species or flowering periods. The stigma of *D. speciosum* is receptive at anthesis and remains receptive until flower senescence (Slater and Calder 1988). Stigma of *D. secumdum* becomes receptive after the flowers have been open for 10–12 d (Kerr 1909). The best pollination periods of *D. hercoglossum* and *D. nobile* are 6–10 d and 1–6 d after flowering respectively (Wang et al. 2006; Li et al. 2009b).

The success of pollination is determined by stigma receptivity and pollen vitality, and pollen vitality is easily affected by environmental factors including temperature, humidity, and storage time. Pollen can be stored for a relatively longer period in optimum temperature condition, but its effective storage duration varies among *Dendrobium* species. Previous studies have indicated that pollen vitality can keep longer under dry conditions and decrease with storage duration. For instance, under dry conditions, the optimum effect for pollen storage of *D. candidum* is at 4°C. The proportion of pollen with vitality is 48.7% after 8 d and 21.2% after 20 d respectively. In addition, the rate of decrease of pollen vitality is larger in early storage than later, for example, *D. officinale* (Zhu et al. 2011).

To improve the rate of fruit set of *Dendrobium* by artificial pollination, pollen with higher vitality should be collected and stored in optimum conditions. Pollen of *Dendrobium*, as a two karyotype, theoretically is easy to save due to thick outer wall, desiccation tolerance and long life (Shi 1994).

Pollinia collected during disaccord flowering season of the parents, may be stored for artificial pollination (Wang et al. 2006; Zhu et al. 2011).

Visitors and their Behavior

Dendrobium species attract pollinators by a variety of ways, such as color, smell (*D. speciosum*), rest and mating place (*D. loddigesii*), providing reward: nectar (*D. setifolium*, *D. devonianum*, *D. finisterrae*), pseudopollen (*D. jiajiangense*, *D. unicum*), and shelter (*D. jiajiangense*) (Kjellsson and Rasmussen 1987; Inoue et al. 1995; Davies and Turner 2004; He 2008; Li et al. 2009a; Kamińska and Stpiczyńska 2011; Pang et al. 2012). However, some species such as *D. infundibulum*, have neither nectar nor any other reward for pollinators, and no odour could be perceived. They can simulate surrounding plants at the same flowing period in order to attract pollinators (Kjellsson et al. 1985). The flower of *D. sinense* mimics the alarm pheromone of honey bees in order to attract prey-hunting hornets for pollination (Brodmann et al. 2009). Other species such as *D. speciosum*, can take advantage of bonding factors: visual and olfactory, to attract pollinators. Potential pollinators of *D. speciosum* are attracted to the plant by large, bright, finely segmented, highly aromatic flowers, and the flowers on all plants in an area open almost synchronously (Slater and Calder 1988).

A larger number of studies in different research areas have shown that there may be a variety of visitors in some areas and also rare or no visitors in other areas. The types and characteristics of visitors and the position of pollen carried by pollinators vary among species (Table 6) (Kjellsson and Rasmussen 1987; Inoue et al. 1995; He 2008; Kamińska and Stpiczyńska 2011; Pang et al. 2012).

Visiting time of pollinators is affected by many factors. The specific climatic conditions during which pollinators visit the flowers are important for visitors, some visitors' visit activities become intensive in warm weather (Pang et al. 2012). For example, in *D. specious*, osmophores scattered over the perianth produce a strong, sweet scent in sunny weather, this has an indirect effect on the pollinators' behavior (Slater and Calder 1988). Some pollinators only visit flowers on sunny days, and the others have visiting action in rainy days until heavy rain (Slater and Calder 1988; Pang et al. 2012). There are differences in the peak period of visiting time of flowers among species, such as *D. jiajiangense*: 14:00–15:00, *D. loddigesii*: 11:00–14:00. The frequency of visitors is also affected by flowering phase, such as in *D. loddigesii*, there are more visitors at the full-blossom time than the end of the flowering period (He 2008; Pang et al. 2012).

The behavior of visitors is different. Some visitors coming from a distance fly into flowers directly and leave quickly. They have no observation and testing behavior and don't return to the same flower or circumjacent

Table 6. Visitors of *Dendrobium* species.

Species	**Visitors**	**Pollinator**	**Position of the pollinia**
D. loddigesii	ant, *Apis cerana*, Fabricius, Hymenoptera, Diptera, Coleoptera, Orthoptera, Araneae, Phasmida	*Ctenoplecta florisomnis*	on the pronotum of the insects observation
D. jiajiangense	*Pieris rapae* (Pieridae), *Delia platura* (Anthomyiidae), *Syrpyhus serarius* (Syrphidae), *Apis cerana* (Apidae), *Andrena parvula* (Andreniae)	*Andrena parvula*	
D. sinense	bees, wasps, butterfly	*Vespa bicolo*	on the pronotum of the insects observation
D. infundibulum	*Anthohora himalayensis*, *Apis dorsata* (bees), *Clinteria* sp. (a Cetoninae beetle) and two species of hawkmoth	*Bombus eximius* (main pollinator), *B. rotundiceps*	
D. setifolium	*Braunsapis* sp. (Anthophoridae)	*Trigona melanocephala* (Teragonula)	attached to the corbicula of the hind legs of the stingless bee
D. simth	hoverflies (Syrphidae)	*Trigona carbonaria*	

Data from (Kjellsson et al. 1985; Slater and Calder 1988; Inoue et al. 1995; He 2008; Brodmann et al. 2009; Pang et al. 2012)

flowers. The others have more testing behavior before visiting the flower and repeatedly visit the same flower (He 2008). Generally, visitors seldom fly into the flower passages and only pollinators will fly into the flower passages (Kjellsson et al. 1985; He 2008; Pang et al. 2012). The differences in visiting behavior will lead to variation of visiting time. For example, when *Andrena parvula* visit *D. jiajiangense,* visiting-time of a flower for seeking rewards is 37 ± 5 s, for rest or exercise on labellum is 33 ± 9 s, and for feeding the labellum fluff is 6 ± 1.5 s. Pang et al. (2012) found that *Andrena parvula* spend the night in the flower passage of *D. jiajiangense* to avoid rain or the heat from the sun, with its tails usually placed inwards and its head facing outwards. The flowers of *D. jiajiangense* provide shelter, and their male and female reproductive success rate is higher, 19% and 27% respectively, than those that don't provide shelter (Pang et al. 2012). Different visitors of *Dendrobium* species have different visiting frequencies and efficiency as well as flower numbers (Kjellsson et al. 1985; He 2008; Pang et al. 2012).

Of all the visitors, only those who removed pollinia and spread it to the stigma effectively are potential pollinators (Kjellsson et al. 1985; He 2008; Pang et al. 2012). It has been reported that the majority of *Dendrobium* species have specific pollinators (Richards 1997; Brodmann et al. 2009). The

body size and structure of the pollinator form an ideal mechanical fit with the height of flower passage and flower morphology (Table 7), and thus resulting in skillful removal of pollinia and accurate pollination (Figs. 1 and 2) (Slater and Calder 1988; He 2008; Pang et al. 2012).

Body structure of the pollinator can also adapt to pollination, except for the adaptation between body size and flower passage. For example, pollinator of *D. infundibulum*, the mesonotum is usually hairy in bumble bees, but in *B. eximius* the hairs are absent from a narrow median area. The pollinia exactly fit the bald patch on the back of the bee. The attachment of the pollinia to the bee appears to be very effective: bees were frequently observed flying around with *D. infundibulum* pollinia (Kjellsson et al. 1985).

Table 7. Flower and pollinator structure.

Species	Wh of flower passage (mm)	Ht of flower passage (mm)	Wh of body (mm)	Ht of thorax (mm)
D. jiajiangense	4.01 ± 0.47	1.73 ± 0.26	3.41 ± 0.09	3.11 ± 0.09
D. loddigesii	3.01±0.24	3.89±0.22	2.73±0.02	2.57±0.01
D. sinense	5.44 ± 0.10	5.67 ± 0.06	5.38 ± 0.03	5.54 ± 0.02

Data from (He 2008; Brodmann et al. 2009; Pang et al. 2012)

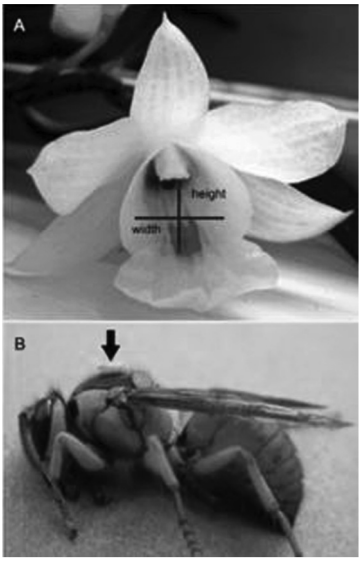

Figure 1. *D. sinense* flower and *Vespa bicolor* forager. *D. sinense* flower (A) and *V. bicolor* forager with pollinia stuck onto the thorax (B) (Brodmann et al. 2009).

Color image of this figure appears in the color plate section at the end of the book.

Theoretically, pollination can be completed effectively via ingenious cooperation between flowers and the behavior of pollinator (Figs. 3 and 4). However, few researches have focused on the whole process of pollination, which is only reported in *D. infundibulum* by Kjellsson (1985). The gynostemium of *D. infundibulum* is short and erect. The functional stigma is a cavity formed mainly by the median stigma lobe, referred to as the rostellum. The dorsal side of the apex of the rostellum, the rostellum projection, carries a vesicle containing a sticky substance, which is released when the pollinator pushes the apex of the anther upwards. When a visiting bee with pollinia on its mesonotum starts backing out of the flower, the pollinia are caught by the slightly emarginated apex of the rostellar projection and are scraped off into the stigmatic cavity (Kjellsson et al. 1985). Flowers vary in size within the six recognized varieties of

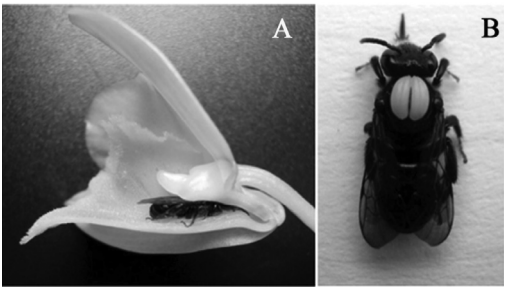

Figure 2. *D. jiajiangense* and its pollinator. (A). *Andrena parvula* pollination method, the black arrow expresses the direction of the anther cap pushed by *A. parvula.* (B). *A. parvula* carrying pollinia (Pang et al. 2012).

Color image of this figure appears in the color plate section at the end of the book.

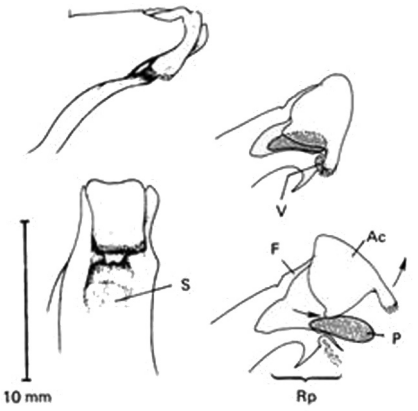

Figure 3. Gymostemium of *D. infundibulum*. ac = anther cap, f = filament, p = pollinia, rp = rostellar projection, s = stigma, v = viscidium (Kjellsson et al. 1985).

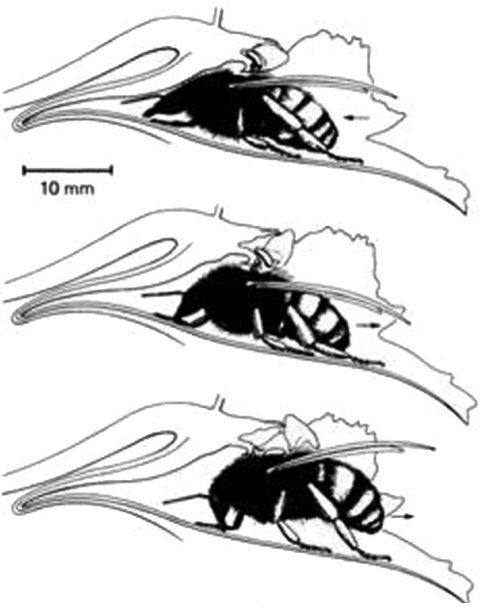

Figure 4. Pollination of *C. insigne* (Kjellsson et al. 1985).

D. speciosum (vars *speciosum*, *curvicaule*, *grandiflorum*, *hillii*, *capricornicum* and *pendunculatum*) and are pollinated when visited by bees of appropriate size (Slater and Calder 1988).

Although obligate pollination can improve the pollination efficiency in plants, the natural fruit set of *Dendrobium* is lower on account of scarce pollinators in the field conditions. The reproductive fitness of *Dendrobium* species, such as *D. infundibulum* will be increased through extended flowering season to make up lacking pollinators (Kjellsson et al. 1985).

Post-pollination Response

Generally, when pollinia is removed or spread into stigma, the perianth closes around the column and the flower color may change. This response, by hiding the central target area, prevents prospective visitors being specifically attracted to previously pollinated flowers. The senescent perianth intensifies or dulles in color, increasing the visual contrast between the inflorescence and the surrounding vegetation (Kjellsson et al. 1985; Slater and Calder 1988; Slater 1991; Jin 2009).

Breeding System and Fruit Set

Previous studies have indicated that *Dendrobium* showed high incompatibility in interspecific pollinations (Table 8). The majority (72%) of the 61 species that were self-pollinated showed self-sterility. Self- and interspecific incompatibility is expressed by flower abscission and not by inhibition of pollen germination or pollen tube growth (Johansen 1990). Till now, self-compatible and partly self-compatible *Dendrobium* species include 19 species, i.e., *D. albosangnineum, D. bilobuatum, D. brymerianum, D. crystallinum, D. erostelle, D. exile, D. formosum* v. *giganteum, D. gibsonii, D. heterocarpum, D. infundubulum, D. nathanielis, D. pendulum, D. phalaenopsis, D. salaccense, D. senile, D. tetrondon, D. tortile, D. fimbriatum,* and *D. jiajiangense* (Johansen 1990; Pang et al. 2012).

Spontaneous self-pollinatione exists in *D. brymerianum, D. erostelle* and *D. telrdon* (Johansen 1990), but most *Dendrobium* species have no self-pollination and apomixes. Therefore, these species are pollinator-dependent for fruit set in wild state. *Dendrobium* species have more flowers and less fruit. Except higher fruiting rate in *D. jiajiangense* (46.1%), the others are

Table 8. Self-incompatible species of *Dendrobium.*

Species	Abscission time in days	Species	Abscission time in days	Species	Abscission time in days
D. aciculare	3–4	*D. keithii*	4–5	*D. ellipsophyllum*	14
D. acinaciforme	5–7	*D. lamellatum*	7–15	*D. falconeri*	14
D. aloefolium	6	*D. levnis*	5–8	*D. farmeri*	4–7
D. alterum	5–11	*D. lindeyi*	7–8	*D. gratiolissimum*	9
D. aphyllum	5–8	*D. linguella*	5–13	*D. griffithianum*	10–12
D. bicameralum	7–9	*D. mannii*	6–7	*D. hendersonii*	5
D. blumei	6–7	*D. moschatum*	7–9	*D. primulinum*	9–10
D. brevimentum	5–8	*D. mucronatum*	10–15	*D. pulchellum*	7–8
D. cariniferum	11	*D. pachyglossum*	8–10	*D. secundum*	5–12
D. chrysotoxum	10–11	*D. pachyphyllum*	3–6	*D. setifolium*	3–7
D. crumenatum	4–7	*D. panduriferum*	6–7	*D. subulatum*	4–6
D. denudans	12–14	*D. parcum*	8–10	*D. thyrsiflorum*	6–10
D. devoniannum	5–18	*D. parishii*	7	*D. virgineum*	21–28
D. disticum	5–6	*D. planibulbe*	4–7	*D. indivisum* v. *pallidum*	4–10
D. draconis	18–21	*D. podagraria*	4–5		
There are many species exist self-incompatible but their abscission time is unclear, including *D. infundibulum, D. speciosum* var. *speciosum, D. speciosum* var. *curvicaule, D. speciosum* var. *grandiflorum, D. speciosum* var. *hillii*					

Data from (Kjellsson et al. 1985; Slater and Calder 1988; Johansen 1990)

relatively lower, such as *D. sinense* (13%), *D. fimbriatum* (11.11%), *D. candidum* (0.31% and 0.86%) and *D. loddigesii* (0.48%) (Lian and Li 2003; He 2008; Zhu et al. 2011; Pang et al. 2012). The main reasons of lower fruiting rate of *Dendrobium* species are as follows: (1) special flower structure: four pollinia are covered by two anther cap that drop through external forces; (2) lacking or even no pollinators in the field conditions; (3) there are spatial segregation between stigma and androecium and some species have well developed rostellar projections that autogamy seems mechanically impossible; (4) under natural conditions, few *Dendrobium* species have self-pollination and apomixes; (5) It is also considered to be a combined result of self-infertility and the absence of rewards offered by the flower (Kjellsson and Rasmussen 1987; Johansen 1990; Liang and Li 2009; Pang et al. 2012).

Many factors impact the fruit set of *Dendrobium* species, such as nutritional status of the female parent, parental attributes and pollination time (Lian and Li 2003; Pan et al. 2010; Zhu et al. 2011). *Dendrobium* species is a package plant. It can grow vigorously and accumulate more nutrients than individuals (Lian and Li 2003). The nutritional status of *Dendrobium* may affect fruiting rate. In the study of artificial pollination on *D. devonianum* and 4 medicinal *Dendrobium* (*D. crepidatum*, *D. chrysotoxum*, *D. monilifome* and *D. nobile*), fruiting rate was significantly associated with the number of tree/individual and the number of inflorescences and flowers. Within a certain range, more plants and less inflorescences and flowers result in higher fruiting rate and larger pods (Liang and Li 2009; Pan et al. 2010). Zhu et al. (2011) observed that fruiting rate of direct crossing and reciprocal crossing (I, mother × G, father) were 85.0% and 89.5%, on the contrary, were 88.5% and 92.0%. The fruiting rate of *D. candidum* is 100% when pollination is timely carried out (Zhu et al. 2011). In four medicinal *Dendrobiums* (*D. crepidatum, D. chrysotoxum, D. monilifome, D. nobile*), the fruiting rate of artificial selfing and hybridization in flowering period is higher than that in bud period, e.g., flowering period: selfing (25%–75%), hybridization (16.6%–50%); bud period: selfing (14.2%–50%), hybridization (16.6%–33.3%) (Pan et al. 2010).

Most of *Dendrobium* plants are cross–pollinated. In *D. chrysanthum*, if the pollinator fails in removing the pollen-masses when visiting the flower, the plant can skillfully finish fertilization through self-pollination (Darwin 1862). Artificial pollination experiments found that different pollination modes have different effects on fruit set and seed quality. Selfing can bring various degree of inbreeding depression (Wang et al. 2009).

The different pollination modes can lead to different fruiting rate. In general, there are significant differences between selfing and hybridization (Wang et al. 2009). In *D. devonianum*, fruiting rates of different plexus cross-pollination, the cross-pollination, inflorescence pollination, as well as selfing are 50%, 11.11%, 4.12% and 3.33% respectively (Lian and Li 2003).

In *D. fimbriatum*, fruiting rates of the cross-pollinations in interspecific populations, intraspecific populations, the same tree and selfing are 76.4%, 58.62%, 21.74% and 11.11% respectively (Wang et al. 2009). In *D. candidum*, fruiting rates of artificial hybridization and selfing are 82.6% and 7.3% respectively (Zhu et al. 2011). In *D. hercoglossum*, fruiting rate of selfing is only 4.3% (Li et al. 2009b). Fruit abortion will happen after pollination, whose differences depend on pollination modes. In *D. candidum*, the numbers of fruit abortion after selfing and hybridization are 153 and 37 respectively. The peaks of abortion after selfing and hybridization are within 5 d and 20 d. The numbers of fruit abortion after 5 d of selfing and hybridization account for 96.1% and 45.9% of the total numbers in *D. candidum*, respectively (Zhu et al. 2011). The self-pollinations are monitored and after 10 d four varieties of *D. speciosum* (*D. speciosum* var. *speciosum*, *D. speciosum* var. *curvicaule*, *D. speciosum* var. *grandiflorum* and *D. speciosum* var. *pendunculatum*) aborted all developing capsules (Slater and Calder 1988).

Seed quality is affected by the pollination modes. Normally, seed quality of hybridization is superior to that of selfing (Wang et al. 2009; Pang et al. 2012). In fruit obtained from selfing of *D. fimbriatum*, seeds with no or imperfect embryos account for about 2/3 of the total, only 1/3 of them have vitality. In fruit obtained from hybridization, the percentage of seeds with activated embryos is significantly higher than those obtained from selfing. Seeds with activated embryos obtained from interspecific populations are significantly higher than intraspecific populations; their percentages are 96.70% and 70.05% respectively (Wang et al. 2009). Seed viability of *D. jiajiangense* under the treatment of hand-cross-pollination was the highest, and that of seeds resulting from hand-self-pollination was lowest (Pang et al. 2012). In *D. fimbriatum*, seed germination rates of self-pollinations, cross-pollinations, interspecific populations and intraspecific populations cross-pollinations are respectively 77.42%, 72.73%, 98.50% and 92.17% (Wang et al. 2009).

Artifical-assited-pollnation strongly improve fruiting rate of *Dendrobium*, and artificial selective pollination also enhance qualities of fruit and seed (Pan et al. 2010; Zhu et al. 2011).

Fruit and Seed

After successful pollination of *Dendrobium*, the sepal usually closes within 2 d; the ovary turns green and expands after a certain period, e.g., *D. devonianum* 7 d, *D. officinale* 4–5 d and *D. loddigesii* 4–5 d. However, flowers become withered, ovary turns yellow and fall off after unsuccessful pollination (He 2008; Liang and Li 2009; Wang et al. 2009). The growth and development of ovary change with time (Table 9). For example, after pollination of *D. officinale*, the prototype of fruit will be observed after 6

Table 9. Maturity time of capsule in different species.

Species	Maturity time (mon)	Time range (mon)	Fruit set (mon)	Seed collecting time (mon)
D. candidum	4–6	7–11		
D. chrysotoxum	12		5–11	11–12
D. aphyllum	12			
D. linguella	20			
D. devonianum	3	12–2		
D. crepidatum			4–10	10–11
D. moniliforme			4–10	10–11
D. nobile	4	11–2	4–10	10–11

Data from (Lian and Li 2003; Pan et al. 2010; Zhu et al. 2011)

d, grow rapidly within 20 d, keep stable after 60 d and the fruit mature after 70 d (Zhu et al. 2011). After pollination of *D. devonianum*, fruit grow rapidly within 7–15 d (width increase faster than length), and then almost stop growing and enter maturity period (Liang and Li 2009). The maturity time of the capsule of the same *Dendrobium* species is different at different years, e.g., the average time of *D. secundum* was 67 d in 1986 and 87 d in 1987 (Johansen 1990).

Fruit of Dendrobium: Capsule with edges, oval or strip shape, gynostemium-tipped fruits. Containing 0.1–1 millions seeds, yellow, fine as dust, 0.3–0.4 μg each seed (Wang et al. 2007). The capsule automatically cracks after maturity and spreads the seeds out. The seeds, no endosperm, having after-ripening phenomena, germinate with difficulty under natural conditions, and their germination rates are usually less than 5% (Zhang et al. 2000; Wang et al. 2006). Seed germination of *Dendrobium* need mycorrhizal symbiosis (Guo et al. 2000; Song et al. 2004; Swamy et al. 2007; Li et al. 2010), which vary at different growth stages in natural state. *Epulorhiza* sp., *Mycena dendrobii, Rhizoctonia* sp., *Mycena orchidicola,* and *Gliocladium* sp. can improve seed germination of *D. candidum*. In addition, its seedlings are enhanced by *Cephalosporium* sp., *Epulorhiza* sp. and *Gliocladium* sp. (Guo et al. 2000). Guo et al. (2000) also found that *Gliocladium* sp., *Epulorhiza* sp. and *Mycena dendrobii* can form mycorrhiza structure with roots of *D. nobile*, thus improving the growth of the seedlings.

Seed of Dendrobium: Under light microscope they appear as irregular spindle, mid-intamescentia, both ends attenuate: one end is broad obtuse and the other is apiculate. There are also differences in the length of both ends among species. Seed structure is formed by testa and embryo. Yellow embryo is ellipsoidal and occupies the middle part of seed. When the fruit is ripe, there are no differentiations of radical, hypocotyl, germ and cotyledon in the original embryo period. Testa is paper like and transparent, located in

the middle of seed. It wraps up the embryo which stores gas at both ends (Wang and Xiao 2010). There are great differences in characteristics of seeds among different species, such as the size of seed and embryo as well as the proportion of the gas chamber (Table 10) (Swamy et al. 2007).

The qualities of fruit and seed of *Dendrobium* are affected by pollination modes, plant hormones and nutritional status (Johansen 1990; Zhang et al. 2008; Wang et al. 2009). Generally, fruit and seed from hybridization are superior to those from selfing (Wang et al. 2009). Moreover, plant hormones have effects on fruits and seeds. Earlier studies found that, fruit treated by 2,4—D after one month of artificial pollination, needed 6 month until maturity, significantly enlarged, e.g., length and width of fruit treated were 1.5–3 cm and 0.5–1 cm larger than those that are untreated. Meanwhile, fruit treated by 2,4—D can produce more seeds than those that are untreated (Fig. 5) (Zhang et al. 2008). After application of NAA or IAA on *D. phalaenopsis*, parthenocarpic fruit formation occurred. No seeds were formed in these parthenocarpic developed capsules (Johansen 1990).

The seed longevity will be shortened by high humidity and temperature conditions. In order to gain a longer store life, the specific procedures are as follows: (1) the capsule is put into sterile tubes with 0.1% mercuric chloride for 10 min and disinfected; (2) the capsule is carefully taken out using sterile

Table 10. Characteristics of seed.

Species	Length (mm)	Width (mm)	Diameter (mm)	Time of seed maturity	Color
D. nobile	0.75–1.10	0.09–0.20		6–8	White-faint yellow
D. candidum	0.2		0.04		
D. candidum	0.4	0.09			
D. chrysanthum				8	golden yellow

Data from (Song et al. 2004; Luo et al. 2006; Tang et al. 2007; Jin 2009; Wang et al. 2009)

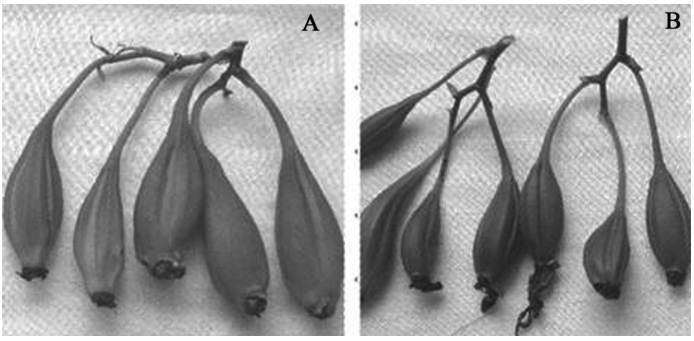

Figure 5. Fruits treated (A) and untreated (B) by 2, 4—D (Zhang et al. 2008).

tweezers and washed 3 times with sterile water; (3) immediately, it is dried for 1–3 d in a sterile petri dish, transferred into sterile tubes and tamponed; (4) the tubes are placed into desiccators and stored in the refrigerator at 10°C or below 0°C (Wang et al. 2006).

Perspective

Dendrobium, as the second largest genera of Orchidaceae, consists of 1000–1400 original species with higher ornamental and medicinal values (Kuehnle 2006; Wang et al. 2007; He et al. 2008). However, only about 70 species reproductive biological characteristics have been reported to date. Therefore, there are lots of species whose reproductive biological characteristics are unknown and worthy of researching in the future. It is most important to probe the following interesting questions.

Flower morphology: diversity and function. Flower morphology of *Dendrobium* varies remarkably among species. For example, there exist great differences in the flower morphology between *D. aphyllum* and *D. brymerianum*. It is unclear why they have various flower morphologies and what is the specific value in propagation adaptation?

Pseudopollen: reward or deception. Many studies have paid attention to the morphological characteristics and chemical composition of pseudopollen covering in labellum. It is speculated that pseudopollen may be a reward for pollinators because it contains starch and protein. However, till date there has been no direct experimental evidence. Therefore, further studies are also needed in order to fully understand how the pseudopollen plays a role in pollination.

Anther Cap: Structure and Function

The structure of anther cap obviously varies among the *Dendrobium* species though why this is so, is still unknown. Darwin reported that pollinators can pollinate the flowers of *D. dixanthum*, but *D. chrysanthum* will complete self-pollination skillfully, when pollinators do not take away the pollinia. Moreover, Liang and Li (2009) also found that abscission of anther cap needed external force. However, the mechanism of anther cap-releasing-pollinia is rarely mentioned in the current study. Moreover, Pang et al. (2012) observed that flower passage would be blocked up by the fallen anther cap; whose adaptive significance in reproductive biology remains mysterious.

Lower Fruiting Rate: Causes and Countermeasures

Dendrobium species have very low fruiting rates, for example, *D. sinense* (13%), *D. fimbriatum* (11.11%), *D. candidum* (0.31% or 0.86%) and *D. loddigesii* (0.48%) (He 2008; Liang and Li 2009; Zhu et al. 2011; Pang et al. 2012). Although some scientists analyze the reasons resulting in lower fruit set, corresponding achievements are insufficient to some extent. Most of *Dendrobium* species are endangered plants in nature. Why are those rare and how will they be protected l are still worthy of investigation.

References

Brodmann, J., R. Twele, W. Francke, Y.B. Luo, X.Q. Song and M. Ayasse. 2009. Orchid mimics honey bee alarm pheromone in order to attract hornets for pollination. Curr. Biol. 19: 1368–1372.

Cheng, X.M. and S.X. Guo. 2001. Study progress of the dendrobium plants in chemical constituents and pharmaceutical activity. Nat. Prod. Res. Dev. 13: 70–74.

Darwin, C. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects: and on the good effects of intercrossing. 1st edn. London: John Murray.

Davies, K. and M. Turner. 2004. Pseudopollen in *Dendrobium unicum* Seidenf. (Orchidaceae): reward or deception? Annals of Botany 94: 129–132.

Guo, S.X., W.Q. Cao and W.W. Gao. 2000. Isolation and biological activity of mycorrhizal fungi from *Dendrobium candidum* and *D. nobile*. China J. Chin. Mater. Medica. 25: 333–341.

He, P. 2008. Study on orchids diversity in Northern Hainan island and pollination biology of *Dendrobium loddigesii*. M.S. Thesis, Guangxi Normal Univ, Guilin, Guangxi.

He, P., X.Q. Song, Y.B. Luo and M.G. He. 2009. Reproductive biology of *Dendrobium officinale* (Orchidaceae) in Danxia landform. China J. Chin. Mater. Medica. 34: 124–127.

He, T., Z. Chun, A.X. Luo, Y.J. Fan, J. Liu and M.Z. Hu. 2008. Wild resources and conservation of dendrobium in Sichuan, China. Chin. J. Appl. Envir. Biol. 14: 710–715.

Hou, P.Y. and S.X. Guo. 2005. Studies on transplanting suspension-cultured protocorms of *Dendrobium candidum* onto solid culture medium. China J. Chin. Mater. Medica. 30: 729–732.

Inoue, K., M. Kato and T. Inoue. 1995. Pollination ecology of *Dendrobium setifolium*, *Neuwiedia borneensis*, and *Lecanorchis multiflora* (Orchidaceae) in Sarawak. Tropics 5: 95–100.

Ji, Z.H., X.Q. Chen and Y.B. Luo. 1999. The flora of China. Science Press 19.

Jiang, B., C.L. Yang, J. Huang, Y.Q. Zhan and X.B. Li. 2005. Study on protocorm differentiation and rhizogenesis plantlet strengthening of *Dendrobium candidum* Wall. ex Lind. J. Yulin Teach. Colg. (Narural Sci.) 26: 66–69.

Jiang, L., P. Ding and Y.D. Zheng. 2003. Effects of additives on tissue culture and rapid propagation of *Dendrobium canducum*. J. Chin. Medic. Mater. 26: 539–541.

Jin, Y.B. 2009. Study on biological characteristics and flowering pollination technology of *Dendrobium candidum*. J. Anhui. Agri. Sci. 37: 5280–5282.

Johansen, B. 1990. Incompatibility in dendrobium (Orchidaceae). Bot. J. Linnean Soc. 103: 165–196.

Kaiser, R. 1993. The scent of orchids: olfactory and chemical investigations, Elsevier Science Publishers BV.

Kamińska, M. and M. Stpiczyńska. 2011. The structure of the spur nectary in *Dendrobium finisterrae* schltr. (Dendrobiinae, Orchidaceae). Acta Agrobotanica. 64: 19–26.

Kerr, A.F.G. 1909. Notes on the pollination of certain Dendrobium. Scientific Proceedings of the Royal Dublin Society 12: 47–53.

Kjellsson, G. and F.N. Rasmussen. 1987. Does the pollination of *Dendrobium unicum* Seidenf. involve pseudopollen" ? Orchidee. 34: 183–187.
Kjellsson, G., F.N. Rasmussen and D. Dupuy. 1985. Pollination of *Dendrobium infundibulum*, *Cymbidium insigne* (Orchidaceae) and *Rhododendron lyi* (Ericaceae) by *Bombus eximius* (Apidae) in Thailand: a possible case of floral mimicry. J. Trop. Ecol. 1: 289–302.
Kuehnle, A. 2006. Orchids. Flower Breeding and Genetics. 539–560.
Li, G.F., J.J. Li, J.Y. Xu and J.J. Liao. 2010. Review of *Dendrobium candidum*. J. Chin. Medi. Mater. 33: 150–153.
Li, J.J., J.J. Liao, J.Y. Xu and Y.L. Mai. 2007. Effects of $AgNO_3$ on caducity-resistant from tissue culture of *Dedrobium officinale*. Chin. Tradi. Herb. Drugs 38: 914–917.
Li, J.L. and X.H. Xiao. 1995. An invetigation on medicinal plant resources of dendrobium in Sichuan province. Chin. Tradi. Herb. Drugs 20: 7–9.
Li, Z.J., X.P. Kang, Y. Wang, Y. Yu and K. Miao. 2009b. Studies on the pollination biology and microscopic structure of *Dendrobium hercoglossum* Rchb.f. (Orchidaceae). Acta Botanica-Borea1i-Occidentalia Sinica 29: 1804–1810.
Li, Z.J. and K. Miao. 2009. Introduction and *ex situ* conservation of endangered native dendrobium orchid. Chin. Wild Plant Res. 28: 67–69.
Li, Z.J., Y. Wang, Y. Yu, Y. Zhang and K. Miao. 2009a. Studies on floral and pollination biology in endangered dendrobium orchid. Guangdong Agri. Sci. 43–45.
Liang, Q. and J. Li. 2003. Artificial pollination of *Dendrobium devonianum*. Trop. Agri. Sci. Tech. 32: 35–36.
Liao, J.J., J.Y. Xu, J.J. Li, Y.L. Mai, Q.H. Xu and A.F. Zhou. 2006. Analysis of technical factors of test-tube seedlings industrialization production of *Dendrobium candidum*. J. Chin. Med. Mater. 29: 533–535.
Liu, H. and Z.G. Zhang. 1998. Study on culture medium of test-tube seedling of *Dendrobium candidum*. Chin. Tradit. Herb. Drugs 23: 654–656.
Luo, J.F., Z.Y. Cheng and C.L. Long. 2006. Studies on the rapid propagation and *in vitro* storage of *Dendrobium candidum*. Guihaia 26: 69–73.
Ma, Z.X. and G. Wang. 2008. Research on *Dendrobium nobile*. J. Zunyi Med. Colg. 31: 536–538.
Ng, T.B., J. Liu, J.H. Wong, X. Ye, S.C. Wing Sze, Y. Tong and K.Y. Zhang. 2012. Review of research on dendrobium, a prized folk medicine. Appl. Microb. Biotechnol. 93: 1795–1803.
Pan, C.X., Y. Bai, Y.H. Bao and M.L. Fang. 2009. Study of pollen viability of medicinal dendrobium. J. Anhui. Agri. Sci. 37: 16841–16842.
Pan, C.X., Y. Bai, Y.H. Bao and M.L. Fang. 2010. Preliminary report on interspecific Sexual hybridization breeding of medicinal dendrobium. Li Shi Zhen Med. Materia Medica Res. 21: 1214–1215.
Pang, S., K. Pan, Y.J. Wang, W. Li, L. Zhang and Q.B. Chen. 2012. Floral morphology and reproductive biology of *Dendrobium jiajiangense* (Orchidaceae) in Mt. Fotang, southwestern China. Flora-Morphology, Distribution, Funct. Ecol. Plants 207: 469–474.
Richards, A.J. 1997. Plant breeding systems. Garland Science.
Shi, L. 1994. Breeding method of flower. Chinese Flowers Miniascape. 19.
Slater, A. 1991. Interaction of the stigma with the pollinia in *Dendrobium speciosum*. Austral. J. Bot. 39: 273–282.
Slater, A. and D. Calder. 1988. The pollination biology of *Dendrobium speciosum* Smith: a case of false advertising? Austral. J. Bot. 36: 145–158.
Song, X.Q., N. Gong, X.C. Zhan, L. Nie and Q.Q. Song. 2004. Research of non-symbiotic sproution of *Dendrobium nobile* Lindl and germplasm storage. J. Guizhou Normal Univ. (Natural Sci.) 22: 13–16.
Swamy, K., H. Kumar and S. Ramaswamy. 2007. Studies on seed morphometry of dendrobium species. Phytomorphology 57: 33–43.
Tang, D.Y., C.Y. Yang, L.S. Duan and X.L. Li. 2007. Study on the biological characteristics of *Dendrobium nobile.* Li Shi Zhen Med. Mater. Medica Res. 18: 2586–2587.

Wang, L.Z. and D.Q. Wang. 1989. Observation of pollen of three species of dendrobium using the scanning electron microscope in Anhui Mount Holyoke. J. Anhui. Tcm. Colg. 8: 53.
Wang, S.Y. and F.H. Xiao. 2010. Seed morphology of 17 dendrobium species from Yunnan. China J. Chin. Mater. Medica. 35: 423–426.
Wang, S., X. Song, R. Cai, L. Nie, H. Wu and J. Zhao. 2006. The study of *Dendrobium nobile* Lindl pollination biology and the property of seed sprouting. Seed. 25: 23–26.
Wang, X.J., L. Yu and S.H. Yin. 2009. Pollintion modes effects on fruit set and seed viability of *Dendrobium fimbriatum* (Orchidaceae). J. Yunnan Univ. 31: 374–377.
Wang, Y., Z.J. Li and H.M. Peng. 2007. Dendrobium: resources, production, application. China Forestry Publishing House.
Wei, X.Y. 2005. Research progress of alkaloids in dendrobium Plants Chinese Pharmaceutical Affairs. 19: 445–447.
Zhang, G.N., Z.M. Bi, Z.T. Wang, L.S. Xu and G.J. Xu. 2003. Advances in studies on chemical constitutents from plants of dendrobium. Sw. Chin. Tradit. Herb. Drugs 34: 5–8.
Zhang, M., H.X. Xia, L.Q. Zhu and Y.J. Zhang. 2000. Advances in tissue culture of dendrobium. China J. Chin. Mater. Medica. 25: 323–326.
Zhang, Y.L., Y.Q. Rao and G.L. Rong. 2008. Preliminary study on effects of wild dendrobium fruit setting rate of artificial pollination. Biol. Teach. 33: 39–40.
Zhu, B., H. Yuan, Q. Yu and J. Si. 2011. Pollen vigor and development of germplasm of *Dendrobium officinale*. China J. Chin. Mater. Medica. 36: 755–757.
Zou, C.Y. and Y. Liu. 2010. Advances in dendrobium research in China. J. Anhui. Agri. Sci. 38: 6164–6166, 6223.

11

Biotic Pollination: How Plants Achieve Conflicting Demands of Attraction and Restriction of Potential Pollinators

K.R. Shivanna

ABSTRACT

In flowering plants, pollination, transfer of pollen grains from the anther to the stigma, is a pre-requisite for seed set and plays a critical role in their reproductive success. Over 85% of the flowering plants are pollinated by animals (biotic pollination) and the remaining are pollinated by wind or water. Amongst animals, insects are the major pollinators. Birds and bats are the other important pollinators. Plant-pollinator interactions are largely mutualistic, but there are a good number of species that achieve pollination by deceit.

Biotic pollination essentially involves a sedentary partner (plant) and a mobile partner (animal). This imposes two conflicting demands on plants. 1) They have to develop effective devices to attract animals to visit their flowers in a sustainable way and use them effectively for pollination services. 2) They have to apply some degree of

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discrimination to restrict the number of animal species visiting the flowers. In the absence of such discrimination, all animal species that reside in the habitat may visit all synchronously flowering plant species and bring about extensive hetero-specific pollination. As pollination success depends on the transfer of pollen to conspecific stigma, hetero-specific pollination compromises reproductive fitness of plant species. Most of the studies on pollination ecology have so far concentrated on floral attraction; only limited studies have been carried out on the details of restriction of floral visitors. This chapter gives a brief introduction on the origin of biotic pollination and its significance in diversification of flowering plants, and discusses our current understanding on how plants have been able to achieve conflicting demands of attraction and restriction of floral visitors for efficient pollination.

Keywords: Biotic pollination, deceptive pollination, diversification of angiosperms, floral attractants, floral rewards, fragrance cues, fragrance filters, morphological filters, nectar filters, nocturnal pollination, nursery pollination, pollen filters, pollination syndromes, restriction of pollinators, visual cues

Introduction

Flowers are the units of sexual reproduction that involves a series of sequential events—production of functional pollen grains (male partners) and ovules (female partners), transfer of pollen grains from the anther to the stigma (pollination), pollen-pistil interaction leading to the entry of pollen tubes into the ovules and delivery of male gametes near the female gamete, fertilization, development of fruits and seeds, dispersal of seeds, and growth of seedlings into adults. Pollination is one of the most critical events in sexual reproduction of seed plants and plays a crucial role in both fundamental and applied aspects of reproductive biology. Pollination is the only means of gene flow between conspecific plants and populations, and is thus the basis of recombination. Successful pollination is a prerequisite for fruit and seed development, and thus plays an important role in reproductive success of both cultivated and wild plant species. Pollination limitation reduces the yield of cultivated species (Roubik 1995; Knight et al. 2005; James and Pitts-Singer 2008) and often acts as a driving force for species vulnerability in natural habitats (Spira 2001; Wilcock and Neiland 2002; Biesmeijer et al. 2006; Potts et al. 2009; Shivanna 2012a; Burkle et al. 2013; Tylianakis 2013). Information on pollination ecology is essential for the release of genetically transformed plants (Armstrong et al. 2005). Pollination services are needed to sustain pollinators particularly those that depend exclusively on floral resources (nectar/pollen).

Pollination is simply the deposition of pollen grains from an anther to the stigma. Based on the source of pollen, pollination is of three types: i) autogamy: transfer of pollen grains from the anthers to the stigma of the *same* flower, ii) geitonogamy: transfer of pollen grains from the anthers to the stigma of *another* flower of the *same plant or another plant* of *the same clone* and iii) xenogamy: transfer of pollen grains from the anther to the stigma of *another* non-clonal plant.

Sexuality of the flower and of the plant affects pollen flow within and between flowers/plants. The flowers may be hermaphrodite (producing both functional pollen and ovules) or male (producing only functional pollen) or female (producing only functional ovules). The plant may be bisexual (producing only hermaphrodite flowers) or monoecious (each plant producing both male and female flowers) or dioecious (each plant producing only male or only female flowers). Thus, all three types of pollinations can occur in plants producing hermaphrodite flowers; only geitonogamy and xenogamy can occur in monoecious species while only xenogamy is possible in dioecious species. Expression of male and female phases in hermaphrodite flowers may be synchronous or show temporal (dichogamy) or spatial (herkogamy) separation. When the female phase in dichogamous flowers is expressed earlier than the male phase, the condition is called protogyny and when the male phase is expressed earlier than the female phase, it is called protandry. In herkogamous flowers, the anthers and the stigma are spatially separated and are located at different levels in the flower. Herkogamy and dichogamy prevent autogamy but are not effective in preventing geitonogamy. Many hermaphrodite species are self-incompatible that prevents both autogamy and geitonogamy completely and permits only xenogamy.

Over 85% of the flowering plants are pollinated by a variety of animals (biotic-pollination) and the remaining by wind or water (Ollerton et al. 2011). Thus, biotic pollination provides one of the crucial ecosystem services both in agricultural fields and natural habitats. These services are threatened by habitat destruction and global warming which lead to reduction of pollinator diversity and abundance. Several recent studies have highlighted decline of pollinators and also parallel decline of plant species (that depended obligately on biotic-pollination) with their pollinators (Biesmeijer et al. 2006; Aguilar et al. 2006; Memmott et al. 2007; Potts et al. 2009; Garibaldi et al. 2013; Burkle et al. 2013 see also Tylianakis 2013).

Plant-pollinator interactions are largely mutualistic and result in reciprocal benefits. It is a form of 'biological barter' and involves exchange of resources of the plant such as pollen and nectar with the services of the pollinator (Ollerton 2006). There are, however, a good number of species that achieve pollination through deceit without offering any rewards to the pollinators. The ultimate strategy of plants is to deliver and receive

conspecific pollen with minimum allocation of resources while that of the pollinator is to harvest maximum reward with minimum use of energy and time.

Evolution of biotic pollination provides several advantages to plants over wind and water pollination: i) biotic-pollination is more efficient as animals seek out conspecific flowers and often transport pollen for longer distances, ii) it can thrive even in habitats with minimum wind as in tropical forests with closed canopy and iii) plant species need to allocate lesser resources for pollen production than wind/water-pollinated species, as the latter involves extensive pollen wastage (see Pellmyr 2002).

Many evidences indicate that abiotic pollination by wind and water is a derived condition from biotic pollination: i) Anemophily is present in families such as Poaceae, Cyperaceae, Betulaceae and Juncaceae that are highly evolved and whose ancestry can be traced to animal-pollinated species (Faegri and van der Pijl 1971). ii) Anemophily is rare in tropical rain forests where pollinating animals are abundant. iii) Several wind-pollinated species show a combination of wind- and biotic-pollination. For example species of *Salix* produce nectar and their flowers are pollinated by insects; they also produce large amount of pollen that are carried by wind. iv) Insect pollination seems to be ancestral in basal angiosperms which represent the first flowering plants to diverge from the ancestral angiosperms (Hu et al. 2012). Anemophily seems to have evolved as an adaptation to overcome the scarcity of pollinators. Hydrophily seems to have evolved in response to aquatic habitat which is considered to be a derived one for higher plants (Faegri and van der Pijl 1971). Most of the aquatic plants in which the flowers emerge above the water level are pollinated by animals similar to their terrestrial ancestors. Only those aquatic species in which flowers remain inside the water have developed devices to use water for pollen transport.

Two Conflicting Demands of Biotic Pollination: Attraction and Restriction of Pollinators

Animal-mediated pollination essentially involves a sedentary partner (plant) and a mobile partner (animal). This imposes two conflicting demands on plants. 1) They have to develop effective devices to attract suitable animals to visit their flowers in a sustainable way and use them effectively for pollination services. This requires substantial investment from plants in the form of attractants and rewards. 2) Plants have to apply some degree of discrimination to restrict the number of animal species visiting the flowers of each plant species. In the absence of such discrimination, all animal species that are present in the habitat may visit all synchronously flowering plant species. This would result in (i) unhealthy competition

between plant species, (ii) enormous wastage of pollen and (iii) extensive heterospecific pollination (with pollen of other species). These features seriously compromise reproductive fitness of plant species (see Pellmyr 2002). Therefore, plants have to devise ingenious ways to achieve these conflicting demands of attraction and restriction of floral visitors for efficient pollination. Studies on pollination ecology so far have concentrated largely in understanding the details of attraction; only limited studies have been carried out to understand the details of restriction. Even the reviews and books on pollination ecology hardly discuss the restriction aspect of pollination, except some aspects of morphological filters (Pellmyr 2002; Roubik et al. 2005; Dafni et al. 2005; Bronstein et al. 2006; Waser and Ollerton 2006; James and Pitts-Singer 2008; Anonymous 2009; Willmer 2011; Patiny 2012). In this review an attempt is made to give a brief introduction on the origin of biotic pollination and its significance in diversification of flowering plants and pollinators, and bring together available information on how the plants have been able to achieve these conflicting demands of attraction and restriction of pollinators to flowers. As the literature in the area is extensive, the coverage tends to be subjective with an emphasis on recent studies.

Origin of Biotic-Pollination

Biotic pollination evolved in two groups of Gymnosperms (Cycadales and Gnetales) as early as the Permian period (about 250 million yr ago) and is present even in several extant species of these groups. Beetles, flies, thrips and wasps are the most common pollinators in Gymnosperms (Seymour et al. 2004; Terry et al. 2007; Marler 2010). Fragrance is the basis of attraction of pollinators. The chemical composition of their fragrance is similar to general herbivore deterrents (Pellmyr and Thien 1987). It is suggested that early attractants for pollinators evolved from herbivore deterrents (Pellmyr et al. 1991). The pollinators frequently utilize male cones for nursery purpose (for mating and obtaining food for larvae and adults) and female cones mimic olfactory cues of male cones to attract pollinators. In some species, the pollination drop present at the tip of the ovule may serve as a reward for pollinators. Some cycads show obligate mutualism with their specialist pollinators, and have evolved mechanisms to generate heat (4–12°C above ambient temperature) in the cones. The heat increases the emission of volatiles and improves pollinators' attraction (Terry et al. 2007; Marler 2010).

In Angiosperms, animal-mediated pollination was prevalent from the beginning of their evolution in the early Cretaceous period (over 100–120 million yr ago). The oldest fossil of the flower discovered in the early Cretacieous (about 120 million yr ago) is related to extant Nymphaeales.

Another fossil found in the upper Cretaceous (about 90 million yr ago) is similar to the extant *Victoria* species. Based on molecular phylogeny, earliest angiosperms, termed basal angiosperms, representing the first flowering plants to diverge from the ancestral angiosperms, have been recognized (Soltis et al. 2000). Pollination ecology of many species of basal angiosperms has been studied (Pellymr and Thien 1987; Endress 2010, 2011; Thien et al. 2009). The flowers of basal angiosperms are generally small, simple and radially symmetrical. Bisexual flowers are more common and all of them are protogynous. Most of the species show apocarpous (free carpels) condition and well-defined style is generally absent. They seem to attract generalized pollinators prevalent in animal-pollinated Gymnosperms and show many similarities to the pollination system in Gymnosperms (Pellymr and Thien 1987). Beetles and flies are the major pollinators and pollen is the major reward; many of them show floral thermogenesis (see p. 243). Bee pollination is found only in Nymphaeaceae. Abiotic pollination is rare in basal angiosperms.

Animal Partners

Amongst animals, insects are the principal pollinators. Hymenoptera (bees, wasps and ants), Lepidoptera (butterflies and moths), Coleoptera (beetles) and Diptera (flies) are the major orders of insects involved in pollination. In recent years, thrips (Thysanoptera) have been reported to bring about pollination in a number of species (Ananthakrishnan 1993; Williams et al. 2001; Garcia-Fayos and Goldarazena 2008). Thrips are very small (< 1 mm long) and are poor flyers; they can, however, be carried for long distances by the wind. Thrips largely bring about autogamy and geitonogamy. Birds and bats are the other important pollinators. They are generally confined to tropical and sub-tropical areas that provide floral resources throughout the year. Bird-pollinated (ornithophilous) species have been reported in over 60 families (see de Wall et al. 2012). In the New World, hummingbirds are the principal bird pollinators and in the Old World and Pacific regions, sunbirds and sugarbirds are the major bird pollinators. In Australia the honeyeaters and in Hawaii the honeycreepers are the main bird pollinators. Bird-pollinated species are more prevalent in Australia when compared to other continents. According to an old survey (Ford et al. 1979) 111 species of birds have been reported to visit flowers of about 250 species of plants.

Only two families of bats, Pteropodidae which occurs throughout tropical and subtropical regions of the Old World (including Australia and Pacific islands) and Phyllostomidae which inhabits tropical and subtropical regions of the New World contain species that are specialized in nectar feeding (see Fleming et al. 2009). Old World bats are larger with shorter tongues and do not hover whereas those of the New World are smaller

with longer tongues and hover. Bat-pollination (chiropterophily) has been recorded in over 500 species belonging to 67 families and 28 orders of tropical and semitropical plant species (Gibson 2001; Flaming et al. 2009). Bat and bird pollinations are considered to be derived conditions and have evolved independently in many advanced families of flowering plants. Some unusual pollinators have also been reported in some plant species: cockroaches (Nagamitsu and Inoue 1997—*Uveria*), mice (Wester et al. 2009 —Pagoda lily), squirrels (Tandon et al. 2003—*Butea*), snails (Sarma et al. 2007—*Volvulopsis*) and lizards (Olesen and Valido 2003; Ortega-Olivencia 2012—*Scrophularia;* Hansen et al. 2006—*Trochetia*).

Bumblebees and honeybees have trichromatic vision with UV, blue and yellow as primary colours and their spectral range extend from about 550 nm to 336 nm. Human vision is also trichromatic (blue, green and red as primary colours) and the spectral range is confined to visible wavelengths (from 400 nm to about 700 nm). All Lepidoptera and flies so far examined have the ability to see objects in UV range (see Kevan 1983; Kearns and Inouye 1993). Those insects which can distinguish UV from longer wavelengths have a separate UV absorbing pigment. Some butterflies can see red while others cannot. Honeybees are also red-blind. Hummingbirds and a few other birds also see in the near UV region of the spectrum (Chen et al. 1984). Bats are generally red-blind although they can distinguish white vs black even in extremely low light (Gibson 2001).

Pollinating insects may be social or solitary (Anonymous 2012). All species of ants and termites, and some species of bees (honeybees, bumblebees and stingless bees) and wasps are social insects. Truly social (eusocial) insects are characterized by: i) all individuals of a colony share a common nest site, ii) reproduction in the colony is restricted to only one or a few females, iii) the individuals of a colony are made up of overlapping generations and iv) brood care within the colony is a co-operative function. Most of the other bees involved in pollination (such as *Xylocopa, Megachile, Osmia* and *Nomia*) are solitary. Solitary bees do not form colonies. A solitary female constructs her own nest, stores food for her brood in the form of pollen or nectar and then dies or departs without further care to her offspring. The adult life of these bees is generally short, spanning for a few weeks. They nest alone or in aggregations.

Invertebrate pollinators have limited memory and their visits are largely confined to just one type of flower at a given time (Minckley and Roulston 2006). In a complex flower with hidden rewards, insects have to learn by trial and error the best approach to harvest the reward with minimum time. When they change host species, they have to learn again to handle the second flower (Lewis 1986; see also Raine and Chitka 2007). Pollen-collecting bees generally have a limited foraging range and frequently commute between the nest and the host plants to unload the pollen sample. Bats have

stronger memory than bees. Nectar-feeding bats can retain up to 40 food locations in their working memory, facilitating their foraging in tropical nocturnal environments (Winter and Stich 2005). Vertebrate pollinators are homoeothermic and need a continuous supply of food to maintain their high metabolic rate. They are able to visit flowers even during low temperature conditions under which insects are unable to fly. Insects, on the other hand, are able to survive long periods of unfavourable conditions in larval stages or as hibernating adults. Vertebrate pollinators and some butterfly species can cope with unfavourable conditions during some parts of the year through local and regional migrations (see Abrahamczyk et al. 2011).

Diversification of Flowering Plants and Pollinators

There was a dramatic increase in the number of angiosperm species soon after their origin in the Cretaceous period. Darwin recognized this increase and termed this diversification as an "abominable mystery" (see Friedman 2009). Paleontological evidences indicate: i) slow diversification of insect pollinator groups (although they arose in the early Mesozoic or even before) until they became associated with angiosperms and ii) rapid diversification of the angiosperms and pollinating insect groups simultaneously in the latter half of the Cretaceous and tertiary period. On the basis of these evidences evolutionary biologists suggested that biotic pollination acted as a catalyst for reciprocal diversification of pollinating insects and flowering plants in the latter half of the Cretaceous period (see Crane et al. 2000; Pellmyr 2002; Magallon and Castillo 2009; Smith et al 2011). A recent study suggests that the rise of bees coincided with the largest flowering plant clade, the eudicots which comprises 75% of the flowering plants (Cardinal and Danforth 2013; see also Cappellari et al. 2013).

Some evolutionary biologists, however, argue that biotic pollination acted not as an exclusive factor but as a co-factor in the diversification of angiosperms (Sanderson and Donoghue 1994; see Pellmyr 2002). This argument is based on: i) angiosperm diversification increased not at the beginning of their evolution when animal pollination arose but rather at a later stage and ii) animal-pollinated Gymnosperm groups (such as *Zamia, Welwitschia* and *Gnetum*) have not diversified more than their wind-pollinated sister groups.

Several evidences indicate that apart from biotic pollination, evolution of the flower and some of its innovations seem to be the contributing factors for the evolutionary diversification of flowering plants. The visual and fragrance components of the flower and the secretion of the nectar as a carbohydrate-rich reward increased dramatically the attraction of pollinators and their sustainability, thus increasing pollination efficiency of flowering plants when compared to the cones of Gymnosperms. The other

innovations of the flower that seem to have contributed to the diversification of angiosperms are the evolution of the carpel and floral zygomorphy. Evolution of the carpel, as the result of covering of the ovule(s) with a sporophytic tissue differentiated into the ovary (enclosing the ovules), style and stigma, had profound effects on the operation of sexual reproduction of angiosperms. Unlike in Gymnosperms, pollen grains in Angiosperms do not have direct access to the ovules in which female gametes are located; they land on the stigma placed far away from the ovules. Evolution of the pistil resulted in the addition of another step in pre-fertilization events—growth of pollen tubes through the tissues of the stigma and style—before pollen tubes enter the ovules and release the male gametes for fertilization. All the carpels of the flower together are referred to as the pistil. In most of the eudicots and monocots, all the carpels of the flower are united (syncarpous) with a well defined style. The transmitting tissue (through which pollen tubes grow) of the styles of individual carpels is also fused in syncarpous pistils enabling pollen tubes originating from one carpel to cross-over to the ovules of other carpels (see Armbruster et al. 2002; Endress 2010). Thus, pollen tubes originating from any stigma or a part of the stigma can enter the ovules of all the carpels in syncarpous pistils. This increases the efficiency of pollination and the extent of seed set even under pollination constraint.

More importantly, the pistil screens pollen grains for quality and compatibility before pollen tubes reach the ovules (see Shivanna 2003). As the number of pollen grains that land on the stigma is generally many times more than the number of ovules available for fertilization, pollen grains are subjected to intense competition during pollen germination and pollen tube growth in the pistil. Those pollen grains that germinate early and pollen tubes that grow faster in the style, enter the ovules earlier than the slow growing pollen tubes and effect fertilization. Pollen grains which germinate later and pollen tubes with slower growth rate are eliminated in this competition. Since cross-pollination takes place to a limited or greater extent in most of the species, there is considerable genetic variability in the pollen population on which pollen competition can operate. There is no scope for such a competition among male gametes in lower groups of plants including gymnosperms. The adaptive significance of pollen competition in increasing the fitness of the progeny is documented in several species through experimental manipulation of pollen competition (Mulcahy 1979; Mulcahy and Mulcahy 1987; Davis et al. 1987; Schlichting et al. 1987; Tejaswini et al. 2001; Lankinen and Madjidian 2011). Seedlings resulting from intense pollen competition show more vigorous and uniform growth when compared to those resulting from no competition or limited competition.

The pistil also plays a critical role in the breeding system of angiosperms. Self-incompatibility (SI) controlled by one or a few genes, each with two or multiple alleles, is one of the common outbreeding devices in plants (de Nettencourt 2001). When the SI allele present in the male gamete matches that of the female gamete, fertilization is prevented. In lower groups of plants where male and female gametes come in direct contact with each other, SI cannot prevent completely the fusion of gametes produced by the same individual. For example S_1 and S_2 gametes produced by the S_1 S_2 self-incompatible individual can fuse although fusion of $S_1$with S_1 and S_2 with S_2 gametes is prevented (see Shivanna 2003). In flowering plants SI recognition is established between the gametophytic pollen (each carrying one of the S alleles) and the sporophytic tissues of the pistil (carrying both the S alleles of the parent). In S_1S_2 plants, for example, pollen grains carrying both S_1 and S_2 alleles are recognized and inhibited in the pistil. Evolution of the pistil has made self-incompatible flowering plants the most efficient outbreeders since all male gametes that originate from the same plant or any other plant with the same genotype are effectively prevented from fertilization.

Yet another floral innovation that appeared in some major lineages in Cretaceous period was the evolution of bilateral zygomorphic flowers from radial actinomorphic flowers (Friedman 2009; Mach 2012; Vamosi and Vamosi 2012). As zygomorphic flowers have one plane of symmetry, effective pollen transfer depends on the accuracy in functional fitness between the flower and the pollinator leading to specialized pollination systems (Sargent 2004). Such specialized interactions facilitate reproductive isolation and speciation. Phylogenetic analyses have shown that lineages with zygomorphic flowers tend to contain more number of species than their actinomorphic sister groups (Sargent 2004; see Vamosi and Vamosi 2012). Thus, biotic pollination together with the evolution of the flower with the pistil and further floral elaboration in the form of zygomorphy would have acted as catalysts for the reciprocal diversification of flowering plants and pollinating insects.

The details of floral morphology in the basal angiosperms are in agreement with the above concept (Thien et al. 2009). As pointed out earlier, flowers in basal angiosperms are radially symmetrical and the carpels are largely free (apocarpous) with ill-defined style. In apocarpous species pollen grains that land on the stigma of a carpel cannot cross-over to the ovules located in other carpels of the flower. This reduces pollination efficiency and the extent of seed set under deficient pollination environment as the seed set is confined only to pollinated carpel. Also, short style prevalent in basal angiosperms is likely to be less effective in screening pollen quality when compared to eudicots and monocots with well-developed styles (see Mulcahy 1979; Armbruster et al. 2002). These floral features explain limited diversification of basal angiosperms.

Floral Attractants

Plants have to invest considerable resources to attract pollinators and sustain their visits by providing rewards to the visitors. Floral attractants advertise the presence of rewards that provide motivation for animals to visit flowers in a sustainable way. Flowers and inflorescences and their fragrance act as advertisers through visual and olfactory cues (Dafni et al. 2005). Flowers exhibit a remarkable diversity in colours, sizes, shapes, scents, and sexual systems; no other plant organ can match flowers in their diversity. This has enabled them to attract a range of animals to the flowers and use them for pollination services. A standard method used to study differential effects of the colour and fragrance in pollinator attraction is to hide the colour of the flower by covering the flowers/inflorescences with a non-transparent material allowing the scent to escape through fine holes and record pollinator responses to the fragrance (Dobson et al. 2005). To study the effect of fragrance, the flowers/inflorescences are sealed with hermetic transparent paper to prevent scent emission and study the responses of pollinators to the colour. The responses of the above two treatments are compared with un-manipulated flowers (controls). Several investigators have used artificial flowers to study the behavior of floral visitors (Kearns and Inouye 1993; Johnson and Dafni 1998; Cresswell and Smithson 2005; Leonard and Papaj 2011). Visual cues are prepared from coloured paper folded suitably by incorporating the landing area, if necessary; sugar solution is provided as the reward. Many animals forage readily from artificial flowers with the most basic similarity to actual flowers (Raguso et al. 2002).

Visual Cues

Visual cues are in the form of size, shape and colour of the pollination unit (flower/inflorescence). Floral colours are essentially pigment-based; anthocyanins, anthoxanthins, carotenoids, flavones, flavonols and betaxanthins impart a wide range of colours to the flowers (see Pellmyr 2002). Amongst different floral organs, petals are the major attractants; occasionally other parts of the flower such as bracts become conspicuous.

Nectar guides. Flowers with hidden nectar generally have contrasting patterns on the petals, termed nectar guides, pointing toward the source of the nectar. The size and shape of nectar guides are highly variable (Medel et al. 2003). Many studies have shown that nectar guides do help the visitors to locate the source of nectar. A white mutant of blue-flowered *Delphinium nelsonii* resulted in the loss of nectar guides. Bumblebees avoided such mutant flowers; even when they visited such flowers, they took longer time to find the nectar site (Waser and Price 1983). Flowers of *Linum pubescens* (Linaceae) are pollinated by the bee-fly, *Usia bicolor* (Johnson and

Dafni 1998). In flower models with nectar guides, the pollinator tended to follow the lines toward the centre of the flower while on the plain models they showed undirected behavior, often moving to the edge of the model. Similarly bumblebees (*Bombus impatiens*) discovered the rewards more quickly on artificial flowers with star-like pattern when compared to plain flowers (Leonard and Papaj 2011).

In a recent study, using natural flowers, Hansen et al. (2012) studied the role of nectar guides in an iris, *Lapeirousia oreogena.* The flowers of this species have nectar guides in the form of white narrow markings pointing towards the narrow entrance of the long corolla tube and long-proboscid nemestrinid fly is its sole pollinator. Painting of the nectar guides with ink that matched the colour of the corolla background dramatically reduced proboscis insertion into the corolla tube, although it did not affect the approaches of the flies to the flowers from a distance. As expected, removal of nectar guides significantly reduced pollen export as well as fruit set.

Change of colour in older flowers and its role in pollinator attraction. In a number of species, older non-rewarding flowers change colour (instead of senescing) and are retained on the plant for several days. This phenomenon has been reported in over 200 species belonging to 74 families (Gori 1983; Weiss 1991). In all the 26 such species investigated (Weiss 1991) fresh flowers offered nectar and pollen rewards, and older flowers contained little or no nectar and lacked pollen. Retention of older flowers increased plant's attractiveness to pollinators from a distance. However, the pollinator discriminated the colour of the flower from a close range and mostly visited rewarding flowers. In *Lantana camara*, a classical example of post-pollination changes in colour, the flowers are yellow on the day of anthesis and offer pollen and nectar to the pollinator. They turn orange and then red on subsequent days. Red flowers are retained on the plant for several days although they do not offer rewards. In a caged experiment, Weiss (1991) reported that the pollinator butterfly (*Agraulis vanilla*) visited yellow inflorescences significantly more often than red ones. Discriminating ability of several pollinators (belonging to Diptera, Hymenoptera and Lepidoptera) between rewarding and non-rewarding flowers was recorded in a number of other colour-changing plant species (Weiss 1991).

Olfactory Cues

Olfactory cues are in the form of volatile fragrance compounds emitted by flowers. Floral fragrance, in most of the species, is produced by petals but in several species other floral organs, particularly pollen grains (Dobson 1988; Falara et al. 2013), also contribute to the fragrance. The fragrance is in the form of complex mixtures of a large number of volatile compounds, which

give each species a characteristic fragrance. Maximum emission of fragrance generally coincides with the activities of their pollinators (Ando et al. 2001) and fragrance emission often follows endogenous rhythm (Dudareva et al. 1999). For example, methyl benzoate is one of the most abundant fragrance compounds in flowers of snapdragon, tobacco and petunia. Its emission follows circadian rhythm (Kolosova et al. 2001); in diurnally pollinated snapdragon, maximum emission occurs during the day coinciding with the forging activity of its pollinator (bumblebees) and in nocturnal pollinated tobacco and petunia, maximum emission of methyl benzoate is during the night. However, nocturnally pollinated flowers of *Clarkia breweri* in which S-linalool (acyclic monoterpene) is a major component of the fragrance (Dudareva and Pichersky 2000) and *Dianthus inoxianus* in which aliphatic 2-ketones and sesquiterpenoids are the major components (Balao et al. 2011) do not show such differences in emission rate between the day and night. In Australian *Chiloglottis* orchids the levels of active compounds, chiloglottones, remain stable not only during the day and night but also over the lifetime of the flower lasting 2 to 3 wk (Falara et al. 2013). In these orchids, UV-B light is required for their synthesis (Falara et al. 2013).

In general, the fragrance acts as long-distance attractant and at closer range both colour and fragrance act synergistically to guide the visitor to the flower. In an interesting study on long-distance attraction of fragrance, (Ackerman 1983; see Williams 1983) took a canoe on a lake in Costa Rica and exposed floral odour of an orchid species, pollinated by a euglossine bee, at a distance of 50, 200 and 1000 m from the shore, and recorded the arrival of euglossine bees up to 1000 m to the odour source. For insects involved in nocturnal pollination, fragrance is the major or the sole attractant (Jurgens et al. 2002; Balao et al. 2011).

Analysis of floral fragrance. The chemical composition of floral fragrance is one of the most extensively investigated areas of floral biology since long because of their commercial value in perfume industry. The role of floral volatiles in pollinators' attraction is comparatively recent. Earlier studies on the composition of floral fragrance and the role of its constituents in attracting pollinators have been reviewed by Williams (1983) and Knudsen et al. (1993). The fragrance is generally extracted through headspace extraction and analyzed using gas chromatography and mass spectrometry (GC-MS). In the head-space extraction method (Dobson et al. 2005), the flower, intact on the plant. is sealed inside a glass chamber and its emitted volatiles are collected by continually purging the glass chamber through a polymer mesh that binds the volatiles. The volatiles are then extracted from the polymer with an organic solvent.

There is great variation among species in chemical composition of floral scents, and the number and relative concentration of constituent volatiles.

Floral scents are complex mixtures of small volatile molecules largely made up of monoterpenoid (such as linalool, limonene, myrcene, geraniol, cineole and menthol), sesquiterpenoid, phenylpropanoid, and benzenoid compounds (see Williams 1983; Knudsen et al. 1993, 2006; Dudareva and Pichersky 2000). In many species, fatty acid derivatives and a range of other chemicals containing nitrogen or sulphur are also present.

In a number of species, the responses of pollinators to various fragrance compounds have been analyzed by using either electroantennogram, a technique commonly used to measure the output of the antenna of an insect to the brain for the given odour and/or a bioassay. In the bioassay, insects are exposed to floral scents or fractions of floral scents or synthetic chemicals, and the responses of insects are studied in a cage of suitable size. Chemicals are generally applied to filter paper discs which are placed in glass vials. Some workers have used killed female insect dummies for the assay in a few orchid species that show sexual deception (Ayasse et al. 2003). To remove innate odour from the dummies, they are Soxhlet-extracted in dichloromethane, dried and fixed on insect pins. Such odourless dummies are impregnated with various odour samples to be tested and offered to the pollinators (Ayasse et al. 2003).

The mixture of volatiles emitted by each species is different; no two species, even if they are closely related, have been shown to produce identical mixture of volatiles. The pollinator is attracted to some of them but not to all of them. For example, Huber et al. (2005) analyzed floral fragrance compounds in two closely related orchids, *Gymnadenia conopsea.* and *G. odoratissima.* They identified 45 volatiles in the flowers of *G. conopsea* of which three were physiologically active. In *G. odoratissima*, 44 volatiles were identified of which seven were physiologically active. The specificity of fragrance of a species is established not by individual fragrance compound but a combination of compounds. Insects are able to distinguish complex mixtures of floral scent volatiles from different species and respond accordingly. The emission of scent is markedly reduced after pollination (Schiestl et al. 1997).

Only a limited number of studies have been carried out on the fragrance of vertebrate-pollinated plants. Bat-pollinated flowers generally emit strong fruity smell. Bats are sensitive to odours of esters, alcohols, aldehydes and aliphatic acids (Gibson 2001). Analysis of floral fragrance of 11 bat-pollinated species showed 49 compounds of which 11 were suplhur compounds (Bestmann et al. 1997). In a recent study, two aliphatic ketones, 3-hexanone and 1-hexane-3-one, have been shown to be dominant compounds in the scent of a rodent pollinated species, *Cytinus visseri* (Johnson et al. 2011). The aliphatic ketone-rich scent in rodent-pollinated plants is in contrast to insect-pollinated plants in which terpenoids, aromatic or non-ketone aliphatic compounds are the dominant scent compounds (Bestmann et al. 1997).

In general, the response of pollinators to floral scent is based on innate abilities of the pollinators superimposed by learning experience (Andersson and Dobson 2003). The fragrance bouquets of flowers elicit strong, innate species-specific attraction to pollinators. However, many pollinators can learn the fragrance bouquets of other species through their association with the reward (Riffell 2011). This learning ability provides a means of flexibility to the pollinators in a changing floral habitat; it enables pollinators to harvest alternative sources of rewards when their preferred species are not in flowering (see Riffell 2011).

Floral Rewards

Flowers offer a range of rewards to pollinators. These include food sources such as pollen, nectar and seeds as well as specialized non-food rewards such as oils and resins, fragrance and brood sites (see Armbruster 2012).

Pollen and Nectar

Pollen and/or nectar are the major rewards for pollinators in most species of flowering plants. Many floral visitors such as honeybees, bumblebees and stingless bees depend solely on floral resources. For such bees, pollen and nectar serve as the exclusive food source not only for adults but also for their larvae. For other floral visitors that feed on other resources also, floral resources only complement their diet. Pollen is highly nutritious and is a rich source of proteins, vitamins, amino acids and minerals (Schmidt and Buchmann 1992; Roulston and Cane 2000). In several species pollen grains, apart from serving as rewards, emit volatiles and attract pollinators (Dobson 1988; Dobson and Bergstrom 1996, 2000). Pollen-foraging insects use pollen odours not only to discriminate between plant species but also between rewarding and non-rewarding flowers on the basis of pollen availability; this enables pollinators to restrict their visits to the rewarding flowers (Schmidt 1982; Dobson et al. 1999). For example, in nectarless *Rosa rugosa* pollinated by bumblebees, application of pollen volatiles, particularly geranyl acetate to emasculated, freshly opened flowers increased landing frequency of bees significantly (Dobson et al. 1999). When anthers were exchanged between first-day flowers (with pollen reward) and second-day flowers (without pollen reward), flowers with first day anthers received more bee visits.

The nectar is an aqueous solution made up of sugars (largely of sucrose, fructose and glucose) generally ranging from 15 to 45% and small amounts of amino acids (see Nicolson and Thornburg 2007; Heil 2011). Secondary metabolites such as alkaloids and phenolics are also present in the nectar of a range of species (Baker 1977). The nectar contains bacteria (Fridman et al.

2012; Alvarez-Perez et al. 2012) or yeast (de Vega et al. 2009; Herrera et al. 2009) in a number of species. Yeast metabolism in the nectar is thought to contribute to the floral scent (Pozo et al. 2009). Interestingly, the proportion of plant species that contain yeasts in the nectar was found to be highest in those pollinated by birds while those visited only by Hymenoptera showed the lowest values (de Vega et al. 2009). Recently de Vega and Herrera (2013) reported significant changes on sugar composition in flowers visited by nectarivorous ants; it contained significantly more glucose and fructose, and less sucrose when compared to the nectar of ant-excluded flowers. These changes were correlated with the density of yeast cells in the nectar indicating that changes in nectar composition is brought about by yeasts transported by ants.

As the nectar and pollen are harvested by visiting animals, the quality and quantity of available rewards get depleted during the life of the flower. Many pollinators are able to discriminate nectar rewarding flowers from non-rewarding flowers by making use of floral cues that honestly indicate nectar availability. Change of colour of older flowers mentioned earlier is one such cue. The nectar in many species is coloured and has so far been documented in 67 taxa belonging to a wide taxonomic and geographic ranges (Hansen et al. 2007). The colour as well as its intensity is highly variable between species. Pollinators can assess the presence and quantity of nectar in the flowers based on visual cues. Dark brown nectar of *Aloe vryheidensis* is due to the presence of phenolic compounds (Johnson et al. 2006). Dark purple nectar of *Leucosceptrum canum* has been shown to be due to the presence of an anthocyanidin, 5-hydroxyflavylium (Zhang et al. 2012). As 5-hydroxyflavylium inhibits the growth of bacteria and fungi, it may have a role in preventing growth of bacteria and fungi in the nectar of long-lived flowers (96 h in this species). Two Mauritian plant species, *Trochetia boutoniana* and *T. blackburniana*, with coloured nectar are pollinated by *Phelsuma ornata* and *P. cepediana* geckos, respectively (see Hanson et al. 2006). *P. ornata* geckos prefer coloured nectar over clear nectar in artificial flowers (Hanson et al. 2006). Thus, the coloured nectar in these species increases its visibility and acts as a foraging signal to increase pollination efficiency.

Floral nectar of several species is scented (Raguso 2004). Nectar-foraging bees are able to discriminate between flowers that contain nectar from nectar-depleted flowers based on nectar fragrance. In a study on solitary *Osmia* bees on *Penstemon* flowers, Howell and Alarcon (2007) reported that nectar-containing flowers received several times more bee visits when compared nectar-depleted flowers. Bees in which antennae were covered with non-toxic silicon (which blocked the olfactory capabilities of bees) did not discriminate between the two types of flowers indicating that bees are

able to discriminate nectar-rewarding flowers from nectar-depleted flowers based on nectar volatiles.

Many other pollinator species are able to discriminate nectar-containing and nectar-depleted flowers on the basis of other floral cues. The flowers of *Datura wrightii* pollinated by a hawkmoth (*Manduca sexta)* show above ambient emissions of up to 200 ppm CO_2 peak at dusk soon after they open (when the flowers are rich in nectar) but floral CO_2 diminishes rapidly after pollinator visit, although visual and fragrance cues persist for a much longer time (Guerenstein et al 2004). The pollinator moth seems to detect such differences with their CO_2 sensing organ. In a dual choice assay using artificial flowers, moths preferred to feed from the flowers emitting higher levels of CO_2 over those emitting ambient level of CO_2 (Thom et al. 2004). Another instance of such floral cue that honestly indicates nectar availability has been reported in night blooming *Oenothera cespitosa* pollinated by another species of hawkmoth (*Hyles lineate*). The relative humidity of the headspace of the flowers of this species reaches about 4% above ambient relative humidity during the first 30 min after flower opening, although the other floral traits such as visual and fragrance cues may persist for up to 24 h (von Arx et al. 2012). As floral humidity gradients are largely produced by the evaporation of the nectar, these gradients indicate the amount of reward available to floral visitors. Hawkmoth pollinators can distinguish the differences in RH; they consistently visited flowers with higher humidity levels when compared to those with ambient humidity levels (von Arx et al. 2012). These abilities of pollinators to discriminate rewarding flowers from non-rewarding flowers increase the fitness of both the plants and pollinators.

Fragrant Compounds

Many neotropical orchids (over 600 spp.) produce fragrant compounds largely terpinoids and aromatics to attract as well as reward male euglossine bees (over 130 spp.). Euglossine bees collect fragrant compounds and store them in their modified hind legs (Dressler 1982). The bees are thought to use these compounds to produce sex pheromones that are released to attract females. Attraction is species-specific in many of these orchids. Species of *Bulbophyllum* orchid which do not produce nectar are pollinated by fruit flies (*Bactrocera* spp.) (see Tan 2006). Methyl eugenol is a major fragrance compound in several *Bulbophyllum* spp. Methyl eugenol attracts its pollinator, male *Bactrocera* fruit flies, even at a very low concentration. The number of flies visiting the flowers in the morning hours was high and the number dwindles in the afternoon. This is correlated with the release of high methyl eugenol peak in the morning and absence of detectable peak from 14.00 hr onwards in the afternoon (Tan et al. 2002; Tan 2006). Methyl

eugenol is eventually converted into sex pheromone and is released by males to attract females (see Tan 2006). Thus, the fragrance in these orchids acts as an attractant as well as a reward. Apart from orchids, fragrance has been reported to act as attractant as well as reward in some six other neotropical species such as *Anthurium* (Araceae), *Gloxinia* (Gesneriaceae) and *Crinum* (Liliaceae) (Dresler 1982; Pellmyr 2002; Armbruster 2012).

Oils and Resins

Several plants belonging to some 11 families such as Malpighiaceae, Krameriaceae and Scrophulariaceae provide oils as reward. Solitary bees harvest oils and use them for nest building and as a provision for their larvae (Buchmann 1987; Steiner and Whitehead 1991; Pauw 2006; Renner and Schaefer 2010; Armbruster 2012). Species of *Dalechampia* (Euphorbiaceae) and *Clusia* (Clusiaceae) and a few orchids produce resins as rewards. Solitary bees use them for nest building and for antifungal purpose (Pemberton and Liu 2008; Armbruster 2012).

Young Seeds

Many species reward pollinators with nutrients for their larvae in the form of seeds. Fig and fig wasps, and *Yucca* and yucca moths are classical examples of providing seeds as rewards for pollinators (nursery pollination). Pollination in *Ficus* and *Yucca* is highly specialized; each plant species is generally pollinated by a specific species of insect (see Cook and Rasplus 2003; Machado et al. 2005). These pollination systems are examples of obligate relationships as neither the plant nor the pollinator is able to reproduce without the other. They represent insect-plant co-evolution. A few exceptions to this obligate mutualism in figs, however, have been reported recently (see Cruaud et al. 2012; Wang et al. 2013). Senita cactus (*Lophocereus schottii*) and senita moth (*Upiga virescens*) is another example of nursery pollination; it is not, however, an obligate system as two species of halictid bees also bring about pollination of Senita cactus to a lesser extent (Fleming and Holland 1998). Seed reward has also been reported in a few other species (see Armbruster 2012). In nursery pollination systems the pollinators lay their eggs in the ovary of a proportion of flowers and the larvae consume developing seeds after they emerge. Viable seeds develop from uninfected ovaries/ovules.

Extensive studies have been carried out on the fig and fig-wasp mutualism. Most of the *Ficus* species produce monoecious flowers in special inflorescences called syconia. The female wasps enter the receptive syconium through the terminal pore, termed ostiole; they bring about pollination with pollen collected from male flowers of the syconium visited earlier, and lay

their eggs in a proportion of female flowers. The larvae of the wasp feed on the gall formed in oviposited flowers and the remaining pollinated flowers develop into seeds. The emergence of adult wasps developed from the larvae coincides with the maturation of male flowers. The wingless male wasps are short-lived; they mate with the females and cut an exit hole in the syconium for the females to escape. The females loaded with pollen come out through the exit tunnel and enter another receptive inflorescence (which is in the female phase) through the ostiole to reproduce; they bring about pollination during their movement inside the syconium.

The trees in receptive phase generally occur at low densities. As the adult female wasps live only for a few days, it is critical that the female wasps fly to a receptive syconium usually of a different tree within a short time. Reproductive success of both fig and wasp depends on transmission of a very strong signal by receptive syconia (Harrison and Shanahan 2005). Several studies have shown that volatile compounds emitted by the receptive syconia are responsible for attraction of their specific pollinator (Khadari et al. 1995; Proffit et al. 2008, 2009). The syconia emit volatile compounds attractive to pollinating wasps only during the period of receptivity. Pentane extracts of receptive syconia of *Ficus carica* have been shown to attract its pollinator *Blastophaga psenes* from distances of at least 5 m in the field (Hossaert-McKey et al. 1994). Even non-receptive syconia when their ostioles were painted with pentane extracts of receptive syconia elicit the entry of pollinator wasps (Hossaert-McKey et al. 1994).

Several studies have shown species-specificity of fragrant compounds and their role in attracting specific pollinators. The fragrance emitted by receptive figs of three sympatric *Ficus* species, namely, *F. hispida*, *F. racemosa*, and *F. tinctoria* could be clearly distinguished by the composition of their fragrance (Proffit et al. 2008, 2009). Specific odours of these three species were tested for attraction of the pollinator (*Ceratosolen solmsi marchali*) of one of the fig species, *F. hispida*. Behavioural bioassays showed that the pollinator was attracted only to the specific odour of *F. hispida* but not to the fragrance of other *Ficus* species (Proffit et al. 2009). In another dioecious fig, *Ficus semicordata.* 4-methylanisole was shown to be the predominant (94–98%) volatile compound emitted by receptive male and female syconia (Chen et al. 2009). This compound was absent in receptive volatiles emitted by two other sympatric fig species, *F. racemosa* and *F. hispida*. 4-methylanisole attracted its pollinator, *Ceratosolen gravelyi*, in a wide range of concentrations. The fragrance was totally absent in the volatiles emitted by syconia 4 days after pollination. Chemical blends lacking 4-methylanisole were unattractive to *C. gravelyi*. Receptive syconia of two non-host fig species, *F. racemosa* and *F. hispida*, repelled *C. gravelyi.* These results on different fig species clearly indicate that active volatiles are the species-specific signal compounds that attract their obligate pollinator to the host syconia at the receptive stage.

Recent studies of Wang et al. (2013) have indicated that apart from long range volatiles, contact cues after the wasp lands on the syconium also plays a role in determining pollinator specificity.

There is an optimum balance between the number of wasps that enter a syconium and available resources. Entry of too many wasps into the syconium results in oviposition in most of the ovaries; this would lead to eventual collapse of the syconium due to the development of very few or no seeds, compromising the fitness of the plant as well as the wasp species. Several hypotheses have been put forward to explain the stability of this mutualism by preventing wasps from overexploiting the figs (see Dunn et al. 2008). According to one of the hypotheses, the wasps seem to have developed mechanisms to discriminate wasp load in the syconium on the basis of the number of wings left behind by the wasps that have already entered the syconium at and around the ostiole (Ramya et al. 2011). In *F. benghalensis* and *F. microcarpa*, Ramya et al. (2011) have reported a strong correlation between the wing load on the ostiole and the wasp load in the syconium. The wasps preferentially entered those syconia from which the wings were removed rather than those on which the wings were retained. Adding wings artificially to the empty syconia also deterred the wasps from entering them, suggesting that residual wings serve as negative feedback regulators for the entry of wasps. In another fig species, *F. racemosa,* however, wasps did not use leftover wings as cues. A rapid post-pollination decrease in pollinator attractant as has been reported in some other species may act as a cue and limit pollinator visits, thus minimizing overexploitation of ovaries by mutualistic wasps.

The fig-fig wasp pollinator mutualism is also exploited by several parasites of pollinating fig wasps. The parasites generally do not enter the syconium but insert their ovipositors into the syconium from outside and deposit their eggs in some of the flowers; the offspring of the parasites feed pollinator larvae already present in the galls. For more information on pollinator and parasitoid interaction in figs please refer Nedft and Compton (1969); Dunn et al. (2008); Jousselin et al. (2008) and Ghara et al. (2011) and references therein.

Similarly, each species of yucca is pollinated by a specific species of moth (*Tegeticula* or *Parategeticula*) (see Powell 1992; Pellmyr and Huth 1994; Pellmyr 2003). The scent from virgin flowers of the host *Yucca glauca* was sufficient to attract its obligate pollinator *Tegeticula yuccasella* (Svensson et al. 2011). A female yucca moth mates with a male in yucca flower and collects pollen in its specialized mouthparts. She carries pollen to another flower, lays its egg in the ovary wall and deposits pollen grains on the stigma. The larva after its emergence in the ovary consumes only a proportion of the seeds from the developing fruit and the remaining seeds develop normally. After the fruits dehisce, the larvae drop to the ground, burrow into the soil

and construct cocoons. The larvae remain in cocoons during the winter. Following spring rains, adult moths emerge from the cocoon. By this time yucca plants would be in flowering and initiate new pollination cycle. Some pupae may remain dormant in the soil for more than a year.

Nocturnal Pollination

In a number of species, pollination takes place at night. As visual attraction is not reliable during the night, nocturnally pollinated species relay more on olfactory cues. Nocturnally pollinated flowers are generally drab in colour but emit strong smell. Some species show both diurnal and nocturnal pollination (Young 2002; Dar et al. 2006; Muchhala et al. 2009; Dafni et al. 2012). Moths and bats are the most important nocturnal pollinators. Moths have superposition compound eyes, where each rhabdom receives light through a wider aperture consisting of hundreds of facets. This increases the number of photons caught in dim-light conditions and thus facilitate nocturnal vision (Warrant 2004).

Bees in general are diurnal; their foraging activity begins at dawn and ends at dusk. However, several bees have developed abilities to forage in dim light. Many of them such as *Xenoglossa fulva* (Linsley et al. 1955), *Xylocopa tabaniformis* (Janzen 1964), *Ptiloglossa guinnae* (Roberts 1971) and *Megalopta genalis* (Warrant et al. 2004) are able to forage during crepuscular (twilight) periods. Some bee species such as *Apis dorsata* and *Apis mellifera* (Dyer 1985) have been reported to forage nocturnally on moonlit nights. However, *Xylocopa tranquebarica* has been shown to be a true nocturnal bee (Somanathan and Borges 2001; Kelber et al. 2006; Somanathan et al. 2008); it can fly and navigate even during the moonless parts of nights and so far is the only known bee to be able to do so. Unlike typical nocturnal insects such as moths which have superposition compound eyes, all bees including the nocturnal species, have apposition compound eyes that are well adapted to diurnal vision. *X. tranquebarica* has also been shown to exhibit colour vision under moonlight, twilight, and even starlight conditions and is the only insect with apposition eyes to exhibit colour vision under such dim light conditions. Opposition eyes of *X. tranquebarica* are larger with large facets and very wide rhabdoms which make them very sensitive to dim light (Somanathan et al. 2009).

Bats involved in pollination generally have longer tongues when compared to insectivorous species. Bat-pollinated flower syndrome includes nocturnal anthesis, drab coloration and musty, fetid odour. Their flowers are generally placed away from the foliage at the tips of branches or are borne directly on the trunk or branches (cauliflory). In many deciduous trees flowering is initiated after defoliation. Flowers of bat-pollinated species may be tubular or radially symmetrical and produce relatively large amounts

of hexose-rich nectar (Flemings et al. 2009). In flowers of bat-pollinated *Ochroma* species as much as 7–15 ml of nectar has been reported (Faegri and van der Pijl 1971). Amongst floral traits, nocturnal anthesis, placement of flowers away from foliage and production of larger amount of nectar are found in almost all bat-pollinated flowers (Fleming 2009). Bats are long distance pollen dispersers. They use vision, olfaction and echolocation to locate flowers. They have keen sense of smell that helps in long-distance location of flowers. Bat-pollinated species generally occur in low density and bats play a crucial role in maintaining genetic continuity of their population (Fleming et al. 2009).

New World Bats (Phyllostomidae) can produce ultrasonic sound to locate flowers. The sound is reflected by the petals of bat-pollinated flowers and bats have the ability of recognize this reflected sound (echolocation). The erect petals of the flowers of many species such as *Mucuna holtonii* have been shown to reflect sound pulses produced by echolocation bats that visit the flowers for their copious nectar (von Helversen and von Helversen 1999). Echolocation ability is not developed in most of the old world bats (Pteropopodidae); they depend largely on olfactory and visual cues to locate the flowers. The eyes of all bats are well adapted to low illumination. Old World fruit bats have colour vision whereas New World bats can see only in black-and-white.

Pollination by Ants: A Rare and Ineffective Pollination Syndrome

Ants are among the most abundant insects on earth and visit flowers frequently. Also, the fossil record shows that ants were present during the explosive radiation of the angiosperms in the late cretaceous period even before the presence of bees (Beattie 1985). Surprisingly, ant pollination has not evolved as a major pollination syndrome in angiosperms. So far, ant pollination has been documented in less than 20 species of herbs and shrubs (Shivanna 2010). Although ants are active 24 hr a day, ant-pollination does not seem to have evolved in any of the night blooming plant species (Beattie et al. 1984; Beattie 1985; Sharma et al. 2009; see Shivanna 2010). Interestingly, ant-pollination has not been reported so far in any tree species.

The following are some of the hypotheses put forward to explain the rarity of ant pollination:

1. Ants bring about largely geitonogamous self-pollination as their movements are usually restricted to within the plants.
2. Secretion of antibiotics from metapleural glands present in most of the ant species (as a defense against bacteria and fungi) reduces pollen viability.

3. Pollen transfer is not efficient as their body surface is smooth and pollen grains do not adhere well to their bodies.
4. Ants groom their bodies too frequently with the result very little pollen is left on the body for transfer to the stigma.

Most of the observations recorded in various plant species do show that the movements of ants are restricted within the plant and most of the pollinations are geitonogamous. They may move between plants and bring about cross-pollination infrequently only in small herbaceous species that grow in high density in small patches (Hickman 1974). Studies on the effects of antibiotics show differential potency among ant species and differential sensitivity among pollen species (Beattie et al. 1984). Thus, inhibition of pollen function by exposure to the surface of ants may provide only a partial explanation for the rarity of ant pollination systems. Smoothness of the body surface of ants is not universal. Many ant species are as hairy as bees or are covered with bristles suitable for carrying pollen load. Although pollen load recorded on ants are generally low (10–30), pollen load of over 200 grains per ant has been recorded in a few species. Many investigators have discounted the suggestion that ants groom their body frequently since bees also groom their body frequently (Beattie et al. 1984). Thus, available evidences support, to some extent, the first two hypotheses to explain the rarity of ant pollination.

There is very little information on floral attractants for which ants are responsive. Although some investigators have suggested that floral scent could play a key role as floral attractant, there are no experimental evidences. The nectar acts as the reward for ants. For most of the species, ants are not exclusive pollinators; they share pollination services with several winged insects. For example, *Balanophora* flowers are visited by a variety of flying insects besides ants (Peakall and Beattie 1989). However, specialized, exclusive ant-pollination has been reported in two Australian orchids. *Leporella fimbriata* (Peakall 1989) is exclusively pollinated by male winged ants of *Myrmecia urens.* The other orchid, *Microtis parviflora* (Peakall and Beattie 1989) is pollinated by flightless worker ants of *Iridomyrmex gracilis,* through sexual deceit.

Non-Mutualistic Pollinations

In a number of plant species, pollination is achieved through non-mutualistic interaction (Dafni 1984; Renner 2006; Bernklau 2012). Such plants advertise the presence of reward without offering the reward and achieve pollination through deceit. Non-mutualistic interactions have evolved in all major groups of flowering plants. Orchids form the most important group of non-mutualistic pollination; about one third of the orchids (over 10,000 species)

are estimated to be deceptive (Renner 2006; Jersakova et al. 2006). Most of the species producing reward-less flowers are pollinated by insects; however, two of the reward-less species have been reported to be bird-pollinated and one bat-pollinated (Renner 2006).

Many animal visitors also harvest the rewards of the flower without effecting pollination. Such visitors are referred to as pollen or nectar robbers. Nectar robbers generally enter the flower from the side by piercing the corolla tube and harvest the nectar without coming in contact with the anthers and stigma. Pollen robbers forage the pollen from the anthers but do not come in contact with the stigma.

Food Deception

Plants of non-rewarding species (mimic) coexist with rewarding species (model) and the flowers of the mimic resemble those of the model. Floral visitors draw rewards from the model but do not discriminate against non-rewarding flowers of the mimic. Food deceptive orchids generally attract both male and female pollinators. In many food deceptive orchids, floral colour seems to play more dominant role than the extent of morphological similarity of the model and the mimic (Gigord et al. 2002; Streinzer et al. 2010). A South Africa orchid, *Disa ferruginea* (Johnson 1994; Newman et al. 2012), for example, depends on a butterfly species, *Aeropetes tulbaghia* for pollination. In the Cape Province (western part of its range) a red flowered form of this orchid occurs and attracts butterfly visits by imitating red, nectar-producing flowers of *Tritoniopsis triticea* (Iridaceae). The butterfly does not discriminate between the nectarless orchid flower and the nectar-producing model flower in sympatric populations. Interestingly, in the Langeberg Mountains (eastern part of its range) an orange-flowered form of *D. ferruginea* occurs and mimics the orange, nectar-producing flowers of *Kniphofia uvaria* (Asphodelaceae). The pollinator butterfly preferred red flowers in the western part where its main nectar model has red flowers while in the east it preferred orange flowers, where its main model has orange flowers. These results indicate that the flower colour in *D. ferruginea* is adaptive and driven by local colour preference of its pollinator.

Several studies have indicated that the food deception is largely mediated by visual signals and olfactory signals do not seem to play any major role. Studies of Galizia et al. (2005) on a food deceptive orchid, *Orchis israelitica* and its model *Bellevalia flexuosa* (Liliaceae) showed that fragrance compounds in the mimic were quite different and weak when compared to the model. The odour of the model and the mimic elicited distinct activity patterns in the brain of its pollinator bee, indicating that the bee can easily distinguish the flowers of the model and the mimic on the basis of their odors. As the bee does not discriminate between the flowers of the two

species, odor obviously does not play any role in signaling. In another study in which both the mimic and the model are orchids, Salzmann et al. (2007) reported that both the model (*Anacamptis coriophora*) and the mimic species (*A. morio*) emit complex odour bouquets. Several components of the fragrance of the model triggered electrophysiological responses in olfactory neurons of honey-bees and bumble-bees. The scent of the mimic, however, was too weak to elicit any electrophysiological responses (Salzmann et al. 2007), negating the role of olfactory signals in deception. The fragrance of the model, however, may still play a role in long distance attraction of the pollinators to the location where the model and the mimic grow.

Sexual Deception

A large number of orchids achieve pollination by sexual mimicry. Most of the species showing sexual mimicry are pollinated by species of Hymenoptera. Pollination through sexual deception is often highly specific and attracts a particular species of pollinator. Odor signal plays a dominant role in sexual mimicry (see Galizia et al. 2005). Flowers of non-rewarding species mimic visual, olfactory and tactile cues of conspecific females of the pollinator. Olfactory cues are similar to sex pheromones of receptive females. For example, sexually deceptive Australian orchid, *Chiloglottis trapeziformis* attracts males of its pollinator wasp, *Neozeleboria cryptoides*, by emitting a unique volatile compound, 2-ethyl-5-propylcyclohexan-1, 3-dione which is also produced by female wasps as a sex pheromone (Schiestl et al. 2003). The visitor lands on deceptive flowers and tries to copulate (pseudocopulation) and during this process, brings about pollination. In sexual deception, pollinators are first attracted to the fragrance signals from a distance but in close vicinity they seem to be guided exclusively by visual signals (Streinzer et al. 2009).

Kullenberg (1956) was the first to show the role of fragrance in sexual deception in orchids. Until then it was thought that sexual mimicry in orchids was mediated only by visual cues. Kullenberg (1956) covered the flowers *Ophrys lutea* with a piece of cloth for a few hours and showed that the cloth attracted its pollinator, *Adrena* bees. Subsequent studies confirmed that both visual and volatile chemicals are involved in attraction of male bees. Flowers showing sexual mimicry produce complex odour bouquets of a large number of volatiles that are similar to those produced by the females of their pollinators (see Ayasse et al. 2003). *Ophrys* is one of the well-investigated orchids showing sexual mimicry. Different species of *Ophrys* produce distinctive qualitative and quantitative blends of floral fragrance compounds. The fragrance compounds produced by *Ophrys* flowers of several species have been shown to be similar to those found in the mandibular and/or Dufour's glands of their pollinators (see Williams 1983;

Borg-Karlson 1990). Ayasse et al. (2003) studied the details of fragrance in the flowers of *O. speculum* and its pollinator, *Campsoscolia ciliata.* In field trials, they used female dummies as control. The male insects hardly responded to odourless female dummies but were attracted in high frequency to freeze-killed virgin females and intact *O. speculum* flowers; Labellum extracts of *O. speculum* flowers and cuticular extracts of virgin females. They identified eight electrophysiologically active compounds. Of these, 9-hydroxydeconic acid was the major one in both the labellum and the insect.

In *Ophrys sphegodes,* pollination has been shown to result in changes in odour components (Ayasse et al. 2000; Schiestl et al. 1997, 2003) and thus the attractiveness of pollinated flowers to its pollinator male bees (*Andrena nigroaenea*) decreases. Pollinated flowers showed a significant increase in farnesyl hexanoate. Flowers treated with farnesyl hexanoate were found to be less attractive to males indicating that this chemical acts as a repellent and guides pollinators to unpollinated flowers (Schiestl and Ayasse 2001).

In another study (Stokl et al. 2009), the odour compounds were analyzed in three species of *Ophrys, O. lupercalis, O. bilunulata*, and *O. fabrella.* All the species grow sympatrically and are pollinated by three species of *Andrena.* These *Ophrys* species use the same odour compounds for pollinator attraction, but in different proportions. Thus a change in the concentration of the constituents of floral odour can result in the attraction of a new pollinator species that acts as an isolation barrier towards other sympatrically occurring *Ophrys* species (Stokl et al. 2009).

Brood Site Deception

Several species mimic brood sites and attract insects whose larvae feed or lay their eggs on decaying organic matter such as dung, decaying feces and carrion. Brood site mimicry has been documented in species belonging to some ten families such as Annonaceae, Araceae and Aristolochiaceae (see Wiens 1978; Bernclau 2012). Their flowers mimic the odors of dung and/or carrion to attract coprophilous beetles and flies that oviposit or feed on dung or carrion. These odours are very unpleasant to humans. The odours are composed of sulfide compounds, ammonia, alkylamines, cadaverine and putrescine. Fecal-like odors are also produced by skatole and indole compounds (Meeuse 1978; Dettner and Liepert 1994).

In many species showing brood site mimicry, the odours are enhanced by the production of heat (thermogenesis). Floral thermogenesis appears to be more common in basal angiosperms and Eumagnoliids when compared to other angiosperms. It has been reported in 3 families (Nymphaeaceae, Schisandraceae and Illiciaceae) of basal angiosperms, 6 families (Annonaceae, Araceae, Arecaceae, Aristolochiaceae, Cyclanthaceae and Magnoliaceae) of Eumagnoliids, and only 2 families (Nelumbonaceae

and Refflesiaceae) of Eudicots (see Thien et al. 2009). Detailed studies have been carried out on *Victoria amazonica*, a thermogenic basal angiosperm (Prance and Arias 1975) in which beetles are the major pollinators. Flowers are protogynous; they are in the female phase on day 1 and in the male phase on day 2. Flower buds open in the evening of day 1, and produce heat (>11ºC above ambient) and strong odour that attract beetles. Flowers close by the next morning trapping the beetles followed by gradual loss of heat and odour. The flowers gradually turn from white to deep purplish-red. By evening of day 2, the stamens release pollen and the flowers reopen. The escaping beetles pick up pollen and fly to another flower of day 1 which is thermogenic and scented. When beetles are trapped inside the flowers, they consume starchy stylar appendages. High temperature inside the flower also seems to act as an energy reward for the beetles in maintaining their body temperature at endothermic level (Seymour and Matthews 2006). In general, thermogenesis and trapping mechanism are associated with food deceptive species which do not provide any rewards to the visitor. But in *N. amazonica* the flowers seem to provide rewards in the form of nutrients and heat energy (Seymour and Matthews 2006).

In species of *Aristolochia* (Murugan et al. 2006; Trujillo and Sersic 2006) the flowers are differentiated into an expanded limb, a long narrow tube of various lengths and a basal expanded utricle. The inner surface of the tube is lined with downward-pointed hairs which facilitate the entry of insects into the floral chamber where the sexual organs are located but not their exit. The flower produces an odor similar to decaying plant tissues, mimiking the natural oviposition substrate of their pollinators. Flowers are protogynous and the stigma is receptive at the time of flower opening. The pollinators (mostly Dipterean flies) are attracted by the colour and odour of the flower, enter the floral chamber through the narrow tube and are entrapped for 24–48 h as the downward-pointed hairs on the inner surface of the floral tube prevent their exit. By the time anthers dehisce and the insects get coated by the pollen, the hairs in the tube become flaccid and start senescing allowing the flies to escape. The flies, coated with pollen enter freshly opened flower with receptive stigmas and bring about pollination.

The flowers of some species of Aristolochiaceae, Araceae, Orchidaceae and Saxifragaceae are pollinated by fungus gnats. The flowers or floral parts of many such species mimic oviposition sites (gill fungi) of fungus gnats (the larvae of which feed on gill fungi) (Vogel and Martens 2000; Okuyama et al. 2004 and references therein). Some of them also have trap mechanism. The visual mimicry in combination with emission of odour characteristic of fungi attracts fungus gnats which oviposit and bring about pollination. The pollination in *Mitella* (Saxifragaceae) species differs from other fungus gnat-pollinated species in that the fungus gnats do not lay

their eggs in the flower and seem to consume small amount of the nectar available (Okuyama et al. 2004).

Aphidophagus hoverflies (Syrphidae) generally lay their eggs in places of aphid infestation as their larvae feed on aphids. The orchid *Epipactis veratrifolia* is exclusively pollinated by five species of aphidophagous hoverflies (Ivri and Dafni 1977) and the authors suggested that the flowers in this species mimic the shape and colour of aphids to attract syrphid flies. However, recent studies (Stokl et al. 2010) have shown that the flowers produce α- and β-pinene, β-myrcene and β-phellandrene (which are similar to the alarm pheromone released by several aphid species). The pheromones attract aphidophagus hoverflies. The flies bring about pollination during egg deposition and nectar feeding. As these pheromones are not species specific (they attract several species of syrphid flies) and also the flies consume nectar, Stokl et al. (2010) prefer to call it generalized mimicry rather than typical mimicry.

Evolution of rewardless flowers and their stability has been discussed by some investigators (Pellmyr 2002; Renner 2006). Pollinators constantly encounter rewardless flowers during most of the foraging bouts even within the rewarding species. This regularity in encountering rewardless flowers makes pollinators follow flexible foraging. Because of this foraging flexibility pollinators do not discriminate more strongly against rewardless species (Pellmyr 2002; Renner 2006). It has been suggested that transiently rewardless flowers may have facilitated the evolution of rewardlessness as a stable strategy because their presence lowers the strength of selection on pollinators to consistently discriminate against lack of rewards (Renner 2006).

Restriction of Pollinators

Any natural community is made up of a number of plant species and a range of animal species, many of which are potential pollinators. However, each plant species attracts only a proportion of potential pollinators to visit its flowers in a sustainable way and use them for pollination services but prevents the visit of several others present in the community. So far studies on pollination ecology have largely been on floral attractants and rewards. However, for an efficient pollination system, restriction of visits to a reasonably limited number of pollinator species is important to ensure their visits to largely conspecific flowers. Although there are several studies on the factors which act as filters to prevent the visits of some animal species, there is hardly any discussion on this aspect in reviews on pollination ecology. Available evidence indicate that restriction of floral visitors may act at different levels—morphology of the flowers, species–specific fragrance, and quantitative and/or qualitative features of the nectar and of pollen.

Morphological Filters

The role of morphological elaboration of floral traits that permit some species of pollinators (and prevent some others) was known since long. Faegri and van der Pijl (1971) referred to such adaptations that permit only a particular group of animals as 'harmony' between the visitor and the flower. Lack of harmony prevents floral visitors. One such floral elaboration is the evolution of bilateral symmetry. It enables the flowers to guide the approach pattern of the visitors to harvest the rewards efficiently. Flowers of such species are visited only by those animal species that are able to find and harvest the rewards. Another type of elaboration is the evolution of the flowers with a long corolla tube or a spur in which nectar is located. Plant species with such flowers are very common. In southern Africa for example, long-tube flowers have been reported in about 200 spp. of at least 10 families. A large number of orchids have spurs of various lengths often up to 40 cm. In flowers with long corolla tubes, only insects that have the proboscis of matching length or birds with matching beak length can harvest the reward. Pollinator species with different tongue lengths tend to specialize on plant species with matching spur/corolla tube lengths. Such correlation has been highlighted in several studies on plant-pollinator communities (Pleasants 1983; Pellmyr 2002; see also More et al. 2007; Santos-Gally et al. 2013). In a bumblebee community with different tongue lengths (short, medium and long), a relationship between the corolla length of the plant species and the tongue-length of the bees that visit them has been reported even in earlier literature (Pleasants 1983). Such plant-pollinator relationship reduces competition by restricting the visits of the bees of various tongue lengths to flowers of matching corolla length. The bees with tongue-length shorter than corolla length would not visit the flowers of long corolla tubes since they cannot reach the nectar. Avoidance of the bees with long tongue-length to visit the flowers with shorter corolla tubes has been explained on the basis of less efficient foraging (of long-tongued bees on short corolla tubes) when compared to those with appropriate tongue length. Another reason put forward to explain this avoidance is the lower net profit to long-tongued bees in visiting flowers of short corolla tube as they have to compete with short-tongued bees which would reduce the nectar reward to unprofitable levels (see Pleasants 1983; More et al. 2007).

Pollination Syndromes

The role of morphological traits of flowers and their relationship to the pollinators has been elaborated in the form of pollination syndromes (Faegri and van der Pijl 1971). Since the time of Darwin, the traditional concept on the evolution of pollination systems has been toward specialization leading

to a greater refinement in making the pollinator and the flower mutually inter-dependent. Various pollination syndromes such as beetle-pollinated, bee-pollinated, butterfly-pollinated and bird-pollinated were identified and described (Table 1). A syndrome is a combination of phenotypic traits associated with attraction and utilization of specific types of animals for

Table 1. Pollinator groups and their respective floral syndrome traits (based on Faegri and van der Pijl 1971; Turner 2001 and Ghazoul and Sheil 2010).

Pollinator group	Floral syndrome characteristics and some examples
Beetles (Cantharophily)	Flowers unspecialized, open, generally large, cylindrical or bowl-shaped, sex organs exposed, dull coloured, no nectar guides, rewards easily accessible, strong odour, pollen deposited all over body, thermogenicity often involved in attraction. Members of Annonaceae, Lauraceae and Myristicaceae, Dipterocarpaceae.
Flies (Myiophily)	Flowers small, radial, dull coloured, no nectar guides, decaying odour, sex organs generally hidden, pollen deposited all over the body. Members of Aristolochiaceae, Araceae.
Bees (Melittophily)	Flowers highly variable in morphology, simple, open to complex, often zygomorphic with landing sites, nectar guides present, brightly coloured, odours mild, nectar hidden but not deep, sexual organs concealed, stamens few, precise pollen placement on body, offer nectar and pollen. Members of Fabaceae, Bignoniaceae, Melastomaceae and Euphorbiaceae.
Butterflies (Psychophily)	Flowers tubular, diurnal blooming, mild odour, brightly coloured, flowers erect, nectar ample and deeply hidden in tubes/spurs, pollen deposited on proboscis and head. *Delonix, Caesalpinia, Ixora, Mussaenda.*
Moths (Phalaenophily)	Flowers horizontal or pendent, nocturnal blooming, pale and heavily scented, nectar deeply hidden in long tubular corolla or spurs, pollen deposited on proboscis and head. Members of Rubiaceae, Apocynaceae, Meliaceae, Mimosoidea.
Birds (Ornithophily)	Flowers fairly robust, diurnal blooming, rigid, vividly coloured often red, long tubular corolla, copious nectar with low sugar concentration, odours absent, pollen placed on beak, head or precisely on body, nectar sole reward. *Erythrina, Butea, Spathodea.*
Bats (Chiropterophily)	Flowers nocturnal blooming, flowers generally placed at the tips of branches generally pale and large, strong odour at night, large-mouthed and stiff, copious nectar, anthers often numerous, pollen placed on the head, nectar sole reward. Members of Bombacaceae, Bignoniaceae and Myrtaceae.

Pollination syndromes do not form closed compartments and do not represent exclusiveness of the respective pollinators to the flowers of a particular syndrome. They also do not indicate the presence of all the specific traits of a syndrome in all plant species of that syndrome. Pollination syndromes simply represent traits that are over-represented in flowers that attract specific types of pollinators; they do not exclude other visitors (Faegri and van der Pijl 1971; Pellmyr 2002).

pollination. Specialization in floral traits leading to pollination syndromes was explained on the basis of selection pressure exerted by the pollinators. Different pollination syndromes are often present in closely related species. For example, *Petunia axillaris* (flowers white, nocturnally scented) is pollinated by nocturnal hawkmoth (*Manduca* sp.) while *P. integrifolia* (flowers colourd and unscented) is pollinated by diurnal bee (*Hexantheda* sp.) (Ando et al. 2001). Similarly, unrelated plants also show similarities in floral traits; such similarities are attributed to evolutionary convergence resulting from selection by shared types of pollinators (Faegri and van der Pijl 1971).

Many recent studies have shown that in a majority of species, flowers are visited by a number of animal species and the advanced level of specialization is seen only in a limited number of species. Based on these studies traditional concept of pollination syndromes has been questioned (Waser et al. 1996; Waser and Ollerton 2006). However, many pollination biologists (Pellmyr 2002; Fenster et al. 2004; Bronstein et al. 2006; Fleming et al. 2009; Mitchell et al. 2009) argue that the concept of pollination syndromes helps in understanding the mechanisms of floral diversification and the convergence of floral form across angiosperms pollinated by similar pollinators. The concept of generalization and specialization is used in terms of the number of pollinators a plant species attracts. Most of the plants are pollinated by two or more species, indicating selection against excessive specialization; this gives pollination assurance even under spatio-temporal variation in pollinator abundance.

One of the major limitations of assessing the number of pollinators of a given plant species is the lack of data on actual pollinators based on pollen transfer. In some plant species, the number of animal species visiting the flowers is quite large. Over 70 species of insects have been reported to visit the flowers of *Andira inermis* (Fabaceae) (Frankie et al. 1976; see Bawa 1990). In such plant species true pollinators, on the basis of pollen transfer, are likely to be much less. Some of them may be casual visitors to explore the availability of resources and some others may predate on insects that visit the flowers; and yet others may rob the pollen and/or nectar without bringing about pollination. Further, the frequency of some of the visitors may be too low to have any impact on pollination efficiency of the plant species. The number of effective pollinators on the basis of pollen transfer has been documented only in a limited number of species. In one of the *Syzygium* species, S. *heyneanum*, out of 23 species of floral visitors only three species turned out to be effective pollinators (Giby Kuriokose, P.A. Sinu and K.R. Shivanna, unpublished). In the absence of such detailed information, records of the number of visitors to flowers may be misleading in assessing the number of pollinators of plant species and the extent of its specialization/generalization. In most of the plant species with unusually

large number of visiting species, the number of pollinators is likely to be much less than the number of visitors reported.

Generalization and specialization also applies to pollinators. Generalists are those that forage the flowers of a number of plant species and specialists are those that forage the flowers of a limited number of species. Specialization is not generally in the evolutionary interest of flower-visiting animals. Many animal species specialize when their preferred species is flowering but in the absence of their preferred species, utilize a wide range of plant species.

Co-evolution

Guided by Darwin's concept of co-evolution (reciprocal selection between plant and pollinator), earlier pollination biologists interpreted most of the diversification of plants and pollinators as the result of co-evolution. Subsequently it became apparent that co-evolution is largely restricted to a limited number of species which show obligate relationship between plant species and its pollinator such as figs and fig-wasps, yucca and yucca-moths (Pellmyr 2002; Bronstein et al. 2006), and several orchids. In these highly specialized pollination systems in which each plant species is pollinated by just one animal species, the specificity of pollinators is apparently the result of mutual adaptation of the plant and pollinator species. Attraction of species-specific pollinator is largely mediated through morphological elaboration of the flower and/or species-specific fragrance. Other species do not visit the flowers because these lack matching flower structure or specific fragrance.

The well-recognized example of co-evolution, first proposed by Darwin, is the length of floral spurs, and the length of insect tongues and beaks of birds (see Pellmyr 2002). While explaining the exceptionally long nectar spur (40–50 cm) of the star orchid in Madagaskar, *Angraecum sesquipedale*, Darwin proposed that a co-evolutionary 'race' (between spur length of flowers and tongue length of insects) acted as a driving force for the directional increase in the length of plant's spur (where the nectar is concealed) and the tongue of its pollinator. Floral visitors whose proboscis is longer than the length of the spur/corolla tubes of its host flower, rob the nectar without effecting pollination (as the insect need not push the proboscis deep enough to bring its mouthparts in contact with the anthers and the stigma). For effective pollination it is advantageous for the plant if the length of the spur/corolla tube exceeds the length of its pollinator tongue so that the pollinator is forced to push the proboscis deep into the spur thus bringing the mouthparts in contact with the anthers and the stigma. This would induce directional selection for longer spurs in the population. Evolution of deeply concealed nectar induces reciprocal selection in tongue length in the pollinators. This

process can continue as long as genetic variation is available in both the partners.

Correlation of plant fitness with the length of the spur was supported by studies of Nilsson (1988) in an orchid, *Platanthera bifolia,* in which spur length ranges from 30 to 50 mm. He shortened spur length by pushing the nectar upward and tying the spur with a thread and studied the responses of its pollinator moth. When the spur length was shorter than the length of the proboscis, the moths pushed the proboscis only up to the level of the spur necessary to forage the reward; their mouth parts did not come in contact with the sexual organs of the flower which is necessary for the removal of pollinaria and its deposition on the stigma. Shortening of the length of the spur proportionately reduced both the removal of pollinaria (male fitness) and their deposition on the stigma (female fitness) of visited flowers. Longer the spur length better was the removal of pollinaria and their deposition.

In contrast to many examples documenting the effects of selection pressure exerted by pollinators on plants, plant-mediated selection on pollinators is limited except in those highly specialized obligate pollination systems. This is largely because of the difficulty of measuring life-time fitness of pollinators which are mobile. Also plant-mediated selection on animals is indirect since the plants do not exert direct effects on the pollinator's gene flow but only on their food intake (see Pellmyr 2002). However, plant-mediated selection on animals has been correlated to differences in bill length and shape in male and female individuals of the purple-throated carib (*Eulampis jugularis*) (Temeles et al. 2000). In this species the female has significantly longer and curved bill when compared to the male. The males primarily visit nectar-rich patches of *Heliconia caribaea* which has short, straight flowers corresponding to their bills, whereas females are the primary visitors to flowers of *Heliconia bihai,* which has long, curved flowers matching the size and shape of their bills. On the basis of these studies sexual dimorphism in *E. jugularis* has been suggested to have evolved in response to differences in the floral traits of their primary floral resources. Because of this partitioning, heterospecific pollen transfer is avoided. Their subsequent studies provided evidence to indicate that differences in bill length and curvature between sexes in hermit hummingbirds are also associated with differences in their food plants (Temeles et al. 2010).

Strong selection on bill size has also been documented in Hawaiian honeycreeper in response to dietary shift (Smith et al. 1995). The honeycreeper, *Vestiaria coccinea,* used its long curved beak to access nectar in tubular lobelias. These lobelias have now become extinct or very rare. The bird has now changed its diet to the nectar of *Metrosideros polymorpha,* the flowers of which lack corolla. Comparison of the museum specimens

of *V. coccinea* collected before 1902 with the extant individuals revealed a significant reduction in bill length in extant specimens.

Restriction of Pollinators in 'Open Flowers'

In 'open flowers', which are generally symmetrical without any structural elaboration, pollen and nectar are exposed and practically any animal that visits such flowers can harvest both pollen and nectar rewards. Even in such open flowers, there is some degree of specificity; all animals present in the habitat do not visit the flowers. *Syzygium cuminii* (our unpublished observations) for example has a typical open flower. *Apis dorsata* and one species of ant are the major visitors to the flowers; a few other insects such as *Apis cerana*, *Trigona* sp., a few wasps and a butterfly make occasional visits but their frequency is too low to have any impact on pollination efficiency of the species. Flowers of *Passiflora* spp. also have brightly coloured open flowers with exposed pollen and nectar. However, the number of floral visitors reported in different species of *Passiflora* is also limited (Varassin 2001; Shivanna 2012b). In a study on pollination ecology of annual weeds, Shivanna (unpublished observations) observed that *Apis cerana* was a regular and frequent visitor to the open flowers of *Triumfetta rhomboidea* for pollen, but they never visited open flowers of *Abutilon indicum, Urena lobata* and *Melochia corchorifolia,* which were flowering sympatrically next to *T. rhomboidea*. These studies and several others clearly show that although the rewards in such open flowers are accessible to any visitor, only a limited number of animal species visit the flowers in spite of the presence of a large number of insets in the habitat. In many such species, the number of animal species visiting flowers may not be more than those flowers with some structural specialization. In such species with open flowers, morphological filters do not operate; however, they have other filters to restrict the number of visiting species. Several studies have shown that the fragrance, nectar and even pollen may act as filters to restrict the number of species to visit their flowers. These filters may operate in specialized flowers also.

Fragrance Filters

Most of the studies on floral fragrance are concerned with their role in attracting floral visitors. As pointed out earlier, the composition of fragrance is unique to each plant species and attracts a specific pollinator or a group of pollinators. The fragrance acts as a filter in many plant species as their flowers lack suitable fragrance to attract other species. As pointed out earlier, in species with obligate specialization, fragrance appears to be the main filter. Several studies have highlighted the role of fragrance in repelling some floral visitors. Omura et al. (2000) reported that only a few species

of insects visit the flowers of *Osmanthus fragrans* despite their strong scent and vivid coloration. A butterfly, *Pieris rapae*, a potential visitor, never visits the flowers of this species. They showed that isopentane fraction of floral volatiles, particularly γ–decalactone, is a strong repellent to this butterfly. Willmer et al. (2009) have reported that floral volatiles act as repellents to ants in some species of *Acacia*. A meta-analysis of 18 studies on the response of animals to floral scents by Junker and Bluthgen (2010) have also highlighted the dual function of floral scents; obligate floral visitors are attracted to floral scent while those which are facultative and antagonists are repelled by floral scents.

Nectar Filters

Studies on nectar have highlighted largely the role of its nutritive components, sugars and amino acids, as rewards for the visitors. The amount of nectar present in the flower and its sugar concentration are, to some extent, correlated with the type of animals visiting the flowers (Baker and Baker 1983). Bee-visited flowers generally have lower amount of nectar with higher sugar concentration while bat- and bird-visited flowers have higher nectar volume with lower sugar concentration. These features are well recognized and form a component of pollination syndromes.

Although the presence of non-nutritive metabolites such as alkaloids and phenolics in the nectar was known since long (Baker 1977), only limited number of studies are available on their role in attracting or repelling floral visitors (Stephenson 1981; Adler 2000; Adler and Irwin 2005; Raguso 2004; Wright et al. 2013). In an earlier study Stephenson (1981, 1982) showed that secondary compounds of the nectar of *Catalpa speciosa* are effective in filtering the visitors. The floral nectar of this species contains iridoid glycosides, catapol and catalposide, which adversely affected potential nectar thieves (ants and a skipper butterfly, *Ceratomia catalpa*). The legitimate diurnal bee pollinators were not affected by these glycosides. Some South African species of *Aloe* produce dark brown nectar with a bitter taste because of the presence of phenolic compounds in high concentration (1.2–1.5 mg/ml). Detailed studies of Johnson et al. (2006) on one such species, *A. vryheidensis* clearly indicated that the main effect of phenolics in the nectar is to repel inefficient-/non-pollinators. Bulbuls and white eye, which are effective pollinators are not affected by the bitter taste of nectar. Bees and sunbirds which are not effective pollinators do not even approach the flower as they do not like dark brown nectar. Naive honeybees and sunbirds, however, approach nectar during initial visits but avoid it completely in subsequent visits. Thus, the dark phenolic component of the nectar functions as a floral filter by attracting some animals and deterring others by its taste. In another study Liu et al. (2007) have shown that buck

wheat nectar phenolics in combination with 15–35% sugar syrups attracted *Apis cerana* but acted as deterrents below or above this sugar concentration range. Such synergism between phenolics and sugar may provide a novel mechanism for plants to preferentially select some pollinators and to reduce energy investment in nectar.

Presence of alkaloids in plants is quite common and they act largely as deterrents to herbivores. As mentioned earlier, the nectar of some species contains alkaloids such as nicotine and caffeine. In *Nicotiana atenuata* pollinated by a moth and hummingbirds, presence of nectar nicotine decreased visitation time and the volume of nectar removed for both the pollinators, but increased the number of their visits (Kessler and Baldwin 2006). Interestingly, pollinators removed nearly 70% more nectar from nicotine-silenced plants (through genetic transformation) in which nectar lacked nicotine completely when compared to control plants. On the basis of these results they hypothesized that nectar repellents optimize the number of visits per volume of nectar produced, allowing plants to keep their nectar volumes small. The flowers of *Coffea* and *Citrus* contain low concentration of caffeine which does not deter their pollinator bees (Wright et al. 2013; see also Chittka and Peng 2013). Interestingly, the presence of caffeine in the nectar has been shown to significantly enhance the memory of bees; honeybees rewarded with caffeine were three times more likely to remember the reward when compared to honeybees rewarded with sucrose alone.

Floral nectar of several species is scented. The nectar scent compounds in some species have been shown to be a subset of the compounds emitted by the surrounding floral tissues, while in some others they contain unique scent compounds compared to the floral tissues (Raguso 2004). In a detailed study, Kessler and Baldwin (2006) analyzed secondary metabolites in floral and nectar fragrance of *Nicotiana atenuata* and studied their responses on their pollinators (one moth and two hummingbird species) and a nectar robber (one ant species). Various components of the fragrance showed positive or negative effects on different animals tested. Compounds from the same biosynthetic class tended to evoke similar responses. Benzyl acetone attracted the pollinators (moth and hummingbirds). Methyl salicylate repelled hummingbirds and ants but attracted moths. Although studies on nectar filter so far are limited, they have clearly shown that the nectar can act as one of the effective filters favoring some floral visitors and deterring others (Kessler and Baldwin 2006, 2011).

Pollen Filters

Nutritional quality of pollen is highly variable; some of them lack several essential nutrients and some are poor in proteins (Roulston and Cane 2000; Rasmont et al. 2005) and yet others contain secondary compounds which

are repellent or toxic to insects (Pimentel De Carvalho and Message 2004; see Hargreaves et al. 2009; Sedivy et al. 2011). There is much evidence to indicate that pollen can act as a filter to select floral visitors.

Several studies have analyzed pollen loads of bee species to check the extent of their host specificity. Pollen load analyses of 35 species of the genus *Chelostoma* (Megachilidae) revealed that 33 species to be pollen specialists at the level of plant family or genus (Sedivy et al. 2008). In another study on pollen load analyses, Muller and Kuhlmann (2008) reported that 26 species of the genus *Colletes* (Colletidae) out of the 60 species analyzed, were specialists at the level of plant family, subfamily or genus and the remaining 34 species were pollen generalists to varying degrees, visiting the flowers of up to 15 plant families. Fourteen of the specialist species were found to harvest exclusively or predominantly the pollen of Asteroideae. However of the 34 pollen generalist species Asteroid pollen formed a very small proportion (<3%) of the pollen load of only seven species; pollen loads of the remaining 27 generalist species did not contain pollen of Asteroidae flowers. Thus, pollen of Asteroideae seems to be unfavourable to a majority of generalist bees and those which use Asteroideae pollen may have developed physiological adaptations to digest this pollen. Thus host choices of pollen foragers may be physiologically constrained. The bees obviously select the hosts based on digestibility of their pollen. In a recent study Leonhardt and Blüthgen (2012) investigated the pollen feeding patterns of honeybees and bumblebees. Although the colonies of both the bees were located at the same site, they differed in the range of plant species visited and nutritional quality of the pollen collected. Bumblebees generally collected pollen with higher protein content with more essential amino acids.

Several experimental studies have shown that bees, when offered pollen of several species, prefer pollen of some species over others. In one of the early studies, Schmidt (1982) reported that honeybee species, *Apis mellifera*, preferred pollen of almond over desert broom, saguaro (*Carnegiea* sp.), and Creosote (*Larrea* sp.). In another set of experiments, the pollen of maple was preferred over that of cottonwood (*Populus* sp.), dandelion (*Taraxacum* sp.) and pine. The amount of pollen removed by *A. mellifera* from preferred species was significantly higher when compared to pollen of others. Thus this intrinsic ability of the floral visitors to discriminate pollen of different species can act as a filter.

Some studies have been carried out on the effects of feeding host and non-host pollen to bee species on the development of their larvae. Pollen of *Sinapis arvensis* (Brassicaceae) and *Echium vulgare* (Boraginaceae) failed to support larval development of *Colletes* bee species specialized on pollen of *Campanula* (Praz et al. 2008a). Similarly, pollen of Asteraceae and Ranunculaceae permitted larval growth of only those bee species that are specialized to harvest pollen from plants belonging to these families; their

pollen failed to support larval growth of other bee species. Recognition of host pollen in specialized species seems to be genetically determined (Praz et al. 2008b). They compared the floral preferences of one of specialized bee species, *Heriades fruncorum* (Megachiledae, specialized on Asteraceae pollen), reared on host and non-host pollen. Bees restricted pollen collection to host flowers irrespective of whether they were reared on host or non-host pollen.

Recently, Sedivy et al. (2011) studied larval performance of two generalist solitary bees, *Osmia bicornis* and *O. cornuta* on pollen diet of four plant species. The larvae of *O. bicornis* developed well on pollen diet of *Ranunculus* but failed to develop on *Echium* pollen; it was the reverse for the larvae of *O. cornuta*. Larvae of both the species developed well on pollen of *Sinapis*, but failed to develop on pollen of *Tanacetum vulgare* (Asteraceae). These studies clearly indicate that palatability of pollen can act as an effective filter to restrict the number of floral visitors. Pollen of non-host species may hamper the digestion of the larvae and the bees seem to have adapted their metabolism to digest pollen of their host species.

Concluding Remarks

Enormous information has accumulated over the years on pollination ecology but our understanding on several aspects in this field is far from complete (Mayer et al. 2011). Although biotic pollination evolved in a few gymnosperms and is present in several extant species, it is not refined and may not be more efficient than wind pollination prevalent in a majority of gymnosperms. Lack of specialization in attracting a wider range of pollinators resulting in inefficient pollination may be one of the primary reasons for lack of diversification in entamophilous gymnosperms. Evolution of the flower in angiosperms with nectar as a reward enhanced floral attraction leading to marked improvement in the frequency and consistency of the pollinators' visits. Apart from enhanced pollination efficiency, other innovations of the flower particularly the pistil, and evolution of zygomorphy with its further elaboration leading to floral specialization seems to have contributed to reproductive success and dramatic diversification of angiosperms.

The central focus on biotic pollination is the ability of plants to achieve conflicting demands of attraction and restriction of animal species for pollination services. A major limitation of the enormous data accumulated on biotic pollination is the lack of clear distinction between the pollinator(s) and floral visitors in most of the investigations. It is important for pollination biologists to confirm pollinators and their efficiency amongst floral visitors on the basis of pollen transfer to the stigma. Also, biotic pollination is dynamic and shows temporal and spatial variations. It is desirable to study pollination at different locations and during the entire duration of

the flowering period for a clear understanding of pollinators' variability in time and space and its impact on pollination success. Studies on biotic pollination have become more complicated in the light of the reports that many insect pollinators, in the absence of preferred flowers in the habitat, are able to exploit other floral resources by associative learning of floral odors and/or colours with the reward (Riffell 2011). There are even reports of honeybees visiting the flowers of wind-pollinated species to harvest their nutritive pollen source when insect pollinated plants are not available (Keller et al. 2005; Hocheri et al. 2012).

Our understanding on various floral attractants and their role in pollinators' attraction, and the details of various floral rewards is comprehensive. Considerable information is also available on deceptive pollination. Restriction of floral visitors is as important as their attraction for an efficient pollination system to work. The role of morphological and fragrance filters in specialized flowers that limit the number of animal species with matching features to harvest the reward and consequently eliminate others that are incapable of harvesting the rewards are well documented. The factors responsible for restricting the number of visitors to 'open flowers' seems to be more subtle. Although several recent studies have indicated the role of fragrance, nectar and pollen acting as filters in restricting animal visitors, such studies are limited and they need to be extended to a larger number of species. The role of nectar components particularly the secondary metabolites, which was largely ignored so far, needs to be investigated with more vigour. The role of pollen, its quality, digestibility and toxicity seem to play a important role in attracting/restricting potential pollinators. Interestingly most of the pollen species that have been found to be unfavorable for the development of larvae of several bee species happen to have flowers with fully accessible pollen (Sedivy et al. 2011 and references therein) indicating that pollen filter may be primarily responsible for limiting the number of visitors to open flowers.

Available evidence clearly indicates that a combination of floral traits —floral morphology, its fragrance, the quality and quantity of both nectar and pollen rewards—seem to be involved in achieving the dual function of attraction and restriction of floral visitors. The role of each of these may vary between species. Hopefully future studies would try to dissect these functions with appropriate techniques for a better understanding of the role of different components of the flower, the most innovative organ of angiosperms, in achieving the dual functions.

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References

Abrahamczyk, S., J. Kluge, Y. Gareca, S. Reichle and M. Kessler. 2011. The influence of climatic seasonality on the diversity of different tropical pollinator groups. PLoS ONE 6(11): e27115. doi:10.1371/journal.pone.0027115.

Ackerman, J.D. 1983. Specificity and mutual dependency of the orchid-euglossine bee interaction. Biol. J. Linnean Soc. 20: 301–314.

Adler, L.S. 2000. The ecological significance of toxic nectar. Oikos 91: 409–420.

Adler, L.S. and R.E. Irwin. 2005. Ecological costs and benefits of defenses in nectar. Ecology 86: 2968–2978.

Aguilar, R., L. Ashworth, L. Galettoand and M.A. Aizen. 2006. Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. Ecol. Lett. 9: 986–980.

Alvarez-Perez, S., C.M. Herrera and C. de Vega. 2012. Zooming-in on floral nectar: a first exploration of nectar-associated bacterial communities. FEMS Microbiol. Ecol. 80: 591–602.

Ananthakrishnan, T.N. 1993. The role of thrips in pollination. Curr. Sci. 65: 262–264.

Andersson, S. and H.E.M. Dobson. 2003. Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. J. Chem. Ecol. 29: 2303–2318.

Ando, T., M. Nomura, J. Tsukahara, H. Watanabe et al. 2001. Reproductive isolation in a native population of *Petunia sensu* Jussieu (Solanaceae). Ann. Bot. 88: 403–413.

Anonymous. 2009. Special Issue: Plant-pollinator interactions, Annals of Botany 103: 1355–1600.

Anonymous. 2012. http://en.wikipedia.org/wiki/Eusociality. (accessed on 30 Jan 2013).

Armbruster, W.S. 2012. Evolution and ecological implications of "specialized" pollinator rewards. *In*: S. Patiny (ed.). Evolution of Plant-Pollinator Relationships. The Systematic Association Special Vol. 81, Cambridge Univ. Press, New York, pp. 44–57.

Armbruster, W.S., E.M. Debevec and M.F. Willson. 2002. Evolution of syncarpy in angiosperms: theoretical and phylogenetic analyses of the effects of carpel fusion on offspring quantity and quality. J. Evol. Biol. 15: 657–672.

Armstrong, T.T., R.G. Fitzjohn, L.E. Newstrom et al. 2005. Transgenic escape. What potential for crop-wild hybridization. Mol. Ecol. 14: 2111–2132.

Ayasse, M., F.P. Schiestl, H.F. Paulus, C. Lofstedt et al. 2000. Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes*: How does flower-specific variation of odor signals influence reproductive success? Evolution 54: 1995–2006.

Ayasse, M., F.P. Schiestl, H.F. Paulus, F. Ibarra and W. Francke. 2003. Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. Proc. R Soc. B 270: 517–522.

Baker, H.G. 1977. Non-sugar chemical constituents of nectar. Apidologie 8: 349–356.

Baker, H.G. and I. Baker. 1983. Floral nectar sugar constituents in relation to pollinator type. *In*: C.E. Jones and R.J. Little (eds.). Handbook of Experimental Pollination Biology. Scientific and Academic Editions, Van Nostrand Reinhold Co. Inc., New York, pp. 117–141.

Balao, F., J. Herrera, T.S. Salvador and S. Dotterl. 2011. Spatial and temporal patterns of floral scent emission in *Dianthus innoxia* and electroantennographic responses of its hawk moth pollinator. Phytochemistry 72: 601–609.

Bawa, K.S. 1990. Plant-pollinator interactions in tropical rain forests. Ann. Rev. Ecol. Syst. 21: 399–422.

Beattie, A.J. 1985. The evolutionary ecology of ant-plant mutualisms. Cambridge University Press. Cambridge.

Beattie, A.J., C.L. Turnbull, R.B. Knox and E.G. Williams. 1984. Ant inhibition of pollen function: A possible reason why ant pollination is rare. Am. J. Bot. 71: 421–426.

Biesmeijer, J.C., S.P.M. Roberts, M. Reemer and R. Ohlemuller. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351–354.

Bernklau, E.J. 2012. Chemical mimicry in pollination. http://www.interlibrary.narod.ru/GenCat/GenCat.Scient.Dep/GenCatEcology/400000001/400000001.htm (downloaded 12 February 2012).

Bestmann, H.J., L. Winkler and O. von Helversen. 1997. Headspace analysis of volatile flower scent constituents of bat-pollinated plants. Phytochemistry 46: 1169–1172.

Borg-Karlson, A.K. 1990. Chemical and ethological studies of pollination in the genus *Ophrys* (Orchidaceae). Phytochemistry 29: 1359–87.

Bronstein, J.L., R. Ruben Alarcon and M. Geber. 2006. Tansley review: The evolution of plant–insect mutualisms. New Phytol. 172: 412–428.

Buchmann, S.L. 1987. The ecology of oil flowers and their bees. Ann. Rev. Ecol. Syst. 18: 343–369.

Burkle, L.A., J.C. Marlinand and T.M. Knight. 2013. Plant-pollinator interactions over 120 years: Loss of species, co-occurrence, and function. Science 339: 1611–1615.

Cappellari, S.C., H. Schaefer and C.C. Davis. 2013. Evolution: pollen or pollinators—which came first? Curr. Biol. 23: http://dx.doi.org/10.1016/j.cub.2013.02.049.

Cardinal, S. and B.N. Danforth. 2013. Bees diversified in the age of eudicots. Proc. R Soc. Biol. Sci. 280: 20122686.

Chen, D., J.S. Collins and T.H. Goldsmith. 1984. The ultraviolet receptor of bird retinas. Science 225: 337–340.

Chen, C., Q. Song, M. Proffit, J.-M. Bessiere, Li Z. Zongbo and M. Hossaert-McKey. 2009. Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*. Functional Ecol. 23: 941–950.

Chittka, L. and F. Peng. 2013. Caffeine boosts bees' memories. Science 339: 1157–1159.

Cook, J.M. and J.Y. Rasplus. 2003. Mutualists with attitude: coevolving fig wasps and figs. Trends Ecol. Evol. 18: 241–248.

Crane, P.E., M.F. Friis and K.R. Pedersen. 2000. The origin and early diversification of angiosperms. *In*: H. Gee (ed.). Shaking the Tree: Readings from Nature in the History of Life. Univ. Chicago Press, Ill. USA, pp. 233–250.

Cresswell, J. and A. Smithson. 2005. Artificial flowers and pollinators in pollination research. *In*: A. Dafni, P.G. Kevan and B.C. Husband (eds.). Practical Pollination Biology, Enviroquest, Ltd, Cambridge, Ontario, Canada, pp. 340–344.

Cruaud, A., J. Cook, Y. Da-Rong, G. Genson et al. 2012. Fig-fig wasp mutualism: the fall of the strict conspecific paradigm? *In*: S. Patiny (ed.). Evolution of Plant-Pollinator Relationships. The Systematics Association Special Vol. 81. Cambridge Univ. Press, New York, pp. 68–102.

Dafni, A. 1984. Mimicry and deception in pollination. Ann. Rev. Ecol. Syst. 15: 259–278.

Dafni, A., P.G. Kevan and B.C. Husband. 2005. Practical Pollination Biology. Enviroquest, Ltd. Cambridge, Ontario, Canada.

Dafni, A., T. Marom-Levy, A. Jurgens, S. Dotterl, Y. Shimrat et al. 2012. Ambophily and "super generalization" in *Ceratonia siliqua* (Fabaceae) pollination. *In*: S. Patiny (ed.). Evolution of Plant-Pollinator Relationships. The Systematics Association Special Vol. 81. Cambridge Univ. Press, Cambridge, pp. 345–373.

Dar, S., Ma del C. Arizmendi and A. Valiente-Banuet. 2006. Diurnal and nocturnal pollination of *Marginatocereus marginatus* (Pachycereeae: Cactaceae) in Central Mexico. Ann. Bot. 97: 423–427.

Davis, L.E., A.S. Stephenson and J.A. Winsor. 1987. Pollen competition improves performance and reproductive output of the common zucchini squash under field conditions. J. Am. Soc. Hort. Sci. 112: 712–716.

de Nettencourt, D. 2001. Incompatibility and Incongruity in Wild and Cultivated Plants. Springer, Heidelberg.

Dettner, K. and C. Liepert. 1994. Chemical mimicry and camouflage. Ann. Rev. Entomol. 39: 129–54.
de Vega, C. and C.M. Herrera. 2013. Microorganisms transported by ants induce changes in floral nectar composition of an ant-pollinated plant. Am. J. Bot. 100: 792–800.
de Vega, C., C.M. Herrera and S.D. Johnson. 2009. Yeasts in floral nectar of some South African plants: Quantification and associations with pollinator type and sugar concentration. South African J. Bot. 75: 798–806.
de Wall, C., B. Anderson and C.H. Barrett. 2012. The natural history of pollination and mating in bird-pollinated *Babiana* (Iridaceae). Ann. Bot. 109: 667–679.
Dobson, H.E.M. 1988. Survey of pollen and pollenkitt lipids—chemical cues to flower visitors? Am. J. Bot. 75: 170–182.
Dobson, H.E.M. and G. Bergstrom. 1996. Pollen-advertisement: chemical contrasts between whole flower and pollen odors. Am. J. Bot. 83: 877–885.
Dobson, H.E.M. and G. Bergstrom. 2000. The ecology and evolution of pollen odors. Plant Syst. Evol. 222: 63–87.
Dobson, H.E.M., E.M. Danielson and I.D. van Wesep. 1999. Pollen odor chemicals as modulators of bumble bee foraging on *Rosa rugosa* Thunb. (Rosaceae). Plant Species Biol. 14: 153–166.
Dobson, H.E.M., R.A. Raguso, J.T. Knudsen and M. Ayasse. 2005. Scent as an attractant. *In*: E. Dafni, P.G. Kevan and B.C. Husband (eds.). Practical Pollination Biology. Enviroquest, Ltd, Ontario, Canada, pp. 197–230.
Dresler, R.L. 1982. Biology of the orchid bee (Euglossini). Ann. Rev. Ecol. Syst. 13: 373–394.
Dudareva, N. and E. Pichersky. 2000. Biochemical and molecular aspects of floral scents. Plant Physiol. 122: 627–634.
Dudareva, N., B. Piechulla and E. Pichersky. 1999. Biogenesis of floral scent. Hort. Rev. 24: 31–54.
Dunn, D.W., S.T. Segar, J. Ridley, R. Chan et al. 2008. A Role for parasites in stabilising the fig-pollinator mutualism. PLoS Biol. 6(3): e59. doi: 10.1371/journal.pbio.0060059.
Dyer, F. 1985. Nocturnal orientation by the Asian honey bee, *Apis dorsata*. Animal Behav. 33: 769–774.
Endress, P.K. 2010. The evolution of floral biology in basal angiosperms. Phil. Trans. R Soc. B. 365: 411–421.
Endress, P.K. 2011. Evolutionary diversification of flowers in angiosperms. Am. J. Bot. 98: 370–396.
Faegri, K. and L. van der Pijl. 1971. The Principles of Pollination Ecology. 2 Edition, Pergamon Press, Oxford.
Falara, V., R. Amarasinghe, J. Poldy, E. Pichersky, R.A. Barrow and R. Peakall. 2013. The production of a key floral volatile is dependent on UV light in a sexually deceptive orchid. Ann. Bot. 111: 21–30.
Fenster, C.B., W.S. Armbruster, P. Wilson, M.R. Dudash and J.D. Thomson. 2004. Pollination syndromes and floral specialization. Ann. Rev. Ecol. Syst. 35: 375–403.
Fleming, T.H. and J.N. Holland. 1998. The evolution of obligate pollination mutualisms: senita cactus and senita moth. Oecologia 114: 368–375.
Fleming, T.H., C. Geiselman and W.J. Kress. 2009. The evolution of bat pollination: a phylogenetic perspective. Ann. Bot. 104: 1017–1043.
Ford, H.A., D.C. Patonand and N. Forde. 1979. Birds as pollinators of Australian plants. New Zealand J. Bot. 17: 509–519.
Frankie, G.W., P.A. Opler and K.S. Bawa. 1976. Foraging behavior of solitary bees: implications for outcrossing of a neotropical forest tree species. J. Ecol. 64: 1049–1055.
Friedman, W.E. 2009. The meaning of Darwin's 'abominable mystery'. Am. J. Bot. 96*:* 5–21.
Fridman, S., I. Izhaki, Y. Gerchman and M. Halpern. 2012. Bacterial communities in floral nectar. Environ. Microbiol. Rep. 4: 97–104.

Galizia, C.G., J. Kunze, A. Gumbert, A.-K. Borg-Karlson et al. 2005. Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system. Behav. Ecol. 16: 159–168.

Garcia-Fayos, P. and A. Goldarazena. 2008. The role of thrips in pollination of *Arctstaphyllos uva-ursi*. Int. J. Plant Sci. 169: 776–781.

Garibaldi, L.A., I. Steffan-Dewenter, R. Winfree, M.A. Aizen, R. Bommarco et al. 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science 339: 1608–1611.

Ghara, M., L. Kundanati and R.M. Borges. 2011. Nature's Swiss army knives: Ovipositor structure mirrors ecology in a multitrophic fig wasp community. PLoS ONE 6(8): e23642. doi:10.1371/journal.pone.0023642.

Ghazoul, J. and D. Sheil. 2010. Tropical Rain Forest Ecology, Diversity, and Conservation. Oxford Univ. Press, New York.

Gibson, A.C. 2001. Newsletter Vol. 4, Mildred E. Mathias Botanical Gardens, Univ. Calif., Los Angeles. Fall http://www.botgard.ucla.edu/html/MEMBGNewsletter/Volume4number4/Batsandtheirflowers.html.

Gigord, L.D.B., M.R. Macnair, M. Stritesky and A. Smithson. 2002. The potential for floral mimicry in rewardless orchids: an experimental study. Proc. R Soc. B 269: 1389–1395.

Gori, D.F. 1983. Post-pollination phenomena and adaptive floral changes. *In*: C.E. Jones and R.J. Little (eds.). Handbook of Experimental Pollination Biology, Scientific and Academic Editions, van Nostrand Reinhold Company Inc., New York, pp. 31–49.

Guerenstein, P.G., A.E. Yepez, J. van Haren, D.G. Williams and J.G. Hildebrand. 2004. Floral CO_2 emission may indicate food abundance to nectar-feeding moths. Naturwissenschaften 91: 329–333.

Hansen, D.M., K. Beer and C.B. Muller. 2006. Mauritian coloured nectar no longer a mystery: a visual signal for lizard pollinators. Biol. Lett. 2: 165–168.

Hansen, D.M., J.M. Olesen, T. Mione, S.D. Johnson and C.B. Müller. 2007. Coloured nectar: distribution, ecology, and evolution of an enigmatic floral trait. Biol. Rev. 82: 83–111.

Hansen, D.M., T. van der Niet and S.D. Johnson. 2012. Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. Proc. R Soc. B 279: 634–639.

Hargreaves, A.L., L.D. Harder and S.D. Johnson. 2009. Consumptive emasculation: the ecological and evolutionary consequences of pollen theft. Biol. Rev. 84: 259–276.

Harrison, R.D. and M. Shanahan. 2005. Seventy-seven ways to be a fig: Overview of a diverse plant assemblage. *In*: D.W. Roubik, S. Sakai and A.A.H. Karim (eds.). Pollination Ecology and the Rain Forest: Sarawak Studies. Springer, New York, pp. 111–127.

Heil, M. 2011. Nectar: generation, regulation and ecological function. Trends Plant Sci. 16: 191–200.

Herrera, C.M., C. de Vega, A. Canto and M.I. Pozo. 2009. Yeasts in floral nectar: a quantitative survey. Ann. Bot. 103: 1415–1423.

Hickman, J.C. 1974. Pollination by ants: a low-energy system. Science 184: 1290–1292.

Hocheri, N., R. Siede, I. Illies, H. Gatschenberger and J. Tautz. 2012. Evaluation of the nutritive value of maize for honey bees. J. Insect Physiol. 58: 278–285.

Hossaert-McKey, M., M. Gibernau and J.E. Frey. 1994. Chemosensory attraction of fig wasps to substances produced by receptive figs. Entomologia Experimentalis et Applicata 70: 185–191.

Howell, A.D. and R. Alarcon. 2007. *Osmia* bees (Hymenoptera: Megachilidae) can detect nectar-rewarding flowers using olfactory cues. Animal Behav. 74: 199–205.

Hu, S., D.L. Dilcher and D.W. Taylor. 2012. Pollen evidence for the pollination biology of early flowering plants. *In*: S. Patiny (ed.). Evolution of Plant-Pollinator Relationships. The Systematic Association Special Vol. 81. Cambridge Univ. Press, New York, pp. 165–236.

Huber, F.K., R. Kaiser, W. Sauter and F.P. Schiestl. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). Oecologia 142: 564–75.

Ivri, Y. and A. Dafni. 1977. The pollination ecology of *Epipactis consimilis* Don (Orchidaceae) in Israel. New Phytol. 79: 173–177.
James, R.R. and T.L. Pitts-Singer. 2008. Bee Pollination in Agricultural Ecosystems. Oxford Univ. Press, New York.
Janzen, D.H. 1964. Notes on the behavior of four subspecies of the carpenter bee, *Xylocopa* (*Notoxylocopa*) *tabaniformis*, in Mexico. Ann. Ent. Soc. America. 57: 296–301.
Jersakova, J., S.D. Johnson and P. Kindlmann. 2006. Mechanisms and evolution of deceptive pollination in orchids. Biol. Rev. 81: 219–235.
Johnson, S.D. 1994. Evidence for Batesian mimicry in a butterfly-pollinated orchid. Biol. J. Linnean Soc. 53: 91–104.
Johnson, S.D. and A. Dafni. 1998. Response of bee-flies to the shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. Functional Ecol. 12: 289–297.
Johnson, S.D., L. Anna, A.L. Hargreaves and M. Brown. 2006. Dark bitter-tasting nectar functions as a filter of flower visitors in a bird-pollinated plant. Ecology 87: 2709–2716.
Johnson, S.D., P.M. Burgoyne, L.D. Harder and S. Dotterl. 2011. Mammal pollinators lured by the scent of a parasitic plant. Proc. R. Soc. B 278: 2303–2310.
Jousselin, E., S. van Noort, V. Berry, J.-Y. Rasplus, N. Ronsted et al. 2008. One fig to bind them all: host conservatism in a fig wasp community unraveled by cospeciation analyses among pollinating and nonpollinating fig wasps. Evolution 62: 1777–1797.
Junker, R.R. and N. Bluthgen. 2010. Floral scents repel facultative flower visitors, but attract obligate ones. Ann. Bot. 105: 777–782.
Jurgens, A., T. Witt and G. Gottsberger. 2002. Flower scent composition in night-flowering *Silene* species (Caryophyllaceae). Biochem. Syst. Ecol. 30: 383–397.
Kearns, A. and D.W. Inouye. 1993. Techniques for Pollination Biologists. Univ. Press Colorado, Colarado.
Kelber, A., E.J. Warrant, M. Pfaff, R. Wallen, J.C. Theobald, W. Wcislo and R. Raguso. 2006. Light intensity limits the foraging activity in nocturnal and crepuscular bees. Behav. Ecol. 17: 63–72.
Keller, I., P. Fluri and A. Imdorf. 2005. Pollen nutrition and colony development in honey bees: part I. Bee World 86: 3–10.
Kessler, D. and I.T. Baldwin. 2006. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuate*. The Plant J. 49: 840–854.
Kessler, D. and I.T. Baldwin. 2011. Back to the past for pollination biology. Curr. Opinion Plant Biol. 14: 429–434.
Kevan, P.G. 1983. Floral colours through the insect eye: what they are and what they mean. *In*: C.E. Jones and R.J. Little (eds.). Handbook of Experimental Pollination Biology. Scientific and Academic Editions, van Nostrand Reinhold Company Inc., New York, pp. 3–29.
Khadari, B., M. Gibernau, M.C. Anstett, F. Kjellberg and M. Hossaert-McKey. 1995. When figs wait for pollinators: the length of fig receptivity. Am. J. Bot. 82: 992–999.
Knight, T.M., J.A. Steets, J.C. Vamosi, S.J. Mazer et al. 2005. Pollen limitation of plant reproduction: pattern and process. Ann. Rev. Ecol. Evol. Syst. 36: 467–497.
Knudsen, J.T., L. Tollsten and G. Bergstrom. 1993. Floral scents: a checklist of volatile compounds isolated by head-space techniques. Phytochemistry 33: 253–280.
Knudsen, J., R. Eriksson, J. Gershenzon and B. Ståhl. 2006. Diversity and distribution of floral scent. Bot. Rev. 72: 1–120.
Kolosova, N., N. Gorenstein, C.M. Kish and N. Dudareva. 2001. Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. Plant Cell 13: 2333–2347.
Kullenberg, B. 1956. Field experiments with chemical sexual attractants on aculeate hymenopteran males. I. Zool. Bidrag. Uppsala 31: 253–352.
Lankinen, A. and J.A. Madjidian. 2011. Enhancing pollen competition by delaying stigma receptivity: Pollen deposition schedules affect siring ability, paternal diversity, and seed production in *Collinsia heterophylla* (Plantaginaceae). Am. J. Bot. 98: 1191–1200.

Leonard, A.S. and D.R. Papaj. 2011. 'X' marks the spot: The possible benefits of nectar guides to bees and plants. Functional Ecol. 25: 1–9.
Leonhardt, S.D. and N. Bluthgen. 2012. The same, but different: pollen foraging in honeybee and bumblebee colonies. Apidologie 43: 449–464.
Lewis, A. 1986. Memory constraints and flower choice in *Pieris rapae*. Science 232: 863–865.
Linsley, E.G., J.W. MacSwain and R.F. Smith. 1955. Biological observations on *Xenoglossa fulva* Smith with some generalizations on biological characters of other eucerine bees. Bull. South Calif. Acad. Sci. 54: 128–141.
Liu, F., J. Chen, J. Chai, X. Zhang et al. 2007. Adaptive functions of defensive plant phenolics and a non-linear bee response to nectar components. Functional Ecol. 21: 96–100.
Mach, J. 2012. Transcription factors and Darwin's "abominable mystery": Positive autoregulation in floral zygomorphy. Plant Cell 24: S 1712.
Machado, C.A., N. Robbins, M.T.P. Gilbert and E.A. Herre. 2005. Critical review of host specificity and its coevolutionary implications in the fig-fig-wasp mutualism. Proc. Natl. Acad. Sci. USA 102: 6558–565.
Magallon, S. and A. Castillo. 2009. Angiosperm diversification through time. Am. J. Bot. 96: 349–365.
Marler, T.E. 2010. Cycad mutualist offers more than pollen transport. Am. J. Bot. 97: 841–845.
Mayer, C., L. Alder, W.S. Armbruster, A. Dafni and C. Eardley. 2011. Pollination ecology in the 21st century: Key questions for future research. J. Poll. Ecol. 3: 8–23.
Medel, R., C. Botto-Mehan and M. Kalin-Arrayo. 2003. Pollinator mediated selection on the nectar guide phenotype in the Andean monkey flower *Mimulus luteus.* Ecology 84: 1721–1732.
Meeuse, B.J.D. 1978. The physiology of some sapromyophilous flowers. *In*: A.J. Richards (ed.). The Pollination of Flowers by Insects. Linnean Soc. Symp. No. 6. Academic Press, London, pp. 97–104.
Memmott, J., P.G. Craze, N.M. Waser and M.V. Price. 2007. Global warming and the disruption of plant-pollinator interactions. Ecol. Lett. 10: 710–717.
Minckley, R.L. and T.H. Roulston. 2006. Incidental mutualisms and pollen specialization among bees. *In*: N.M. Waser and J. Ollerton (eds.). Plant-Pollinator Interactions: From Specialization to Generalization. Univ. Chcago Press, Chicago, pp. 69–98.
Mitchell, R.J., R.F. Irwin, R.J. Flanagan and J.D. Karron. 2009. Ecology and evolution of plant-pollinator interactions. Ann. Bot. 103: 1355–1363.
More, M., A.N. Sersic and A.A. Cocucci. 2007. Restriction of pollinator assemblage through flower length and width in three long-tongued hawkmoth-pollinated species of *Mandevilla* (Apocynaceae, Apocynoideae). Ann. Missouri Bot. Gard. 94: 455–504.
Muchhala, N., A. Caiza, J.C. Vizeute and J.D. Thomson. 2009. A generalized pollination system in the tropics: bats, birds and *Aphelandra acanthus.* Ann. Bot. 103: 1481–1487.
Mulcahy, D.L. 1979. The raise of flowering plants: geneecological factor. Science 206: 20–23.
Mulcahy, D.L. and G.B. Mulcahy. 1987. The effects of pollen competition. Am. Sci. 75: 44–50.
Muller, A. and M. Kuhlmann. 2008. Pollen hosts of western palaearctic bees of the genus *Colletes* (Hymenoptera: Colletidae): the Asteraceae paradox. Biol. J. Linnean Soc. 95: 719–733.
Murugan, R., K.R. Shivanna and R.R. Rao. 2006. Pollination biology of *Aristolochia tagala*, a rare species of medicinal importance. Curr. Sci. 91: 795–798.
Nagamitsu, T. and T. Inoue. 1997. Cockroach pollination and breeding system of *Uvaria elmeri* (Annonaceae) in a lowland mixed-dipterocarp forest in Sarawak. Am. J. Bot. 84: 208–213.
Nedft, R.J. and S.G. Compton. 1996. Regulation of seed and pollinator production in the fig-fig wasp mutualism. J. Animal Ecol. 65: 170–182.
Newman, E., B. Anderson and S.D. Johnson. 2012. Flower colour adaptation in a mimetic orchid. Proc. R Soc. B 279: 2309–2313.
Nicolson, S.W. and R.W. Thornburg. 2007. Nectar chemistry. *In*: S. Nicolson et al. (eds.). Nectaries and Nectar. Springer, Heidelberg, pp. 215–263.

Nilsson, L.A. 1988. The evolution of flower with deep corolla tubes. Nature 334: 147–149.
Okuyama ,Y., M. Kato and N. Murakami. 2004. Pollination by fungus gnats in four species of the genus *Mitella* (Saxifragaceae). Bot. J. Linnean Soc. 144: 449–460.
Olesen, J.M. and A. Valido. 2003. Lizards as pollinators and seed dispersers: an island phenomenon. Trends Ecol. Evol. 18: 177–181.
Ollerton, J. 2006. "Biological barter": Patterns of specialization compared across different mutualisms. *In*: N.W. Waser and J. Ollerton (eds.). Plant-Pollinator Interactions; From Specialization to Generalization. Univ. Chcago Press, Chicago, pp. 411–435.
Ollerton, J., R. Winfree and S. Tarrant. 2011. How many flowering plants are pollinated by animals? Oikos 120: 321–326.
Omura, H., H.K. Keiichi and N. Hayashi. 2000. Floral scent of *Osmanthus fragrans* discourages foraging behavior of cabbage butterfly, *Pieris rapae*. J. Chem. Ecol. 26: 655–666.
Ortega-Olivencia, A., T. Rodriguez-Riano, J.L. Perez-Bote, J. Lopez et al. 2012. Insects, birds and lizards as pollinators of the largest-flowered *Scrophularia* of Europe and Macaronesia. Ann. Bot. 109: 153–167.
Patiny, S. (ed.). 2012. Evolution of Plant-Pollinator Relationships. The Systematics Association Special Vol. 81, Cambridge Univ. Press, Cambridge.
Pauw, A. 2006. Floral syndromes accurately predict pollination by specialized oil collecting bee (*Rediviva peringueyi*, Melittodae) in a guild of South African orchids (Coryciinae). Am. J. Bot. 93: 917–926.
Peakall, R. 1989. The unique pollination of *Leporella fimbriata* (Orchidaceae): Pollination by pseudocopulating male ants (*Myrmecia urens*, Formicidae). Plant Syst. Evol. 167: 137–148.
Peakall, R. and A.J. Beattie. 1989. Pollination of the orchid *Microtis parviflora* R. Br. by flightless worker ants. Functional Ecol. 3: 515–522.
Pellmyr, O. 2002. Pollination by animals. *In*: C.M. Herrera and O. Pellmyr (eds.). Plant-Animal Interactions: An Evolutionary Approach. Lackwell Publishers, Malden, MA; Oxford, UK, pp. 157–184.
Pellmyr, O. 2003. Yuccas, yucca moths, and coevolution: A review. Ann. Missouri Bot. Gard. 90: 35–55.
Pellmyr, O. and C.J. Huth. 1994. Evolutionary stability of mutualism between yucca and yucca moths. Nature 372: 257–260.
Pellmyr, O. and L.B. Thien. 1987. Insect reproduction and floral fragrance: keys to the evolution of angiosperms. Taxon 35: 76–85.
Pellmyr, O., W. Tang, I. Groth, G. Bergstrom et al. 1991. Cycad cone and angiosperm floral volatiles: Inferences for the evolution of insect pollination. Biochem. Syst. Ecol. 19: 623–627.
Pemberton, R.W. and H. Liu. 2008. Naturalized orchid bee pollinates resin reward flowers in Florida: novel and known mutualism. Biotropica 40: 714–718.
Pimentel De Carvalho, A. and D. Message. 2004. A scientific note on the toxic pollen of *Stryphnodendron polyphyllum* (Fabaceae, Mimosoideae) which causes sacbrood-like symptoms. Apidologie 35: 89–90.
Pleasants, J.M. 1983. Structure of plant and pollinator communities. *In*: C.E. Jones and R.J. Little (eds.). Handbook of Experimental Pollination Biology. Scientific and Academic Editions, van Nostrand Reinhold Company Inc., New York, pp. 375–393.
Potts, S.G., J.C. Biesmeijer, C. Kremen, P. Neumann et al. 2009. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25: 345–353.
Powell, J.A. 1992. Interrelationships of yucca and yucca moths. Trends Ecol. Evol. 7: 10–15.
Pozo, M.I., C. de Vega, A. Canto and C.M. Herrera. 2009. Presence of yeasts in floral nectar is consistent with the hypothesis of microbial-mediated signaling in plant-pollinator interactions. Plant Signal Behav. 4: 1102–1104.
Prance, G.T. and J.R. Arias. 1975. A study of the floral biology of *Victoria amazonica* (Poepp.) Sowerby (Nymphaeaceae). Acta Amazonica 5: 109–139.

Praz, C.J., A. Müller and S. Dorn. 2008a. Specialized bees fail to develop on non-host pollen: Do plants chemically protect their pollen. Ecology 89: 795–804.
Praz, J.C., A. Muller and S. Dorn. 2008b. Host recognition in a pollen-specialist bee: evidence for a genetic basis. Apidologie 39: 547–557.
Proffit, M., B. Schatz, J.-M. Bessiere, C. Chen, C. Soler and M. Hossaert-Mckey. 2008. Signaling receptivity: comparison of the emission of volatile compounds by figs of *Ficus hispida* before, during and after the phase of receptivity to pollinators. Symbiosis 45: 15–24.
Proffit, M., C. Chen, C. Soler, J.-M. Bessiere, B. Schatz and M. Hossaert-McKey. 2009. Can chemical signals responsible for mutualistic partner encounter promote the specific exploitation of nursery pollination mutualisms?—The case of figs and fig wasps. Entomologia Experimentalis et Applicata 131: 46–57.
Raguso, R.A. 2004. Why are some floral nectars scented? Ecology 85: 1486–1494.
Raguso, R.A., A. Mark and M.A. Willis. 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta.* Animal Behaviour 64: 685–695.
Raine, N.E. and L. Chitka. 2007. Flower constancy and memory dynamics in Bumblebees (Hymenoptera: Apidae: Bombus). Entomol. Gener. 29: 179–199.
Ramya, K.T., R.A. Fiyaz, R. Uma Shaanker and K.N. Gneshaiah. 2011. Pollinators for a syconium: How do wasps choose among syconia. Curr. Sci. 101: 520–527.
Rasmont, P., A. Regali, T.C. Ings, G. Lognay, E. Baudart et al. 2005. Analysis of pollen and nectar of *Arbutus unedo* as a food source for *Bombus terrestris* (Hymenoptera: Apidae). J. Econ. Entomol. 98: 656–663.
Renner, S.S. 2006. Rewardless flowers in the angiosperms and the role of insect cognition in their evolution. *In*: N.W. Waser and J. Ollerton (eds.). Plant-Pollinator Interactions: From Specialization to Generalization. Univ. Chcago Press, Chicago, pp. 123–144.
Renner, S.S. and H. Schaefer. 2010. The evolution and loss of oil-offering flowers: new insights from dated phylogenies for angiosperms and bees. Phil. Trans. R Soc. B 365: 423–435.
Riffell, J. 2011. The neuroecology of a pollinator's buffet: Olfactory preferences and learning in insect pollinators. Integr. Comp. Biol. 51: 781–793.
Roberts, R.B. 1971. Biology of the crepuscular bee *Ptiloglossa guinnae* n. sp. with notes on associated bees, mites and yeasts. J. Kansas Entomol. Soc. 44: 283–294.
Roubik, D.W. (ed.). 1995. Pollination of Cultivated Plants in the Tropics. FAO Agriculture Service Bull. 118.
Roubik, D.W., S. Sakai and A.A.H. Karim (eds.). 2005. Pollination Ecology and the Rain Forest: Sarawak Studies. Springer, New York.
Roulston, T.H. and J.H. Cane. 2000. Pollen nutritional content and digestibility for animals. Plant Syst. Evol. 222: 187–209.
Salzmann, C.C., A.M. Nardella, S. Cozzolino and P. Schiestl. 2007. Variability in floral scent in rewarding and deceptive orchids: The signature of pollinator-imposed selection? Ann. Bot. 100: 757–765.
Sanderson, M.J. and M.J. Donoghue. 1994. Shifts in diversification rate with the origin of Angiosperms. Science 264: 1590–1593.
Santos-Gally, R., R. Perez-Barrales, V.I. Simon and J. Arroyo. 2013. The role of short-tongued insects in floral variation across the range of a style-dimorphic plant. Ann. Bot. 111: 317–328.
Sargent, R.D. 2004. Floral symmetry affects speciation rates in angiosperms. Proc. R Soc. B 271: 603–608.
Sarma, K., R. Tandon, K.R. Shivanna and H.Y. Mohan Ram. 2007. Snail-pollination in *Volvulopsis nummularium*. Curr. Sci. 93: 826–831.
Schiestl, F.P. and M. Ayasse. 2001. Post-pollination emission of a repellent compound in a sexually deceptive orchid: a new mechanism for maximizing reproductive success? Oecologia 126: 531–534.
Schiestl, F.P., M. Ayasse, H.F. Paulus, D. Erdtmann and W. Francke. 1997. Variation of floral scent emission and post-pollination changes in individual flowers of *Ophrys sphegodes* subsp. *sphegodes*. J. Chem. Ecol. 23: 2881–289.

Schiestl, F.P., R. Peakall, J.G. Mant, F. Ibarra et al. 2003. The chemistry of sexual deception in an orchid-wasp pollination system. Science 302: 437–438.
Schlichting, C.D., A.B. Stephenson, L.E. Davis and J.A. Winsor. 1987. Pollen competition and offspring variance. Evol. Trends Plants 1: 35–39.
Schmidt, J.O. 1982. Pollen-foraging preferences of honeybees. South-Western Entomol. 7: 255–259.
Schmidt, J.O. and S.L. Buchmann. 1992. Other products of the hive. *In*: J.M. Graham (ed.). The Hive and the Honey Bee. Dadant & Sons, Hamilton, IL, pp. 927–982.
Sedivy, C., C.J. Praz, A. Muller, A. Widmer and S. Dorn. 2008. Patterns of host-plant choice in bees of the genus *Chelostoma*: the constraint hypothesis of host-range evolution in bees. Evolution 62: 2487–2507.
Sedivy, C., A. Muller and S. Dorn. 2011. Closely related pollen generalist bees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen. Functional Ecol. 25: 718–725.
Seymour, R.S. and P.G.D. Matthews. 2006. The role of thermogenesis in the pollination biology of the Amazon water lily *Victoria amazonica*. Ann. Bot. 98: 1129 –1135.
Seymour, R.S., I. Terry and R.B. Roemer. 2004. Respiration and thermogenesis by cones of the Australian cycad *Macrozamia machinii*. Funct. Ecol. 18: 925–930.
Sharma, I., N. Sharma and H. Kaur. 2009. Studies on the role of ants in reproductive efficiency of three species of *Phyllanthus* L. Curr. Sci. 96: 283–297.
Shivanna, K.R. 2003. Pollen Biology and Biotechnology. Science Publishers, Inc. Enfield, USA; Plymouth, UK.
Shivanna, K.R. 2010. Ant-pollination: A rare and enigmatic mutualism. Botanica 58: 24–28.
Shivanna, K.R. 2012a. Reproductive ecology and conservation of plant genetic resources of the wild. Indian J. Plant Genet. Resources 25: 75–84.
Shivanna, K.R. 2012b. Reproductive assurance through unusual autogamy in the absence of pollinators in *Passiflora edulis* (passion fruit). Curr. Sci. 103: 1091–1096.
Smith, S.A., J.M. Beaulieu, A. Stamatakis and M.J. Donoghue. 2011. Understanding angiosperm diversification using small and large phylogenetic trees. Am. J. Bot. 98: 404–414.
Smith, T.B., L.A. Freed, J.K. Lepson and J.H. Carothers. 1995. Evolutionary consequences of extinctions in populations of a Hawaiian honeycreeper. Cons. Biol. 9: 107–113.
Soltis, D.E., P.S. Soltis, M.W. Chase, M.E. Mort, D.C. Albach et al. 2000. Angiosperm phylogeny inferred from a combined data set of 18S rDNA, *rbcL* and *atpB* sequences. Bot. J. Linnean Soc. 133: 381–46.
Somanathan, H. and R.M. Borges. 2001. Nocturnal pollination by the carpenter bee *Xylocopa tenuiscapa* (Apidae) and the effect of floral display on fruit set of *Heterophragma quadriloculare* (Bignoniaceae) in India. Biotropica 33: 78–89.
Somanathan, H., R.M. Borges, E.J. Warrant and A. Kelber. 2008. Visual ecology of Indian carpenter bees. I. Light intensity and flight activity. J. Comp. Physiol. A 194: 97–107.
Somanathan, H., A. Kelber, R. Wallen, R.M. Borges and E.J. Warrant. 2009. Visual ecology of Indian carpenter bees II: adaptations of eyes and ocelli to nocturnal and diurnal lifestyles. J. Comp. Physiol. A 195: 571–583.
Spira, T.P. 2001. Plant–pollinator interactions: a threatened mutualism with implications for the ecology and management of rare plants. Natural Areas J. 21: 78–88.
Steiner, K.E. and V.B. Whitehead. 1991. Oil flowers and oil bees: further evidence for pollinator adaptation. Evolution 45: 1493–1501.
Stephenson, A.G. 1981. Toxic nectar deters nectar thieves of *Catalpa speciosa*. Am. Midland Nat. 105: 381–383.
Stephenson, A.G. 1982. Iridoid glycosides in the nectar of *Catalpa speciosa* are unpalatable to nectar thieves. J. Chem. Ecol. 8: 1025–1034.
Stokl, J., P.M. Schlüter, T.F. Stuessy, H. Paulus, R. Fraberger et al. 2009. Speciation in sexually deceptive orchids: Pollinator driven selection maintains discrete odour-phenotypes in hybridizing species. Biol. J. Linnean Soc. 98: 439–451.

Stokl, J., J. Brodmann, A. Dafni, M. Ayasse and B.S. Hansson. 2010. Smells like aphids: orchid flowers mimic aphid alarm pheromones to attract hoverflies for pollination. Proc. R Soc. B 278: 1216–1222.

Streinzer, M., H.F. Paulus and J. Spaethe. 2009. Floral colour signal increases short-range detectability of a sexually deceptive orchid to its bee pollinator. J. Exp. Biol. 212: 1365–1370.

Streinzer, M., T. Ellis, H.F. Paulus and J. Spaethe. 2010. Visual discrimination between two sexually deceptive *Ophrys* species by a bee pollinator. Arthopod-Plant Interactions 4: 141–148.

Svensson, G.P., O. Pellmyr and R.A. Raguso. 2011. Pollinator attraction to volatiles from virgin and pollinated host flowers in a yucca/moth obligate mutualism. Oikos 120: 1577–1583.

Tan, K.-H. 2006. Fruit fly pests as pollinators of wild orchids. Proceedings of the 7th International Symposium on fruit flies of economic importance: From basic to applied knowledge, 10–15 September 2006, Salvador, Brazil, pp. 195–206.

Tan, K.-H., R. Nishida and Y.-C. Yock-Chai Toong. 2002. Floral syndrome of a wild orchid, *Bulbophyllum cheiri*, lures *Bactrocera* fruit flies for pollination. J. Chem. Ecol. 28: 1161–1172.

Tandon, R., K.R. Shivanna and H.Y. Mohan Ram. 2003. Reproductive biology of *Butea monosperma* (Fabaceae). Ann. Bot. 92: 715–723.

Tejaswini, K.N. Ganeshaiah and R. Uma Shaanker. 2001. Sexual selection in plants: the process, components and significance. Proc. Indian Natl. Sci. Acad. B 67: 423–432.

Temeles, E.J., J.S. Miller and J.L. Rifkin. 2010. Evolution of sexual dimorphism in bill size and shape of hermit hummingbirds (Phaethornithinae): a role for ecological causation. Phil. Trans. R Soc. B 365: 1053–1063.

Temeles, E.J., I.L. Pan, J.L. Brennan and J.N. Horwitt. 2000. Evidence for ecological causation of sexual dimorphism in a hummingbird. Science 289: 441–443.

Terry, I., G.H. Walter, C. Moore, R. Roemer and C. Hull. 2007. Odor-mediated push-pull pollination in Cycads. Science 318: 70.

Thien, L.B., P. Bernhardt, M.S. Devall, Z. Chen et al. 2009. Pollination biology of basal angiosperms (ANITA grade). Am. J. Bot. 96: 166–182.

Thom, C., P.G. Guerenstein, W.L. Mechaber and J.G. Hildebrand. 2004. Floral CO_2 reveals flower profitability to moths. J. Chem. Ecol. 30: 1285–1288.

Trujillo, C.G. and A.N. Sersic. 2006. Floral biology of *Aristolochia argentina* (Aristolochiaceae). Flora 201: 374–382.

Turner, I.M. 2001. The Ecology of Seeds in Tropical Rain Forests. Cambridge Univ. Press, Cambridge, UK.

Tylianakis, J.M. 2013. The global plight of pollinators. Science 339: 1532–1533.

Vamosi, J.C. and S.M. Vamosi. 2012. Zygomorphy, area, and the latitudinal biodiversity in angiosperms. *In*: S. Patiny (ed.). Evolution of Plant-Pollinator Relationships. The Systematic Association Special Vol. 81. Cambridge Univ. Press, New York, pp. 320–343.

Varassin, I.G., J.R. Trigo and M. Sazima. 2001. The role of nectar production, flower pigments and odour in the pollination of four species of *Passiflora* (Passifloraceae) in south-eastern Brazil. Bot. J. Linnean Soc. 136: 139–152.

Vogel, S. and J. Martens. 2000. A survey of function of lethal kettle traps of *Arisaema* (Araceae) with records of pollination fungus gnats from Nepal. Bot. J. Linnean Soc. 133: 61–100.

von Arx, M., J. Goyret, G. Davidowitz and R.A. Raguso. 2012. Floral humidity as a reliable sensory cue for profitability assessment by nectar-foraging hawkmoths. Proc. Natl. Acad. Sci. USA 109: 9471–9476.

von Helversen, D. and O. von Helversen. 1999. Acoustic guide in bat pollinated flower. Nature 398: 759–760.

Wang, G., S.G. Compton and J. Chen 2013. The mechanism of pollinator specificity between two sympatric fig varieties: a combination of olfactory signals and contact cues. Ann. Bot. 111: 173–181.

Warrant, E.J. 2004. Vision in the dimmest habitats on earth. J. Comp. Physiol. A 190: 765–789.
Warrant, E.J., A. Kelber, A. Gislen, B. Greiner, W. Ribi and W.T. Wcislo. 2004. Nocturnal vision and landmark orientation in a tropical halictid bee. Curr. Biol. 14: 1309–1318.
Waser, N.M. and J. Ollerton (eds.). 2006. Plant-Pollinator Interactions: From Specialization to Generalization. Univ. Chcago Press, Chicago.
Waser, N.M. and M.V. Price. 1983. Optimal and actual outcrossing in plants, and the nature of plant-pollinator interaction. *In*: C.E. Jones and R.J. Little (eds.). Handbook of Experimental Pollination Biology, Scientific and Academic Editions, van Nostrand Reinhold Co. Inc., New York, pp. 341–359.
Waser, N.M., L. Chittka and M.V. Price. 1996. Generalization in pollination systems, and why it matters. Ecology 77: 1043–1060.
Weiss, M.R. 1991. Floral colour changes as cues for pollinators. Nature 354: 227–229.
Wester, P., R. Stanway and A. Pauw. 2009. Mice pollinate the Pagoda Lily, *Whiteheadia bifolia* (Hyacinthaceae)—First field observations with photographic documentation of rodent pollination in South Africa. South African J. Bot. 75: 713–719.
Wiens, D. 1978. Mimicry in plants. Evol. Biol. 11: 365–403.
Wilcock, C. and R. Neiland. 2002. Pollination failure in plants: why it happens and when it matters. Trends Plant Sci. 7: 270–277.
Williams, G.A., P. Adam and L.A. Mound. 2001. Thrips (Thysanoptera) pollination in Australian subtropical rainforests, with particular reference to pollination of *Wilkiea huegeliana* (Monimiaceae). J. Nat. Hist. 35: 1–21.
Williams, N.S. 1983. Floral fragrances as cues in animal behavior. *In*: C.E. Jones and R.J. Little (eds.). Handbook of Experimental Pollination Biology. Scientific and Academic Editions, Van Nostrand Reinhold Co. Inc., New York, pp. 50–72.
Willmer, P. 2011. Pollination and Floral Ecology. Princeton Univ. Press, New Jersey.
Willmer, P.G., C.V. Nuttman, N.E. Raine, G.N. Stone, J.G. Patrick et al. 2009. Pollen volatiles controlling ant behavior. Func. Ecol. 23: 888–900.
Winter, Y. and K.P. Stich. 2005. Foraging in a complex naturalistic environment: capacity of spatial working memory in flower bats. J. Exp. Biol. 208: 539–548.
Wright, G.A., D. Baker, M. Palmer, D. Stabler, J.A. Mustard, E. Power, A.M. Borland and P. Stevenson. 2013. Caffeine in floral nectar enhances a pollinator's memory of reward. Science 339: 1202.
Young, H.J. 2002. Diurnal and nocturnal pollination of *Silene alba* (Caryophyllaceae). Am. J. Bot. 89: 433–440.
Zhang, F.-P., X.-H. Cai, H. Wang, Z.-X. Ren et al. 2012. Dark purple nectar as a foraging signal in a bird-pollinated Himalayan plant. New Phytol. 193: 188–195.

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Nectar: Plant Interface for Complex Interaction with Biotic Environment

Massimo Nepi

ABSTRACT

Angiosperms' nectar, floral and extrafloral, is a valuable energetic alimentary resource for a large variety of animals from insects to small mammals, birds, marsupials and reptiles. It frequently mediates mutualistic relationships between the two partners. In recent years it was clearly demonstrated that this relationships actually often involves other partners: nectar dwelling micro-organisms. Yeasts and bacteria may alter considerably the nectar composition and can be the causal agents of some plant diseases. Nectar has biochemical defences to inhibit micro-organism proliferation, i.e., a heterogeneous arsenal of proteins with anti-fungal and anti-microbial activity that has just been discovered in the last years. Nonetheless yeasts are almost ubiquitous in nectar and their direct and indirect interactions with nectar foragers are almost unknown as well as the consequences for plant reproduction. A recent advance in nectar biology was the recognition of some secondary compounds, especially alkaloids and non-protein amino acids, in modulating the foraging behaviour of nectar feeders through several effects on insect neurophysiology. All these studies demonstrate that nectar has a wider range of more complex interactions than previously thought.

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Keywords: nectar, defence, foragers behaviour, proteins, secondary compounds, non-protein amino acids

Introduction

Nectar is widespread amongst angiosperms and it is exploited as an alimentary resource by a great variety of animals (invertebrates and vertebrates) which may pollinate or defend the plant from herbivores (Nicolson 2007; Heil 2011). Until recently, most studies concentrated on the ability of nectar to attract foragers, largely due to its high concentrations of simple sugars, namely: sucrose, glucose and fructose, as well as its lower content of amino acids. Fundamentally an aqueous solution, nectar is easily ingested, digested and absorbed by the alimentary canal, and is thus a very cost-effective alimentary resource for a wide variety of animals (Nicolson 2007; González-Teuber and Heil 2009). This classical "alimentary" perspective of nectar has recently been challenged by other studies that have both detailed and shown the presence of compounds that are not directly related to the alimentary value of nectar. For example, it was shown that floral and extrafloral nectar may contain a large and heterogeneous assemblage of defence proteins that are active against micro-organisms (Carter and Thornburg 2000, 2004; Naqvi et al. 2005; Carter et al. 2007; Kram et al. 2008; González-Teuber et al. 2009, 2010; Hillwig et al. 2011; Nepi et al. 2011). Some of these micro-organisms are phytopathogens (Bubàn et al. 2003; Farkas et al. 2007). Others are not dangerous for the plant but may interfere with the pollinator's alimentary choice. Yeasts are known to inhabit floral nectar, changing considerably the chemical composition of nectar and consequently affecting the relationships with nectar foragers (Herrera et al. 2008, 2009). It was hypothesized that nectar inhabiting micro-organisms may act as a third party in the mutualistic relationships linking plants and nectar foragers, an emerging tripartite relationship whose ecological and evolutionary significance is still far from being well established (Canto and Herrera 2012).

Moreover, data obtained from recent research that focused on secondary compounds in floral nectar contrasted markedly with the attractiveness of nectar to insects. Generally, nectary alkaloids and phenols are toxic to insects and deter insect foragers that are nectar-thieves, but not genuine pollinators, from visiting the flowers (González-Teuber and Heil 2009 and references therein). However, sometimes, these compounds deter both nectar-thieves and pollinators.

Within this complex context, one particular class of substances that potentially has an important role, namely, the non-protein amino-acids (NPAAs), has received almost no attention. Whereas only twenty amino acids are involved in building proteins, thousands of NPAAs have a myriad

of other functions. Of these amino acids, 250 are found in plants, and it is becoming increasingly clear that these play significant roles both in ecological and physiological processes (Vranova et al. 2011). The existence of non-protein amino acids in floral nectar was first reported in early surveys of nectar composition dating from the 1970s (Baker and Baker 1975; Baker 1977, 1978; Baker et al. 1978). Although several ecological and physiological functions have been attributed to a number of non-protein amino acids derived from both animals and plants, their role in nectar has still received little attention.

Based on the recent works mentioned above, it is evident that floral nectar exhibits an array of potential functions in its interaction with organisms that far exceeds that of a simple alimentary reward. Some of these functions are involved, directly or indirectly, in modulating the foraging behaviour of foragers. In this chapter we discuss the more significant recent advances in floral nectar biology and ecology namely the characterization of nectar proteome and nectar secondary compounds, as well as the interaction with yeasts. The purpose is to stimulate future research and scientific debate in the context of ecological functions of nectar in shaping the complex web of plant-animal interactions.

Nectar Proteins and Interaction with Micro-Organisms

Defence Against Pathogens

Because of its sugar composition, nectar is an excellent environment for the growth of airborne organisms or for those carried by pollinators (Fig. 1).

Some micro-organisms are phytopathogens and may penetrate plant tissues through nectarostomata such as *Erwinia amylovora* and *E. tracheifila* that are the causal phathogen of fire blight and bacterial wild disease respectively (Bubán et al. 2003; Sasu et al. 2010). Thus the plants must defend their nectar from micro-organisms' proliferation by means of a heterogeneous array of anti-microbial substances. The anti-microbial activity of phenolic compounds, as well as alkaloids, substances that may be components of nectar (see next paragraph), is well documented (Adler 2000; González-Teuber and Heil 2009 and references therein). Nectar proteins are known to have a role in defence against fungi and bacteria as well. A new and exclusive metabolic pathway (the so-called Nectar Redox Cycle, NRC) that serves mainly to maintain high levels of hydrogen peroxide and involves 5 enzymes (nectarin I–V) has been found in the nectar of *Nicotiana langsdorffii* × *N. sanderae* (Carter and Thornburg 2000, 2004; Carter et al. 2007). In this way the nectar is maintained in a sterile state. The floral nectar of *Petunia hybrida* contains several RNases, a peroxidase and an endochitinase whose anti-microbial activity has recently been recognized (Hillwig et al.

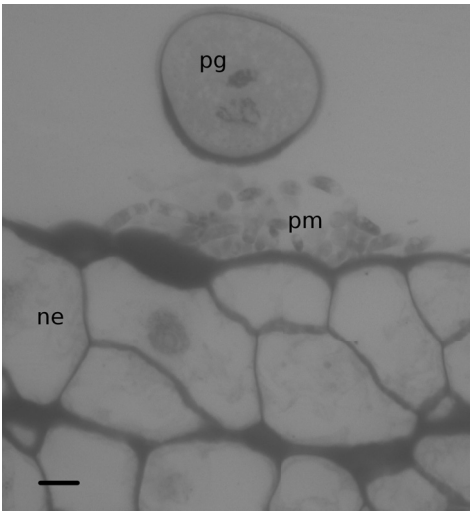

Figure 1. Pollen grain (pg) and proliferating micro-organisms (pm) on the floral nectary surface (ne = nectary epidermis) of *Helleborus foetidus* (Toluidine Blue O staining). Scale bar = 10 µm.

2010, 2011). A lipase (JNP1) was reported for the floral nectar of *Jacaranda mimosifolia* but its anti-microbial activity has not yet been demonstrated (Kram et al. 2008). Four isoforms of β-xylosidases have recently been identified as main proteins in the male and female floral nectar of *C. pepo* and their putative antimicrobial activity has been hypothesized (Nepi et al. 2011). Extrafloral nectar also has its own enzymatic defence against invasion by micro-organisms, the predominant enzymes being chitinase, β-1, 3-glucanase and thaumatine-like proteins (González-Teuber et al. 2009, 2010). Defense proteins such as glucosidases, chitinases, hydrolases, and thaumatine-like proteins have also been detected in pollination drops (Wagner et al. 2007), secretions produced by the ovules of gymnosperms that possess a chemical composition similar to that of angiosperm nectar (Nepi et al. 2009; see also chapter by von Aderkas et al. in this book). It appears that pathogenesis-related proteins are abundant in all the environment-exposed sugary secretions of spermatophytes probably due to a convergent chemical evolution towards protection from invading micro-organisms. Apparently extra-floral nectar, being more exposed and less ephemeral, is characterised by a higher number of proteins than floral nectar (González-Teuber et al. 2009). In all these secretions defence proteins may explain their antibiotic activity in two ways: killing the micro-organisms, for example by degrading their cell wall through chitinases and glucanases, or by reducing their pathogenic potential. Defence proteins may limit the lytic activity of micro-organisms both directly, by inhibiting enzymes responsible of plant

cell wall degradation (Naqvi et al. 2005) and indirectly by reducing the quantity of elicitor molecules that stimulate the production of the degrading enzymes (Nepi et al. 2011).

Yeasts are Frequently Associated with Floral Nectar

Despite this heterogeneous defence "arsenal", nectar is frequently contaminated by yeasts that deeply alter the chemical composition of nectar (Herrera et al. 2009). About half of the samples analyzed in tropical and temperate plant communities have tested positive for yeasts with the predominance of basidiomycetous and ascomycetous species from the genera *Cryptococcus*, *Metschnikowia* and *Candida* (Herrera et al. 2009; Canto and Herrera 2012). Persistence of microbial communities in nectar requires continuous cycles of immigration-multiplication-dispersal as new host flowers constantly appear and disappear in the landscape (Pozo et al. 2012). As nectar chemistry can vary strongly within and between different plant species (Baker and Baker 1975; Nicolson and Thornburg 2007) proliferation depends on the ability of the micro-organisms to cope with a broad range of nectar environments (comprising the heterogeneous presence of antimicrobial compounds) and thus requires the ability to rapidly adapt to different nectar conditions. Pollinators, most often insects, are likely the most important candidates for transferring microbes from one flower to another (Herrera et al. 2009). Recently it was established that ants also, although not generally involved in pollination but rather in plant defence while foraging for extra-floral nectar (Gonzalez-Teuber and Heil 2009), may participate in transferring micro-organisms into floral nectar (de Vega and Herrera 2012, 2013) and possibly they can contaminate extra-floral nectar when moving to extra-floral nectaries.

Immigration and dispersal therefore depend on patterns and frequency of forager visits and movements (Pozo et al. 2012). It was demonstrated that different nectar feeders have different probability to contaminate floral nectar with yeasts. In 22 plant species from Cazorla (southern Spain), yeast frequency and abundance were significantly related to differences in the relative importance of solitary bees vs. bumble-bees in the pollinator assemblage: yeast incidence was more marked in species pollinated mainly by bumblebees (Herrera et al. 2009). Identifications of yeast isolates suggest that the composition of nectar yeast assemblages varies among plant species and that that nectar yeasts impose a detectable imprint on community-wide variation in nectar sugar composition and concentration (Canto and Herrera 2012). This pattern is determined by several factors, including variation among plants species in concentration or effectiveness of antifungal defences in nectar, variability of composition of their associated nectar yeast communities, perhaps as a consequence of differences in pollinator type.

Yeasts are responsible of drastic changes in nectar attributes that may affect forager's behaviour mainly in three ways. First, the presence of yeast decrease the alimentary value of nectar because it causes a drastic changes in sugar and amino acids profiles. Through fermentation they cause a decrease in total sugar concentration and extensive reduction in sucrose percentage (Herrera et al. 2009; Canto et al. 2011; de Vega and Herrera 2013). Since sugars are the dominant chemical constituents of most nectar, providing the key energetic reward for several animals, their variation may influence choices during foraging bouts. Preferences for specific sugar profile are known for different classes of nectar feeding animals (Nicolson 2007). Yeasts cause also degradation of amino acids that they utilize as nitrogen source (Peay et al. 2012). As for sugars, pollinating animals and herbivore-defending ants have preferences for specific amino acid profiles. Thus drastic modifications of nectar composition induced by yeasts may significantly alter nectar attractiveness to specific foragers.

Second, nectar fermentation by yeasts produces ethanol that may rise to levels that are toxic to foragers. The fungus *Cladosporium* is responsible of ethanol production in the nectar of the orchid *Epipactis helleborine* (Ehlers and Olesen 1996). Wasps became very slow and "sluggish" when drinking this nectar and they groomed their body less for pollinia. This, lowering pollinia loss, may enhance pollinia transfer and thus orchid's reproduction (Ehlers and Olesen 1996).

Third, nectar fermentation is also responsible of the emission of volatile compounds that contribute to the scent of flower's headspace perceived by foraging insects (Raguso 2004 and references therein). It is well demonstrated that changes in flower odor may affect plant-animal interactions (Raguso 2009). The nectar of *Agave palmeri* contains sorbic acid that has antimicrobial activity. Sorbic acid is most probably transformed by yeasts into ethyl sorbate that confers to nectar a specific odor (Raguso 2004). While floral nectar scents have been considered in the interaction with pollinators, much less is known about odors in extra-floral nectar although it was recognized that they are likely to play a role in the attraction of beneficial insects to EFN (González-Teuber and Heil 2009).

It is clear that nectar dwelling yeasts may have several important effects in plant-animal interactions mediated by nectar. Very few studies deal with the extent to which these effects may interfere with the behaviour of foraging animals and, in turn, with plant reproduction. Kevan et al. (1988) failed to find evidence of yeasts influencing flower choice by honey bees. More recently Herrera et al. (2013) demonstrated that bumble bees can detect the presence of yeasts in artificial nectar and responded positively by paying proportionally more visits to yeast-containing flowers. This preference is detrimental for the reproduction of *Helleborus foetidus* probably because the longer visits by pollinators to yeast-containing flowers would enhance

autogamy with consequent reduction in number and size of seeds produced (Herrera et al. 2013). Unraveling details of these complex interactions will require further studies.

Nectar Secondary Compounds Interact with Foragers

Secondary metabolites, including tannins, phenols, alkaloids and terpenes, have been found in floral nectar in more than 21 angiosperm families (Adler 2000). These compounds have been known since the 70s and they were considered to be toxic deterrents against predators (Baker and Baker 1983). Recently, researchers have discovered that these compounds may play an important role in managing visitors' behaviour. Flowers face a multidimensional challenge: they need to attract visitors, to compel them to vector pollen with the least investment in rewards, and to repel nectar robbers at the same time. All of this is in the service of maximizing fitness. The bouquet of secondary compounds may serve a number of these objectives.

Alkaloids

Kessler et al. (2008) discovered that both the repellent nicotine and the attractant benzyl acetone were required to maximize capsule production and flower visitation by native pollinators, at the same time, nicotine reduced nectar robbing by non-pollinating animals. The presence of nicotine, a typical insect-repelling alkaloid, is necessary to optimize the number of flower visitors per aliquot volume of nectar produced by flowers of *Nicotiana attenuata*, thereby allowing plants to minimize nectar volumes, whilst maximizing transfer of pollen and seed production events (Kessler and Baldwin 2007).

According to Singaravelan et al. (2005) naturally occurring concentrations of secondary compounds such as caffeine, nicotine, anabasine, and amygadaline did not deter insects. Secondary compounds can be regarded as post-ingestion stimulants to pollinators. Low concentrations of psychoactive alkaloids, nicotine and caffeine, increased nectar feeding significantly. These compounds may have been part of the reward. The presence of psychoactive alkaloids, such as these, in nectar, may also impose a dependence or addiction on pollinators, as well as improving the short-term and early, long-term memory of honeybees (Singaravelan 2010). The latter effect was recently proved by Wright et al. (2013) demonstrating that honeybees rewarded with caffeine, which occurs naturally in nectar of *Coffea* and *Citrus* species, have a higher ability to remember a learned floral scent than honeybees rewarded with sucrose alone. Caffeine concentrations in nectar did not exceed the bees' bitter taste threshold, implying that

pollinators impose selection for nectar that is pharmacologically active but not repellent.

Secondary compounds in nectar are known to have antimicrobial properties (Adler 2000 and references therein). Compounds may not only provide a direct defence from microbial invasion, but can indirectly protect the consuming animal. Manson et al. (2010) demonstrated that consumption of the nectar alkaloid gelsemine found in *Gelsemium sempervirens* reduces pathogen loads in bumblebees. It also protects bees from infection, which, in the long run, improves their foraging efficiency.

Non-protein Amino Acids

Baker et al. (1978) reported the presence of NPAAs in the floral nectar of 35% of 248 broadly distributed species of flowering plants. This percentage increased to 55% when these substances were searched for in the floral nectar of 69 species of tropical trees and lianes (Baker 1978). Guerrant and Fiedler (1981) reported the presence of NPAAs in 13 out of 25 species (52%) growing in dry and wet forests of Costa Rica. Petanidou (2007) found NPAAs in the nectar of 86% of 73 plant species of the phrygana community. While studying the nectar chemistry of the tribe Lithospermeae (Boraginaceae), we found NPAAs in a total of 49 taxa (unpublished data). Surely we can state that NPAAs are not so much uncommon, but rather that they are almost ubiquitous in floral nectar.

Unfortunately, most of the early surveys of nectar chemistry reported only the presence/absence of NPAAs, without any specific determinations. In the years that followed, some NPAAs were detected and determined in floral nectar. These included: β-alanine, γ-amino butyric acid (GABA), α-amino butyric acid (AABA), taurine, ornithine and citrulline, of which GABA and β-alanine appear to be the most common (Baker and Baker 1975; Baker 1977, 1978; Baker et al. 1978; Inouye and Inouye 1980; Guerrant and Fiedler 1981; Gardener and Gillman 2001; Kaczorowski et al. 2005; Petanidou et al. 2006; Nepi et al. 2012; Nocentini et al. 2012; Peay et al. 2012). GABA occurs at the highest concentration, ranging from 0.57 nmoles/ml in *Nicotiana alata* (Kaczorowsky et al. 2005) to about 750 nmoles/ml in *Agrostemma githago* (Gardener and Gillman 2001).

The ecological role of nectar NPAAs

The early report of NPAA in floral nectar (Baker 1977) stated: "it is likely that at least some of them will prove to be toxic to certain kinds of flower-visitor". This hypothesis was later developed in the so-called pollinator fidelity theory applied to nectar secondary compounds. According to this latter theory, toxic compounds encourage foraging by specialist pollinators,

while deterring visits by erratic or undesirable (nectar-thieves) insects that either deliver less or no intraspecific pollen (Adler 2000). Furthermore, it assumes that specialist pollinators are more resistant to specific toxicants than generalists. Unfortunately, the toxicity of NPAAs found in nectar to foraging insects is largely unknown. With regard to plant secondary compounds, the effect of NPAAs has been mainly tested on herbivorous insects, not on nectar feeders. However, a direct toxic effect was reported in *Choristoneura rosaceana* (oblique-banded leaf roller, Lepidoptera) larvae fed on an artificial diet enriched with GABA (Bown et al. 2006). Toxic effects resulting in reduced growth and reduced survival rate were found for a concentration of 2.6 mM, i.e., about three-fold of the highest concentration found in nectar (see above).

The toxic or deterrent effect is dependent on the amino acid concentration of nectar, the rate of intake and the sensitivity of its consumers. It should be noted, however, that deterrent substances are not necessarily toxic, and that substances thought to be toxic are not necessarily deterrents (Singaravelan 2010). According to Inouye and Inouye (1980), the relatively high proportion of NPAAs found in the extra-floral nectar of *Helianthella quinquenervis* suggests that these particular compounds are neither toxic to nor deter ants. Furthermore, they do not appear to have a deterrent effect on wasps, beetles, flies or any other insects that collect nectar in the absence of ants. Ants are also common floral nectar thieves, but NPAAs present in the floral nectar rarely deter these insects from visiting flowers (Guerrant and Fiedler 1981).

Thus, from the scant literature available, it would appear that NPAAs are not obviously involved in the exclusion of undesirable nectar foragers, rather, they seem to be more common in the floral nectar of species pollinated by specific pollinator guilds and in particular, those pollinated by Hymenoptera. Baker and Baker (1978) found that NPAAs are more common in the floral nectar of tropical trees and lianes that are pollinated by Hymenoptera than in those that are visited by Lepidoptera, bats, or birds. Petanidou et al. (2006), who studied the nectar composition of the phrygana community species, presented similar data. These authors found that high levels of GABA could be correlated with long-tongued bees, anthophorid and andrenid bees, as well as anthomyiid and syrphid flies. Interestingly, bees and bumble bees are among the pollinators of those species that have higher GABA concentrations (Gardener and Gillman 2001; Kaczorowski et al. 2005; Petanidou et al. 2006; Nepi et al. 2012; Nocentini et al. 2012).

NPAAs and defence against fungi and bacteria. Extracellular GABA was reported to be involved in plant communication with other organisms (Shelp et al. 2006) and accumulates in response to infection by fungi and bacteria (Chevrot et al. 2006; Oliver and Solomon 2004). It was also

demonstrated that accumulated, extracellular GABA reduced the virulence of *Agrobacterium tumefaciens* in tobacco leaves by inducing the synthesis of enzymes that modulate the infection process (Chevrot et al. 2006). Furthermore, GABA functions in communication between tomato plants and the fungus *Cladosporium fulvum* (Oliver and Solomon 2004). During infection, GABA concentration in the apoplast increases from about 0.8 mM to 2–3 mM, concentrations that resemble and are about three-fold greater, respectively, than the highest concentration found in floral nectar (Gardener and Gillmann 2001). No specific experiments have yet been undertaken to assess the eventual change in GABA concentration following infection by micro-organisms. The only report to date indicates that several species of yeast have little effect on GABA concentration in the floral nectar of *Mimulus aurantiacus*, whereas other amino acids (such as glutamic acid, aspartic acid and proline) were almost completely used as a nitrogen source by these fungi (Peay et al. 2012).

Moreover, ß-aminobutyric acid (BABA), which is structurally related to GABA, but is much less common in nature, including nectar, seems to play a broad role in increasing plant defence against biotic stress, such as invasion by viral, bacterial and fungal pathogens, by priming plants to respond more rapidly and to a greater degree to future stress events (Huang et al. 2011).

This particular biological property of GABA and BABA may contribute to the function of nectar in protecting plants from invasion by pathogenic organisms that based on our present knowledge, it is mainly associated with nectar proteins (Nepi et al. 2011; Park and Thornburg 2009).

NPAAs may affect the foraging activity of nectar feeders. NPAAs may potentially affect the foraging behaviour of insects in three different ways: 1) by directly affecting the insect nervous system; 2) by contributing in regulating the feeding rate (phagostimulation); 3) by increasing the activity of flight muscles.

GABA, taurine and β-alanine are abundant in the nervous systems of insects (Bicker 1991; Gardener and Gillman 2001), where they function as inhibitory neurotransmitters. GABA acts in synergy with taurine, limiting excessive, potentially disruptive excitation states during stressful conditions, probably in antagonism with octopamine in arousal pathways (Stevenson 1999 and references therein). GABA is also the principal inhibitory neurotransmitter in the vertebrate brain and its levels are strictly regulated by transport across the blood–brain barrier. By contrast, GABA-receptors in invertebrates are located peripherally in muscle tissue and neuromuscular junctions, where they are bathed in haemolymph (Bown et al. 2006) and are thus more sensitive to changes in GABA eventually precipitated by GABA-rich nectar feeding. GABA is also important in the

development of the nervous system by acting as a cell-to-cell signal during embryonic and adult neurogenesis in animals, and changes to its synthesis or degradation can cause severe clinical disorders (Bouché et al. 2003) such as seizures, hypotonia, lethargy and severely retarded psychomotor development (Medina-Kauwe 1999). Recently, a colleague observed some bumble bees apparently in a semi-paralyzed condition after visiting the flowers and feeding on the nectar of *Gentiana lutea* (Marta Galloni, personal communication). Analysis of nectar amino acid profile revealed a very high concentration of β-alanine (2.3 mM) two-fold higher than that of proline, which is often the dominant amino acid in nectar. Despite the absence of unequivocal evidence, we suspect that insects accumulate sufficient neurotransmitter to induce lethargic behaviour.

Plants can regulate the feeding behaviour of animals via several metabolites—mainly sugars and amino acids (Shoonhoven et al. 2005). Unfortunately, once again, the feeding behaviour of herbivorous insects has been studied much more than that of nectarivorous species. In the case of locusts, beetles and caterpillars, sucrose and fructose are common and powerful feeding stimulants (Shoonhoven et al. 2005). The two protein amino acids proline and phenylalanine are amongst the most abundant nectar amino acids (Petanidou 2007; Nicolson and Thornburg 2007) and display strong phagostimulatory activity. Interestingly, another potential effect of a particular NPAA, namely, GABA, is the stimulation of chemoreceptors, and this results in the increased feeding behaviour of some caterpillars and adult beetles (Shoonhovet et al. 2005). Furthermore, co- administration of GABA can overcome the antifeedant activity of terpenoids (Passreiter and Isman 1997). Terpenoids can accumulate in the nectar of several species (Raguso 2004), and high levels of nectar GABA may contribute towards the maintenance of an adequate feeding rate (Nicolson and Thornburg 2007).

Several NPAAs appear to be involved in improving muscle performance. For example, β-alanine is a precursor of the dipeptide carnosine that is found in both vertebrate and non-vertebrate skeletal muscles, and acts as a limiting factor to its synthesis (Harris et al. 2006a). It has been demonstrated that carnosine can increase isometric endurance in humans, and β-alanine uptake can be enhanced by the assumption of simple sugars (Harris et al. 2006b; Sale et al. 2012). In fact, β-alanine, taurine and GABA are used by athletes to increase their performance and reduce fatigue (Hill et al. 2007; Zhang et al. 2004; Watanabe et al. 2002).

High concentrations of taurine have been found in several orders of insects, usually in the thoracic region of adults, where they are associated with fully functional flight muscles (Whitton et al. 1987).

The maintenance of good muscle performance by foraging insects is clearly advantageous to the plant as it ensures greater pollinator movements between flowers, plants and populations, thus favouring pollen and gene flow.

Concluding Remarks

Recent researches demonstrated that the relationships between nectar and organisms, from micro-organisms to large animals, are much more complex than we thought before (Fig. 2).

A recent fundamental step forward in this direction was provided by conclusive evidences that nectar sugar composition is not completely controlled by the plant and that this crucial food source for a variety

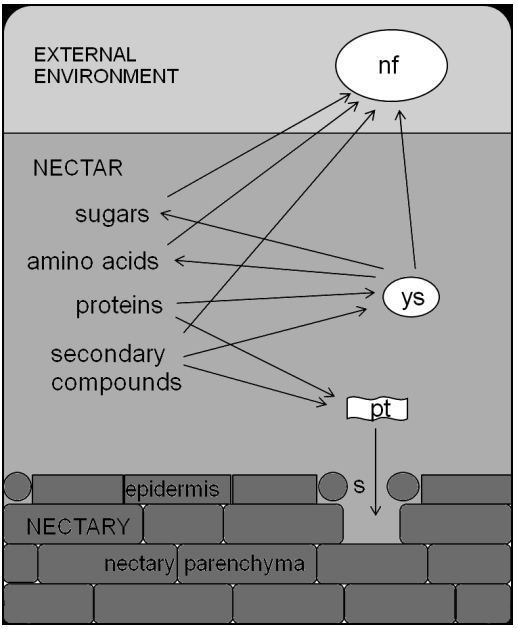

Figure 2. Web of interactions between nectar components and the biotic environment inside (yeasts and bacteria) and outside nectar (nectar feeders). The profile of the main nectar metabolites (sugars and amino acids) determine the alimentary choice of nectar feeders. Nectarins (nectar proteins) are a biochemical defence against micro-organisms (yeasts and bacteria). Secondary compounds (alkaloids and non protein amino acids) have multifunctional activity being involved in the interactions with micro-organisms and nectar feeders. Bacteria can be pathogens that invade the plant tissues penetrating through nectary stomata. Yeasts may affect feeder's behaviour directly, by producing alcohols or volatile compounds, or indirectly by changing considerably sugars and amino acids profile. Nectar feeders are responsible of micro-organism's transport from flower to flower. pt = pathogen bacterium; nf = nectar feeders; s = stoma; ys = yeast.

of organisms may be influenced by external biological factors such as contamination by micro-organisms, particularly yeasts (Canto and Herrera 2012; de Vega and Herrera 2013). Yeasts are also able to interfere with the behaviour of foragers and the functional links between nectar dwelling yeasts, nectar feeders and plant reproduction are going to be discovered (Herrera et al. 2013).

A further step that is awaited in the next future is a better integration between nectar chemistry and insect's physiology and neurobiology. Although specific studies are lacking for most insects, and there is currently little information available on the metabolism of non-protein amino acids and their fate following ingestion by insects, a complex, ecophysiological picture is beginning to emerge: proline powers the take-off of the insect from the flower (Carter et al. 2006), sugars propel its prolonged flight, and taurine, GABA and alanine promote the highly efficient functioning of flight muscles. Meanwhile, insects are forced to move from flower to flower by the combined phagostimulatory activity of proline, phenylalanine and GABA, which maintain a high feeding rate. Alkaloids, such as nicotine and caffeine, may serve to reinforce the fidelity of foragers by improving their memory ability and/or inducing dependence (Singaravelan et al. 2010; Wright et al. 2013). Excessive excitation due to hunger can be reduced by the intake of inhibitory neurotransmitters that keep the insect calm. All these are plausible hypotheses whose confirmation may open up new perspectives on plant-animal relationships.

References

Adler, L.S. 2000. The ecological significance of toxic nectar. Oikos 91: 409–420.

Baker, H.G. 1977. Non-sugar chemical constituents of nectar. Apidologie 8: 349–356.

Baker, H.G. 1978. Chemical aspects of the pollination biology of woody plants in the tropics. *In*: P.B. Tomlison and M.H. Zimmermann (eds.). Tropical trees as living systems. Cambridge University Press, Cambridge, pp. 57–82.

Baker, H.G. and I. Baker. 1975. The study of nectar-constitution and pollinator-plant coevolution. *In*: L.E. Gilbert and P.H. Raven (eds.). Coevolution of plants and animals. University of Texas Press, Austin, pp. 100–140.

Baker, H.G. and I. Baker. 1983. A brief historical review of the chemistry of floral nectar. *In*: B. Bentley and T. Elias (eds.). The biology of nectaries. Columbia University Press, New York, pp. 126–151.

Baker, H.G., P.A. Opler and I. Baker. 1978. A comparison of the amino acid complements of floral and extrafloral nectars. Bot. Gaz. 139: 322–332.

Bicker, G. 1991. Taurine-like immunoreactivity in photoreceptor cells and mushroom bodies: a comparison of the chemical architecture of insect nervous systems. Brain Res. 560: 201–206.

Bouché, N., B. Lacombe and H. Fromm. 2003. GABA signaling: a conserved and ubiquitous mechanism. Trends Cell Biol. 13: 607–610.

Bown, A.W., K.B. MacGregor and B.J. Shelp. 2006. Gamma-aminobutyrate: defence against invertebrate pests? Trends Plant Sci. 11: 424–427.

Bubán, T., Z. Orosz-Kovacs and A. Farkas. 2003. The nectary as the primary site of infection by Erwinia amylovora (Burr.) Winslow et al.: a mini review. Plant Syst. Evol. 238: 183–194.

Canto, A. and C.M. Herrera. 2012. Micro-organisms behind the pollination scenes: microbial imprint on floral nectar sugar variation in a tropical plant community. Ann. Bot. 110: 1173–1183.

Canto, A., C.M. Herrera, I.M. García, R. Pérez and M. Vaz. 2011. Intraplant variation in nectar traits in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental conditions and pollinator exposure. Flora 206: 668–675.

Carter, C., R. Healy, N.M. O'Tool, S.M. Naqvi, G. Ren, S. Park, G.A. Beattie, H.T. Horner and R.W. Thornburg. 2007. Tobacco nectaries express a novel NADPH oxidase implicated in the defence of floral reproductive tissues against micro-organisms. Plant Physiol. 143: 389–399.

Carter, C., S. Sharoni, L.Yehonatan, R.G. Palmer and R.W. Thornburg. 2006. A novel role for proline in plant floral nectars. Naturwissenschaften 93: 72–79.

Carter, C. and R.W. Thornburg. 2000. Tobacco Nectarin I: purification and characterization as a germine-like, manganese superoxide dismutase implicated in the defence of floral reproductive tissues. J. Biol. Chem. 275: 35726–36733.

Carter, C. and R.W. Thornburg. 2004. Is the nectar redox cycle a floral defence against microbial attack? Trends Plant Sci. 9: 320–324.

Chevrot, R., R. Rosen, E. Haudecoeur, A. Cirou, B.J. Shelp, E. Ron and D. Faure. 2006. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. PNAS 103: 7460–7464.

de Vega, C. and C.M. Herrera. 2012. Relationships among nectar dwelling yeasts, flowers and ants: Patterns and incidence on nectar traits. Oikos 121: 1878–1888.

de Vega, C. and C.M. Herrera. 2013. micro-organisms transported by ants induce changes in floral nectar composition of an ant-pollinated plant. Amer. J. Bot. 100: 792–800.

Ehlers, B.K. and J.M. Olesen. 1996. The fruit-wasp route to toxic nectar in *Epipactis* orchids. Flora 192: 223–229.

Farkas, A., Z. Orosz-Kovacs, H. Deri and S.V.S. Chauhan. 2007. Floral nectaries in some apple and pear cultivars with special reference to bacterial fire blight. Curr. Sci. 92: 1286–1289.

Gardener, M.C. and M.P. Gillman. 2001. The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* (Caryophyllaceae). Oikos 92: 101–106.

González-Teuber, M., S. Eilmus, A. Muck, A. Svatos and M. Heil. 2009. Pathogenesis-related proteins protect extrafloral nectar from microbial infestation. Plant J. 58: 464–473.

González-Teuber, M. and M. Heil. 2009. Nectar chemistry is tailored for both attraction of mutualists and protection from exploiters. Plant Signal. Behav. 4: 809–813.

González-Teuber, M., M.J. Pozo, A. Muck, A. Svatos, R.M. Adame-Álvarez and M. Heil. 2010. Glucanases and chitinases as causal agents in the protection of *Acacia* extrafloral nectar from infestation by phytopathogens. Plant Physiol. 152: 1705–1715.

Guerrant, E.O. and P.L Fiedler. 1981. Flower defences against nectar-pilferage by ants. Biotropica 13: 25–33.

Harris, R.C., M.J. Tallon, M. Dunnett, L. Boobis, J. Coakley, H.J. Kim, J.L. Fallowfield, C.A. Hill, C. Sale and J.A. Wise. 2006a. The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. Amino Acids 30: 279–289.

Harris, R., C. Hill, C. Sale, G. Jones, H. Kim, J. Wise and W. Kraemer. 2006b. Effect of 14 days beta-alanine supplementation on isometric endurance of the knee extensors. Med. Sci. Sports Exerc. 38: 125–126.

Heil, M. 2011. Nectar: generation, regulation and ecological functions. Trends Plant Sci. 16: 191–200.

Herrera, C.M., I.M. García and R. Pérez. 2008. Invisible floral larcenies: microbial communities degrade floral nectar of bumblebee-pollinated plants. Ecology 89: 2369–2376.

Herrera, C.M., C. de Vega, A. Canto and M.I. Pozo. 2009. Yeasts in floral nectar: a quantitative survey. Ann. Bot. 103: 1415–1423.

Herrera, C.M., M.I. Pozo and M. Medrano. 2013. Yeasts in nectar of an early-blooming herb: sought by bumble bees, detrimental to plant fecundity. Ecology 94: 273–279.
Hill, C.A., R.C. Harris, H.J. Kim, B.D. Harris, C. Sale, L.H. Boobis, C.K. Kim and J.A. Wise. 2007. Influence of β-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. Amino Acids 32: 225–33.
Hillwig, M.S., C. Kanobe, R.W. Thornburg and G.C. MacIntosh. 2011. Identification of S-RNase and peroxidase in *Petunia* nectar. J. Plant Physiol. 168: 734–738.
Hillwig, M.S., X. Liu, G. Liu, R.W. Thornburg and G.C. MacIntosh. 2010. *Petunia* nectar proteins have ribonuclease activity. J. Exp. Bot. 61: 2951–2965.
Huang, T., G. Jander and M. de Vos. 2011. Non-protein amino acids in plant defence against insect herbivores: representative cases and opportunities for further functional analysis. Phytochemistry 72: 1531–1537.
Inouye, D.W. and R.S. Inouye. 1980. The amino acids of extrafloral nectar from Helianthella quinquenervis (Asteraceae). Amer. J. Bot. 67: 1394–1396.
Kaczorowski, R.L., M.C. Gardener and T.P. Holtsfor. 2005. Nectar traits in Nicotiana section Alatae (Solanaceae) in relation to floral traits, pollinators, and mating system. Amer. J. Bot. 92: 1270–1283.
Kessler, D., K. Gasse and I.T. Baldwin. 2008. Field experiments with transformed plants reveal the sense of floral scents. Science 321: 1200–1202.
Kessler, D. and T. Baldwin. 2007. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors to *Nicotiana attenuata*. Plant J. 49: 840–854.
Kevan, P.G., D. Eisikowitch, S. Fowle and K. Thomas. 1988. Yeast-contaminated nectar and its effects on bee foraging. J. Apic. Res. 27: 26–29.
Kram, B., E. Bainbridge, M. Perera and C. Carter. 2008. Identification, cloning and characterization of a GDSL lipase secreted into the nectar of *Jacaranda mimosifolia*. Plant Mol. Biol. 68: 173–183.
Manson, J.S., M.C. Otterstatter and J.D. Thomson. 2010. Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162: 81–89.
Medina-Kauwe, L.K., A.J. Tobin, L. De Meirleir, J. Jaeken, C. Jakobs, W.L. Nyhan and K.M. Gibson. 1999. 4-Aminobutyrate aminotransferase (GABA-transaminase) deficiency. J. Inherit. Metab. Dis. 22: 414–427.
Naqvi, S., A. Harper, C. Carter, G. Ren, A. Guirgis, W.S. York and R.W. Thornburg. 2005. Tobacco Nectarin IV is a specific inhibitor of fungal xylosidases secreted into the nectar of ornamental tobacco plants. Plant Physiol. 139: 1389–1400.
Nepi, M., P. Von Aderkas and E. Pacini. 2009. Nector and pollination drop: how different are they? Ann. Bot. 104(2): 205–219.
Nepi, M., L. Bini, L. Bianchi, M. Puglia, M. Abate and G. Cai. 2011. Xylan-degrading enzymes in male and female flower nectar of Cucurbita pepo. Ann. Bot. 108: 521–527.
Nepi, M., C. Soligo, D. Nocentini, M. Abate, M. Guarnieri, G. Cai, L. Bini, M. Puglia, L. Bianchi and E. Pacini. 2012. Amino acids and protein profile in floral nectar: much more than a simple reward. Flora 207: 475–481.
Nicolson, S.W. 2007. Nectar consumers. *In*: S.W. Nicolson, M. Nepi and E. Pacini (eds.). Nectaries and nectar. Springer, Dordrecht, pp. 289–342.
Nicolson, S.W. and R.W. Thornburg. 2007. Nectar chemistry. *In*: S.W. Nicolson, M. Nepi and E. Pacini (eds.). Nectaries and nectar. Springer, Dordrecht, pp. 215–264.
Nocentini, D., E. Pacini, M. Guarnieri and M. Nepi. 2012. Flower morphology, nectar traits and pollinators of *Cerinthe major* (Boraginaceae-Lithospermeae). Flora 207: 186–196.
Oliver, R.P. and P.S. Solomon. 2004. Does the oxidative stress used by plants for defence provide a source of nutrients for pathogenic fungi? Trends Plant Sci. 9: 472–473.
Park, S. and W.R. Thornburg. 2009. Biochemistry of nectar proteins. J. Plant Biol. 52: 27–34.
Passreiter, C.M. and M. Isman. 1997. Antifeedant bioactivity of sesquiterpene lactones from *Neurolaena lobata* and their antagonism by gamma-aminobutyric acid. Biochem. Syst. Ecol. 25: 371–377.

Peay, K.G., M. Belisle and T. Fukami. 2012. Phylogenetic relatedness predicts priority effects in nectar yeast communities. Proc. Royal Soc. B 279: 749–758.

Petanidou, T. 2007. Ecological and evolutionary aspects of floral nectars in Mediterranean habitats. *In*: S.W. Nicolson, M. Nepi and E. Pacini (eds.). Nectaries and nectar. Springer, Dordrecht, pp. 343–375.

Petanidou, T., A. Van Laere, W.N. Ellis and E. Smets. 2006. What shapes amino acid and sugar composition in Mediterranean floral nectars? Oikos 115: 155–169.

Pozo, M.I., M.A. Lachance and C.M. Herrera. 2012. Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in community assembly. FEMS Microbiol. Ecol. 80: 281–293.

Raguso, R.A. 2004. Why are some floral nectars scented? Ecology 85: 1486–1494.

Raguso, R.A. 2009. Floral scent in a whole-plant context: moving beyond pollinator attraction. Funct. Ecol. 23: 837–840.

Sale, C., C.A. Hill, J. Ponte and R.C. 2012. β-alanine supplementation improves isometric endurance of the knee extensor muscles. J. Int. Soc. Sports Nutr. 9: 26.

Sasu, M.A., I. Seidl-Adams, K. Wall, J.A. Winsor and A.G. Stephenson. 2010. Floral ransmission of Erwinia tracheiphila by cucumber beetles in a wild *Cucurbita pepo*. Environ. Entomol. 39: 140–148.

Shelp, B.J., A.W. Bown and D. Faure. 2006. Extracellular γ-aminobutyrate mediates communication between plants and other organisms. Plant Physiol. 142: 1350–1352.

Shoonhoven, L.M., J.J.A. van Loon and M. Dicke. 2005. Insect-Plant Biology. Oxford University Press, Oxford.

Singaravelan, N. 2010. Secondary compounds in nectar: the other side of the coin. *In*: W. Zhang and H. Liu (eds.). Behavioural and chemical ecology. Nova Publishers, New York, pp. 217–238.

Singaravelan, N., G. Nee'man, M. Inbar and I. Izhaki. 2005. Feeding responses of free-flying honeybees, to secondary compounds mimicking floral nectars. J. Chem. Ecol. 31: 2791–2804.

Stevenson, P.A. 1999. Co-localisation of taurine- with transmitter-immunoreactivities in the nervous system of the migratory locust. J. Comp. Neurol. 404: 86–96.

Vranova, V., K. Rejsek, K.R. Skene and P. Formanek. 2011. Non-protein amino acids: plant, soil and ecosystem interactions. Plant Soil 342: 31–48.

Wagner, R., S. Mugnaini, R. Sniezko, D. Hardie, B. Poulis, M. Nepi, E. Pacini and P. von Aderkas. 2007. Proteomic evaluation of gymnosperm pollination drop proteins indicates highly conserved and complex biological functions. Sex Plant Reprod. 20: 181–189.

Watanabe, M., K. Maemura, K. Kanbara, T. Tamayama and H. Hayasaki. 2002. GABA and GABA receptors in the central nervous system and other organs. International Review of Cytology 213: 1–47.

Whitton, P.S., R.H.C. Strang and R.A. Nicholson. 1987. The distribution of taurine in the tissues of some species of insects. Insect Biochem. 17: 573–577.

Wright, G.A., D.D. Baker, M.J. Palmer, D. Stabler, J. A. Mustard, E.F. Power, A.M. Borland and P.C. Stevenson. 2013. Caffeine in floral nectar enhances a pollinator's memory of reward. Science 339: 1202–1204.

Zhang, M., I. Izumi, S. Kagamimori, S. Sokejima, T. Yamagami, Z. Liu and B. Qi. 2004. Role of taurine supplementation to prevent exercise-induced oxidative stress in healthy young men. Amino Acids 26: 203–207.

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Hormonal Status of the Pollen-Pistil System: Role after Pollination

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ABSTRACT

Results of studies of hormonal regulation of petunia (*Petunia hybrida* L.) male gametophyte germination and growth *in vivo*, on surface of receiving stigma as well as in conducting style tissue, and also *in vitro*, on the cultivating medium are presented. The contents of IAA, ABA and cytokinins as well as the rate of ethylene production in the pistils and their parts were measured following compatible and self-incompatible pollinations. The data obtained provide evidence suggesting the participation of phytohormones in pollen-pistil interactions controlling uninterrupted pollen tube growth after compatible pollination and its inhibition after incompatible pollination. For two experimental systems studied, *in vitro* germinating male gametophyte and pollen-pistil, it was established that ethylene is able to control the regulation of development, germination, and growth of male gametophyte and also

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to be involved in the mechanism of gametophyte self-incompatibility as one of the main barriers of self-fertilization and to behave as a regulator of the gametophyte-sporophyte interactions. According to our findings germination and growth of petunia male gametophyte *in vitro* is accompanied by changes in the content of endogenous phytohormones and at the same time exhibits sensitivity to exogenous ones. It was shown that the effects of exogenous phytohormones on petunia male gametophyte germination and growth *in vitro* are related to some changes in the organization of its cytoskeleton. In the experiments carried out on germinating pollen grains, the first data indicating a possible mechanism underlying the action of the phytohormones in question were obtained. It was found that IAA, ABA and gibberellin A_3 are capable of significantly changing the cytoplasmic pH of pollen cells and simultaneously resulting in hyperpolarization of the pollen plasma membrane, thereby indicating the phytohormone-induced stimulation of the activity of H^+-ATPase in this membrane. The above results, taken together, indicate a significant impact of phytohormones on various cellular activities of petunia male gametophyte in the course of the gametophyte-sporophyte interactions in the progame phase of higher plant fertilization.

Keywords: Male gametophyte, actin cytoskeleton, ATPase, phytohormones, ethylene, IAA, Petunia

Introduction

Angiosperms have evolved a system of sexual reproduction where water is not required at the point of fertilization, immobile sperm being delivered directly to the egg by a pollen tube. This key innovation, termed 'siphonogamy', allowed them to reproduce sexually in most terrestrial environments. Before a pollen tube (male gametophyte) can access an ovule to release its two sperms into the embryo sac (female gametophyte) it must first penetrate the gynoecium and navigate its way through this material sporophytic tissue to find an ovule. The events and interactions that occur during this prezygotic cellular and molecular 'courtship' between haploid pollen and diploid gynoecium have been termed the pollen-pistil interaction (Heslop-Harrison 1975) and consist of six key stages: pollen capture and adhesion; 2) pollen hydration; 3) germination of the pollen to produce a pollen tube; 4) penetration of the stigma by the pollen tube; 5) growth of the pollen tube through the stigma and style; 6) entry of the pollen tube into the ovule and discharge of the sperm cells. Understanding the physiological and molecular basis of the cellular interactions that occur during the pollen-pistil interactions involving a continuous exchange of signals between the haploid pollen and the diploid maternal tissue of the pistil (sporophyte) is a major goal for plant biologists, because ultimately we all depend on this

fundamental biological process for our food. Despite the recent exponential increase in the number of molecules implicated in pollen-pistil interactions in different model plant species and the significant progress that has been made in elucidating the molecular identity of these signals and the cellular interactions that they regulate, no general consensus has yet emerged of a universal set of pollen-pistil mediation molecules that regulate a common programme of cellular interactions (Hiscock and Allen 2008).

The first experiments on the involvement of phytohormones [indolyl-3-acetic acid (IAA), zeatin, gibberellic acid (GA), and abscisic acid (ABA)] in fertilization (Stanley and Linskens 1974) allowed researchers to suggest that hormones play a regulatory role in pollen tube germination and extension through pistil tissue. The question of phytohormones involvement in controlling post-pollination events had been discussed for a long time but so far remains unstudied.

Here, results of studies of hormonal regulation of petunia (*Petunia hybrida* L.) male gametophyte germination and growth *in vivo*, on surface of receiving stigma and in conducting style tissue, and also *in vitro*, on cultivating medium are presented.

Hormonal Status of Petunia Pollen-Pistil System

The hormonal status of the pollen-pistil system was investigated in two clones of *Petunia hybrida* L. (Kovaleva and Zakharova 2003). The contents of IAA, ABA, and cytokinins, as well as the rate of ethylene production in the pistils and their parts (stigmas, styles and ovaries) were measured over an 8-hr period following compatible and self-incompatible pollination (Figs. 1–3).

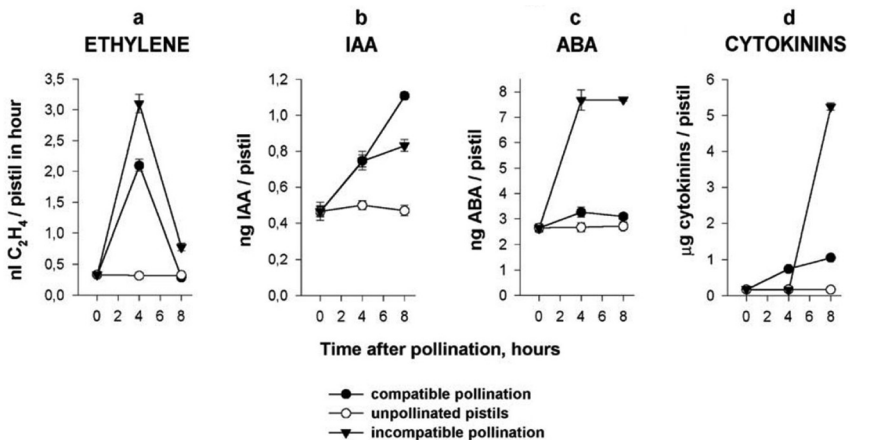

Figure 1. Hormonal status of unpollinated (open circles) and pollinated pistils after compatible (solid circles) and incompatible (solid triangles) pollinations in *Petunia hybrida*.

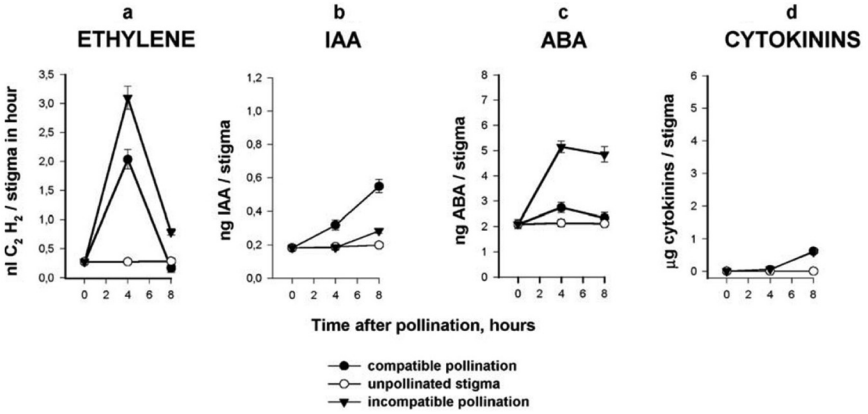

Figure 2. Hormonal status of unpollinated (open circles) and pollinated stigmas after compatible (solid circles) and incompatible (solid triangles) pollinations in *P. hybrida*.

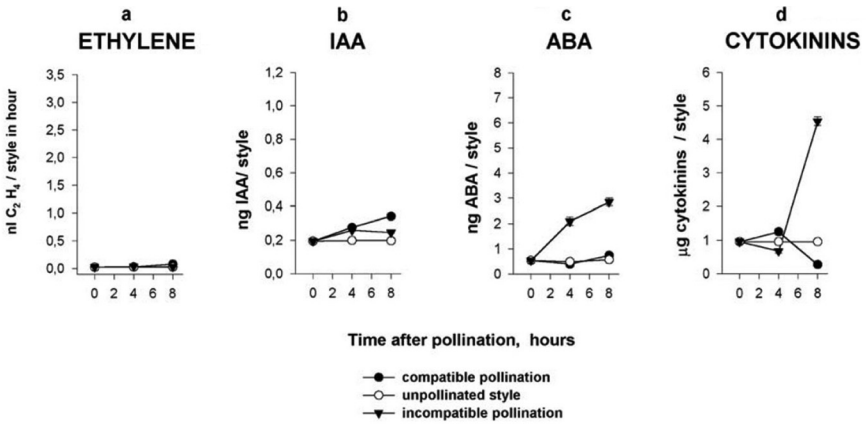

Figure 3. Hormonal status of unpollinated (open circles) and pollinated styles after compatible (solid circles) and incompatible (solid triangles) pollinations in *P. hybrida*.

In both pollinations, the phytohormones were present in various proportions in the stigma, style and ovary: the stigma was the main site of ethylene synthesis and contained 90% of the ABA, while the style contained 80% of the total cytokinin content in the pollinated pistil. Relatively low levels of hormones in the ovary did not influence the hormonal status of the pollen-pistil system. The interaction of the male gametophyte with the stigmatic tissues (adhesion, hydration, and germination of pollen grains) was accompanied by a 7- to 10-fold increase in ethylene production and a 1.5 to 2.0-fold increase in IAA content in the pollen-pistil system over 0–4

hr. Pollen tube germination after self-incompatible pollination, in contrast to compatible pollination, was accompanied by a 3-fold increase in the ABA content in the stigma and style. During the subsequent 4 hr, pollen tube growth was accompanied by some changes in the hormonal status of the pollen-pistil system. Ethylene production by pistil tissues decreased in both cases; however, in the case of incompatible pollination, ethylene content declined more slowly. The IAA content rose in both cases; however, in the case of incompatible pollination, IAA rose more slowly. In both pollinations, the ABA content remained unchanged, but the ABA concentration in the case of incompatible pollination was maintained at the same high level. During incompatible pollination, inhibition of pollen tube growth by 8 hr was accompanied by a 5-fold increase in cytokinin content in style tissues, whereas it remained unchanged during a compatible pollination. The data obtained provide two lines of evidence suggesting the participation of phytohormones in pollen-pistil interactions controlling uninterrupted pollen tube growth after compatible pollination or its inhibition after incompatible pollination. Firstly, a marked difference in the hormonal status of the pollen-pistil system in the course of compatible and incompatible pollination argues in favor of such a hypothesis. Secondly, pollen grain germination on the stigma surface and pollen tube growth in style tissues appears to occur in tissues differing in their hormonal status and to be accompanied by complex alterations of the hormonal concentrations in stigma and style tissues.

Role of Ethylene in the Control of Male Gametophyte Germination and Growth after Self-Compatible and Self-Incompatible Pollination

In flowering plants, pollination of the stigma sets off a cascade of responses in the whole flower that contribute to the successful sexual reproduction in higher plants. Such post-pollination symptoms as petal wilting, pigmentation changes, ovary and ovule development or style and stamen abscission are mediated by ethylene (O'Neill 1997). The responses of the distal floral organs to the pollination event at the stigma surface are regulated by interorgan signaling. Compounds implied in signaling are the gaseous hormone ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Whitehead et al. 1984). Ethylene is produced via a two-step biosynthetic route that starts with the conversion of the Met derivative S-adenosyl-L-Met to ACC and 5'-methyl-thioadenosine by ACC-synthase. Next, ACC is oxidized by ACC-oxidase to form ethylene, CO_2 and HCN (Yang and Hoffman 1984).

Studies performed on the orchid, carnation, tobacco, and petunia indicate that ethylene induced by pollination is necessary for the growth of pollen tubes and successful fertilization (Hoekstra and Weges 1986; Singh et

al. 1992; O'Neill et al.1993; Tang and Woodson 1996; Bui and O'Neill 1998; Holden et al 2003; Kovaleva and Zakharova 2003; Kovaleva et al. 2007, 2011). However, the issue of the physiological role of ethylene in gametophyte-sporophyte interactions in the progamic phase of fertilization both at normal development of the reproductive process and in the presence of genetically determined barriers of self-fertilization is still far from being solved.

The ethylene that is produced upon pollination is characterized by two peaks of its evolving. The first ethylene burst evolves from the stigma and can be attributed mainly to direct conversion of pollen-borne ACC (Hill et al. 1987) by ACC-oxidase that is abundantly present in the stigma (O'Neill 1997). The second peak of ethylene evolving is produced by flower organs that are distal to the stigma, like the style, the ovary, and the petals, and can mainly be attributed to endogenous ACC-synthase and—oxidase activities. These activities correlate closely with the transient increase in expression of the corresponding genes (O'Neill et al. 1993; Tang et al. 1994; Lindstrom et al. 1999; Weterings et al. 2002).

Ethylene production and floral senescence following compatible and incompatible pollinations were studied in a self-incompatible species, *Petunia inflata* (Singh et al. 1992). Both compatible and incompatible pollinations resulted in a burst of ethylene synthesis that peaked 3 hr after pollination. After compatible pollination, a second increase in ethylene synthesis began at 18 hr, and the first sign of senescence appeared at 36 hr. After incompatible pollination, a second increase in ethylene production did not occur until 3d, and the first sign of senescence occurred 12 hr later.

Depending on the type of pollination, germination of petunia (*Petunia hybrida* L.) pollen on the stigma surface and the pollen tube growth in the tissues of style were accompanied by various levels of ACC and ethylene release (Kovaleva et al. 2011). The male gametophyte germination after self-compatible pollination was accompanied by higher content of ACC as compared with the self-incompatible clone, whereas after the self-incompatible pollination we observed a higher level of ethylene production compared with compatible pollination (Fig. 4).

For both types of pollination, ACC and ethylene were predominantly produced in the stigma tissues. Our data indicate the possible participation of ethylene in the mechanism of gametophyte self-incompatibility as one of the main barriers of self-fertilization. This suggestion is confirmed by our data indicating that ethylene at high concentration (10 μl/l) resulted in decreasing by 50% the rate of pollen tube growth on the cultivation medium (Kovaleva et al. 2013). It is believed that one of the reasons for inhibition of the growth of pollen tubes after the self-incompatible pollination can be the programmed cell death (PCD) (Wang et al. 2009) induced by ethylene (Rogers 2006). In this connection, we suggest that intensive production

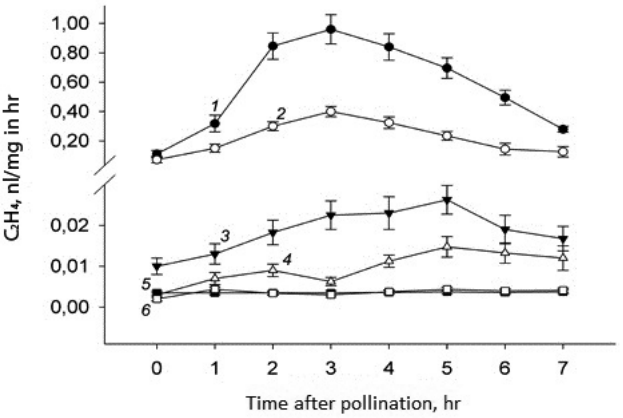

Figure 4. Ethylene evolution by (1, 2) stigmas, (3, 4) styles, and (5, 6) ovaries of the petunia after the self-pollination of the self-compatible (light symbols) and self-incompatible (black symbols) clones.

of ethylene after self-incompatible pollination induces PCD in self-incompatible pollen tubes and thereby leads to retardation of their growth in conducting style tissues.

Very recently, we have obtained preliminary data that may be considered as evidence for ethylene involvement in the mechanism of gametophytic self-incompatibility (K. L.V., unpublished data). In the corresponding experiments it was found that treatment of petunia stigmas of self-incompatible clone with inhibitor of ethylene synthesis resulted in significant stimulation of this incompatible pollen tube growth and the resulting length of pollen tubes appeared to exceed more than two times that of the control pollen tubes. In this connection, it is significant to note that in style tissues where an extension growth of these pollen tubes occurred any signs of programmed cell death (PCD) were not revealed, unlike the control growing pollen tubes (self-pollination of self-incompatible petunia clone) where in 8 h after self-pollination, i.e., during the development of self-incompatibility reaction, clear signs of the PCD involving DNA degradation were observed.

In general, all these results obtained for the two experimental systems studied (anther-male gametophyte and pollen-pistil) (Kovaleva et al. 2007, 2011, 2013) allow us to conclude that ethylene controls the regulation of germination, development and growth of male gametophyte. Our recent results (K.L.V., unpublished data) suggest that ethylene is able to behave as a regulator of the gametophyte-sporophyte interactions in the progame phase of higher plant fertilization by exerting its affect on the base of its interaction with other phytohormones, such as IAA, ABA and gibberellins, in the course of the biosynthesis of ACC. In this connection, recent results

published by Carbonell-Bejerano et al. (2011) and concerning pistil senescence in Arabidopsis is of great interest because here it was found that ethylene is involved in pistil fate by modulating the onset of ovule senescence and the GA-mediated fruit set. Another interesting result very recently obtained is that ethylene is capable of serving a key regulator of autophagy in petunia petal senescence and autophagy is induced by pollination (Shibuya et al. 2013).

Hormonal Status of *in vitro* Germinating Petunia Male Gametophyte

Endogenous level of phytohormones in petunia pollen was shown to undergo pronounced changes in the course of its germinationon the cultivation medium (0.4 M sucrose and 1.6 mM H_3BO_3) (Fig. 5) (Kovaleva et al. 2005).

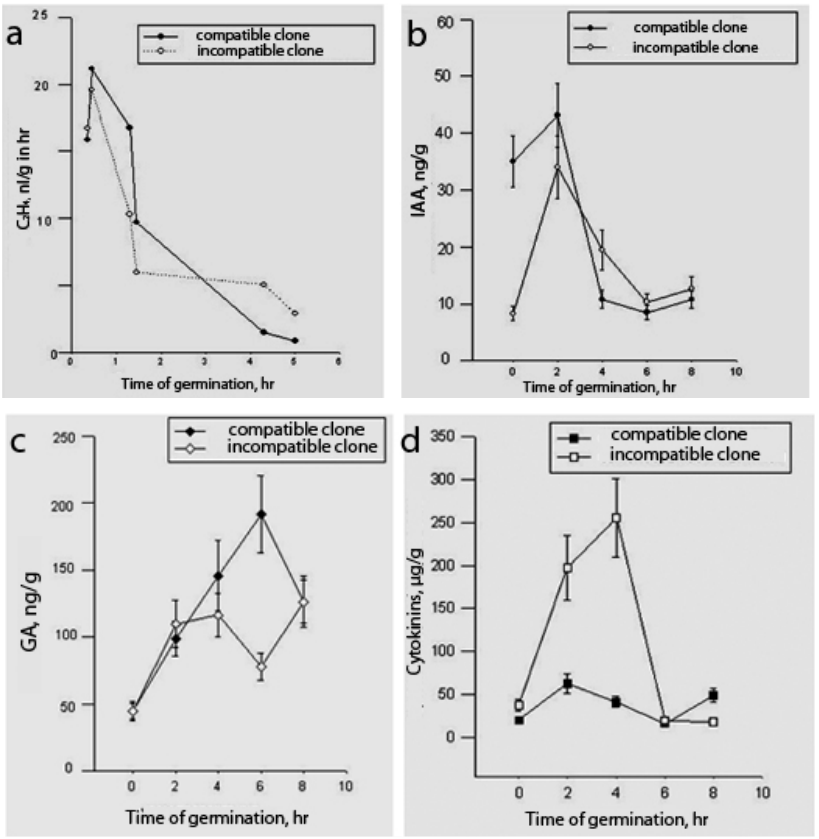

Figure 5. Hormonal balance of *in vitro* germinating petunia male gametophyte (a). Ethylene, (b) IAA, (c) gibberellins, (d) cytokinins.

On stage of hydration and germination of pollen grains ABA content in them declined practically up to zero, while levels of GA, IAA and cytokinins exhibited 1.5–2.0-fold increase. Subsequent growth of pollen tubes was companied by two-fold increase in GA content and marked decrease in IAA and cytokinin levels. Chen and Zhao (2008) in the experiments carried out on *Nicotiana tobacco* found that the IAA content was markedly high in part of the styles before pollen tubes penetrated into them and then gradually decreased when the latter reached the style region.

Application of phytohormones to the cultivation medium significantly influenced the germination and growth of petunia male gametophyte (Table 1 and 2).

ABA and gibberellin A_3 at concentrations of 10^{-12} M to 10^{-3} M markedly stimulated the germination of pollen grains, whereas, IAA at 10^{-12} M to 10^{-10} M concentrations were stimulatory but higher concentrations (10^{-4}–10^{-3} M) were inhibitory. Synthetic cytokinin 6-BAP at concentrations of 10^{-12} M to 10^{-3} M inhibited the germination.

Pollen tube length was measured after 1hr of pollen cultivation in the medium containing 0.4 M sucrose + and 1.6mM H_3BO_3. The data are the means and their standard deviations obtained from three independent experiments carried out in two replicates (n = 6).

The phytohormones that were tested (ABA, gibberellin A_3 and IAA), stimulated the petunia pollen grain germination and pollen tube growth to different extent. Gibberellin A_3 and ABA appeared to be most stimulatory hormones in their action upon pollen germination at concentration of 10^{-12} M. GA_3 exerted a maximal effect on pollen tube growth: after 6-h cultivation

Table 1. Effects of exogenous phytohormones on *in vitro* germination of petunia pollen grains 0.5 hr after their cultivation medium (0.4 M sucrose + 1.6 mM H_3BO_3) (and intensity of this process on the medium = 100% (C)).

Phytohormone	**10^{-12} M**	**10^{-10} M**	**10^{-8} M**	**10^{-6} M**	**10^{-4} M**	**10^{-3} M**
ABA	327	291	198	168	151	112
Gibberellin A_3	278	235	227	219	200	163
IAA	151	136	127	121	101	0
6-BAP	100	83	75	70	65	0

Table 2. Effects of exogenous phytohormones on *in vitro* growth of petunia pollen tube growth (μm) (an intensity of this process on the medium =100% (C).

Phytohormone	**10^{-12} M**	**10^{-10} M**	**10^{-8} M**	**10^{-6} M**	**10^{-4} M**	**10^{-3} M**
Gibberellin A_3	313	296	288	283	244	210
ABA	249	208	209	205	127	100
IAA	204	179	166	156	150	0
6-BAP	100	92	87	75	70	0

their length was 450 μm. IAA at concentration of 10^{-12} M stimulated pollen germination and pollen tube growth 1.5- and 2–2.5-fold, respectively. At the same time, after addition of paclobutrazol, a known inhibitor of gibberellin synthesis, to the cultivation medium the pollen still germinated but under these conditions the length of pollen tubes appeared to be 2–3-fold lower as compared to the control pollen tubes and depended on concentration of the inhibitor.

ABA at concentration of 10^{-12} M exerted a maximal, three-fold stimulation of pollen germination in the first 30 min and 2–3 fold stimulation of pollen germination and pollen tube growth 1.0 h after cultivation. Fluridone, a known inhibitor of ABA synthesis, was found to suppress both pollen germination and pollen tube growth, and intensity of this effect depended on concentration of this inhibitor.

IAA at concentration of 10^{-12} M stimulated pollen germination and pollen tube growth 1.5- and 2–2.5-fold, respectively, with the stimulation of the latter process observed only at low enough concentrations of this hormone, whereas its high concentrations resulted in inhibition of the given process. The 2,4-chlorphenoxy-2-methylpropionic acid, a known inhibitor of IAA transport, at concentration of 10^{-3} M completely blocked the pollen germination in both absence or presence of phytohormones, such as ABA and gibberellin A_3, in the cultivation medium.

In the presence of 6-BAP, in contrast, only suppression of the processes in questions was observed, and such a character of action of this hormone did not depend on its concentration in the cultivation medium.

Based on these results, we put forward the hypothesis that polar transport of IAA impacts the germination and growth of male gametophyte, while ABAis involved in the regulation of their intra-cellular osmotic pressure during these processes. In this connection, an experimental approach for studying the role of ABA in the processes under study is highly intriguing if we take into account the putative signal transduction pathway for this hormone associated with pollen dehydration and leading to corresponding regulation of Rop gene expression (Hsu et al. 2010). Our experiments also showed the involvement of gibberellins in the regulation of pollen tube growth. Earlier, requirement of gibberellins for pollen tube growth was established in the experiments carried out on Arabidopsis mutants characterized by both decreased and increased level of gibberellins (Singh et al. 2002).

Evidence Indicating Impact of Phytohormones on Some Cellular Activities of Petunia Male Gametophyte

It is well known that both pollen germination and maintenance of polar growth of pollen tube require temporal and spatial coordination of many

cell functions including dynamic organization of cytoskeleton elements, intracellular vesicular transport delivering the material for cell wall production during exo- and endocytosis, transmembrane transport of basic physiologically important ions (H^+, Ca^{2+}, K^+), and, in addition, transient changes of some parameters of intracellular ionic homeostasis, such as pH and pCa (Franklin-Tong 1999; Vidali and Hepler 2001; Holdaway-Clarke and Hepler 2003; Certal et al. 2008; Cheung and Wu 2008).

Effects of exogenous phytohormones on the actin cytoskeleton of petunia male gametophyte

In the course of our studies it has been established that the effects of exogenous phytohormones on petunia male gametophyte germination and growth *in vitro* are related to some changes in organization of its actin cytoskeleton (Voronkov 2010). In particular, it was found that IAA at concentrations of 10^{-12} and 10^{-6} M caused the increase by 37% of total amount of actin filaments of pollen tube, and this was expressed in enhancement of fluorescence of these structures stained with FITS-falloidine (Fig. 6), with the largest effect was observed in both apical and subapical regions of pollen tube.

Thus IAA added to the cultivation medium resulted in acceleration of growth of pollen tube through increasing the amount of polymeric actin in the regions in question having the most important significance for maintenance of the polar growth process.

Unlike the above IAA action, stimulating influence of ABA and GA_3 on pollen tube growth was accompanied only by tube zonal redistribution of F-actin, although a total amount of polymer actin in pollen tube remained unchanged (Fig. 6).

Kinetin, unlike IAA, ABA and GA_3, led to suppression of actin polymerization in pollen tubes decreasing a density of actin filaments by about 40% along all the length of pollen tube (Fig. 6), with the strongest effect observed in its basal part.

The above data provide evidence for sensitivity of actin cytoskeleton of germinating petunia male gametophyte to the action of exogenous phytohormones. Here, IAA and kinetin exhibited the most pronounced effects. IAA resulted in accelerating growth of pollen tubes by enhancing the content of polymeric actin in their apical and subapical zones, whereas kinetin, in contrast, inhibited their growth by decreasing the content of polymeric actin in all zones of the tube.

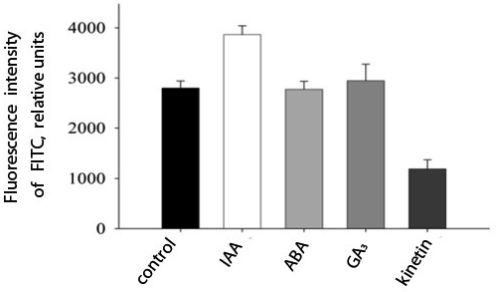

Figure 6. Effects of phytohormones (10^{-6} M) on total amount of polymeric actin in petunia pollen tube. Polymeric actin was stained with phalloidine in phosphate buffer.

Evidence for possible role of phytohormones in petunia male gametophyte in the energization of pollen plasma membrane due to the activity of H^+-ATPase during pollen germination and pollen tube growth

Taking into account the above findings concerning the phytohormone-induced stimulation of germination and growth of petunia male gametophyte in subsequent experiments we attempted to elucidate the mechanism underlying such an action of the phytohormones in question. First of all, it was found that the latter, namely IAA and ABA, are capable of significantly changing cytoplasmic pH (pH_c) of pollen cells, namely resulting in alkalinization of intracellular medium (Fig. 7) (Andreev et al. 2007).

In this connection, it is important to note that the observed increase of intracellular pH appeared to be abolished by orthovanadate, a known inhibitor of P-type ATPases in cell membranes of eukaryotes including H^+-ATPase in the plasma membrane of plant cells. It is known that this

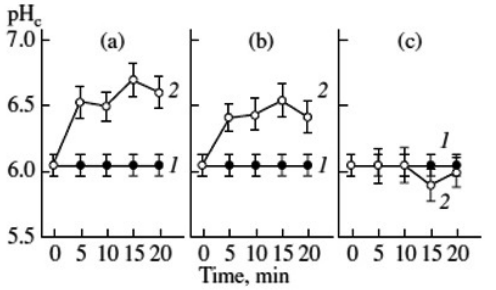

Figure 7. The effects of hormones on pH_c of germinating petunia pollen grains. (a) IAA, (b) ABA, and (c) GA_3 were added to the suspension of pollen grains up to the final concentration of 10 μM 10 min before fluorescence measurements. (*1*) Control; (*2*) hormone.

enzyme acting as proton pump plays very important role in plant cell physiology thanks to its capability to energize the plasma membrane by means of generation of transmembrane proton gradient on it and thereby fueling transport of various ions and metabolites through this membrane. Based on these observations we suggested that the phytohormone-induced shift in pH_c is due to activation of the given proton pump. In order to test further a validity of this hypothesis other series of our experiments had a purpose to elucidate whether the phytohormones used are able to exert a hyperpolarization of plasmalemma of the same pollen cells. In these experiments transmembrane potential ($\Delta\Psi$) changes induced by the phytohormones were followed with voltage-sensitive dyes, the cationic probes Dis-C_3-(5) (not shown) and safranin O, often applied to monitor the membrane potential (negative inside) and ion permeability in a variety of cells of different origin. As follows from Fig. 8, addition of IAA to pollen grain suspension in the K^+-free assay medium immediately initiated their plasma membrane hyperpolarization, as judged by increase in differential

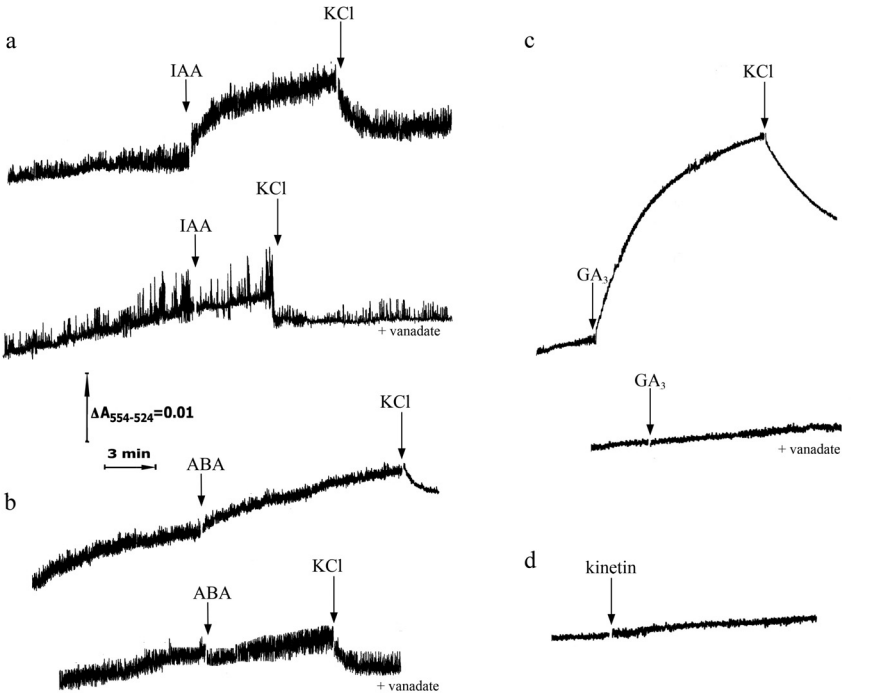

Figure 8. Effects of phytohormones on the membrane potential of petunia male gametophyte. Where indicated, 2, 5 μM IAA(a), ABA(b) or GA_3(c) and kinetin(d) and 60 mM KCl were added to pollen grain suspension. Hyperpolarization of pollen plasma membrane under the action of phytohormones was monitored with potential-sensitive probe safranin O.

absorption of safranin O related to inward flow of the dye across the membrane, with the effect achieving a saturation level in approximately 10–15 min. Reversal of the hormone-induced fluorescence quenching due to leakage of the dye was observed after addition of KCl (60 mM) to pollen grain suspension as a result of the depolarization of the pollen cells, while subsequent addition of the K^+-ionophore valinomycin had practically no effect on the K^+-induced dissipation of the membrane potential. In the given case, a sensitivity of the hormone-induced effect to the external potassium ions can be considered as one of the suitable approaches to specifically select a related dye response to the plasma membrane energization from the total dye signal. To test the contribution of H^+-ATPase pump to the plasma membrane hyperpolarization due to IAA, orthovanadate, the above specific inhibitor of plasmalemma ATP-driven H^+-pump in plant cells, was used. This, as follows from Fig. 8, completely abolished effect caused by IAA indicating that the proton pump was active, exhibited an electrogenic activity and most likely was involved in the IAA-induced hyperpolarization of the plasma membrane. According to our results in fact, similar effects were observed by us in the presence of ABA and GA_3 as well but we did not practically observe any effect in the case of kinetin (Fig. 8).

The fact that the membrane hyperpolarization driven by the phytohormones is indeed affected through the stimulation of ATP-dependent plasmalemma proton pump is additionally confirmed by the data obtained with fusicoccin, a known stimulator of plant plasma membrane H^+-ATPases. This fungus toxin induced pronounced effect greatly resembling the action of IAA or ABA, and in its presence the observed effect of these phytohormones was completely abolished. Recently, we have evidence presented for involvement of both Ca^{2+} ions entering into petunia pollen grain cells from the external medium and active oxygen species in transduction of the hormonal signal triggered by IAA (Voronkov et al. 2010). In addition, it is important to note that all the above effects of phytohormones were observed by us only in the case of viable, germinating pollen grains. To our knowledge, the above data, taken together, indicated, for the first time, one of possible functions of phytohormones in cell of male gametophyte during its germination related to activation of the plasmalemma H^+-ATPase, whose fundamental role in germination and growth of male gametophyte becomes increasingly evident (Certal et al. 2010).

In conclusion, it is important to note that despite the fact that the mechanisms by which IAA and other phytohormones regulate pollen tube growth are still poorly understood. To date it is known that the IAA-induced changes underlying this process involve not only stimulation of the activity of H^+-ATPase but indeed some other effects as well, such as the increase in secretory vesicles, mitochondria and the modification of

pectin and cellulose microfibrils in the tube wall (Wu et al. 2008). In this connection, it cannot be excluded the possibility that the above impact of IAA on pollen plasma membrane H^+-ATPase resulting in corresponding shifts in the membrane potential and the cytosolic pH of pollen cells may serve as a certain stimulus triggering signal transduction pathways, leading to changes in expression of specific genes encoding the proteins required for initiation of the processes involved in the polar growth of pollen tubes. Although it is known that, at present, modeling pollen tube growth largely neglects the roles of phytohormones (Michard et al. 2009; Liu and Hussey 2011), our results may provide a certain base for taking them into account as well as upon developing its future models. It is clear, however, that such modeling will require much more information about the action of phytohormones on different key functions underlying pollen tube growth regulation compared to that accumulated to date.

References

Andreev, I.M., G.V. Timofeeva, Yu.V. Minkina and L.V. Kovaleva. 2007. Effects of exogenous phytohormones on intracellular pH of *Petunia hybrida* pollen grains. Russ. J. Plant Physiol. 54: 626–632.

Bui, A.Q. and S.D. O'Neill. 1998. Three 1-aminocyclopropane-1-carboxylate synthase genes regulated by primary and secondary pollination signals in orchid flowers. Plant Physiol. 116: 419–428.

Carbonell-Bejerano, P., C. Urbez, A. Granell, J. Carbonell and M.A. Ferez-Armador. 2011. Ethylene is involved in pistil fate by modulating the onset of ovule senescence and the GA-mediated fruit set in *Arabidopsis*. BMC Plant Biol. 11: 84–92.

Certal, A.C., R.B. Almeida, L.M. Carvalho, E. Wong, N. Moreno, E. Michard, J. Carmeiro, J. Rodriguezleon, H.M. Wu, A.Y. Cheung and J.A. Feijo. 2008. Exclusion of a proton ATPase from the apical membrane is associated with cell polarity and tip growth in *Nicotiana tabacum* pollen tubes. Plant Cell 20: 614–634.

Chen, D. and J. Zhao. 2008. Free IAA in stigmas and styles during pollen germination and pollen tube growth of *Nicotiana tabacum*. Physiol. Plant. 134: 202–215.

Cheung, A.Y. and H.M. Wu. 2008. Structural and signaling networks for the polar cell growth machinery in pollen tubes. Annu. Rev. Plant Biol. 59: 542–572.

Franklin-Tong, V.E. 1999. Signaling and the modulation of pollen tube growth. Plant Cell 11: 727–738.

Heslop-Harrison, J. 1975. Incompatibility and the pollen-stigma interaction. Annu. Rev. of Plant Physiol. 26: 403–425.

Hill, S.E., A.D. Stead and R. Nichols. 1987. Pollination-induced ethylene and production of 1-aminocyclopropane-1-carboxylic acid by pollen of Nicotiana tabacum cv. White Burley. J. Plant Growth Regul. 6: 1–13.

Hiscock, S. and A.M. Allen. 2008. Diverse cell signaling pathways regulate pollen-stigma interactions: the search for consensus. New Phytol. 179: 286–317.

Hoekstra, F.A. and R. Weges. 1986. Lack of control by early pistillate ethylene of the accelerated wilting of *Petunia hybrida* styles. Plant Physiol. 80: 4–6.

Holdaway-Clarke, T.L. and P.K. Hepler. 2003. Control of pollen tube growth: role of ion gradient and fluxes. New Phytol. 159: 539–563.

Holden, M.J., J.A. Marty and A. Singh-Cundy. 2003. Pollination-induced ethylene promotes the early phase of pollen tube growth in *Petunia inflata*. J. Plant Physiol. 160: 261–269.

Hsu, S.-W., C.-L. Cheng., T.-C.J. Tzen and C.-S. Wang. 2010. RopGTPase and its target Cdc42/Rac-interactive-binding motif-containing protein genes respond to desiccation during pollen maturation. Plant Cell Physiol. 51(7): 1197–1209.

Kovaleva, L. and E. Zakharova. 2003. Hormonal status of pollen –pistil system at the progamic phase of fertilization after compatible and incompatible pollination in *Petunia hybrida* L. Sex. Plant Reprod. 16: 191–196.

Kovaleva, L.V., E.V. Zakharova, Yu.V. Minkina, G.V. Timofeeva and I.M. Andreev. 2005. Male gametophyte germination and growth *in vitro* are sensitive to action of exogenous phytohormones and accompanied by change of endogenous hormone level. Russ. J. Plant Physiol. 52: 584–590.

Kovaleva, L.V., G.V. Timofeeva, A.A. Dobrovolskaya and V.Yu. Rakitin. 2007. Ethylene controls the development, germination, and growth of petunia male gametophytes in the progamic phase of fertilization. *In*: A. Ramina et al. (eds.). Advances in Plant Ethylene Research. Springer, New York, pp. 135–139.

Kovaleva, L.V., G.V. Timofeeva, E.V. Zakharova, A.S. Voronkov and V.Yu. Rakitin. 2011. Ethylenes synthesis in *Petunia* stigma tissues governs the growth of pollen tubes in progamic phase of fertilization. Russ. J. Plant Physiol. 58: 402–408.

Kovaleva, L.V., G.V. Timofeeva, G.B. Rodionova, E.V. Zakharova and V.Yu. Rakitin. 2013. Role of ethylene in the control of gametophyte- sporophyte interactions in the course of the progamic phase of fertilization. Russ. J. Dev. Biol. 44: 69–77.

Lindstrom, J.T., C.H. Lei., M.L. Jones and W.R. Woodson. 1999. Accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) in *Petunia* pollen is associated with expression of a pollen-specific ACC synthase late in development. J. Am. Soc. Hotric. Sci. 124: 145–151.

Liu, J. and P.J. Hussey. 2011. Towards the creation of a systems tip growth model for a pollen tube. Plant Signaling & Behavior 6: 520–522.

Michard, E., R.F. Alves and J.A. Feijo. 2009. The role of ion fluxes in polarized cell growth and morphogenesis: The pollen tube as an experimental paradigm. Int. J. Dev. Biol. 53: 1609–1622.

O'Neill, S.D. 1997. Postpollination regulation of flower development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 58: 47–72.

O'Neill, S.D., J.A. Nadeau, X.S. Zhang, A.Q. Bui and A.H. Halevy. 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell 5: 419–432.

Rogers, H.J. 2006. Programmed cell death in floral organs: how and why do flowers die? Ann. Botany 97: 309–315.

Shibuya, K., T. Niki and K. Ichimura. 2013. Pollination induces autophagy in petunia petals via ethylene. J. Exp. Bot. 64: 111–1120.

Singh, A., K.B. Evensen and T.-h. Kao. 1992. Ethylene synthesis and floral senescence following compatible and incompatible pollination in *Petunia inflata*. Plant Physiol. 99: 38–45.

Singh, D.P., A.M. Ermakov and S.M. Swain. 2002. Gibberellins are required for seed development and polln tube growth in *Arabidopsis*. Plant Cell. 14: 3133–3147.

Stanley, R.G. and H.F. Linskens. 1974. Pollen (biology, biochemistry, management). Springer, Berlin, Heidelberg, New York.

Tang, X.Y., A.M.T.R. Gomes, A. Bhatia and W.R. Woodson. 1994. Pistil-specific and ethylene –regulated expression of 1-aminocyclopropane-1 carboxylate oxidase genes in *Petunia* flowers. Plant Cell 6: 1227–1239.

Tang and Woodson 1996. Reference missing in citation.

Vidali, L. and P.K. Hepler. 2001. Characterization and localization of profiling in pollen grains and tubes of *Lilium longiflorum*. Cell Motil. Cytoskeleton 36: 323–338.

Voronkov, A.S. 2010. Participation of actin cytoskeleton and H+-ATPase in hormonal regulation of petunia male gametophyte growth. M.S. Thesis, Institute of Plant Physiology RAS, Moscow.

Voronkov, A.S., I.M. Andreev, G.V. Timofeeva and L.V. Kovaleva. 2010. Electrogenic activity of PM H^+-ATPase in germinating male gametophyte of *Petunia* and its stimulation by

exogenous auxin: mediatory role of calcium and reactive oxygen species. Russ. J. Plant Physiol. 58: 402–408.

Wang, C.L., G.-H. Xu and X.-T. Jiang. 2009. S-RNase triggers mitochondrial alteration and DNA degradation in the incompatible pollen tube of *Pirus pyrifolia in vitro*. Plant J. 57: 220–229.

Weterings, K., M. Pezzotti, M. Cornelissen and C. Mariani. 2002. Dynamic 1-aminocyclopropane-1-carboxylate-synthase and –oxidase transcript accumulation patterns during pollen tube growth in tobacco styles. Plant Physiol. 130: 1190–1200.

Whitehead, C.S., A.H. Halevy and M.S. Reid. 1984. Roles of ethylene and 1-aminocyclopropane-1 carboxylic acid in pollination and wound-induced senescence of *Petunia hybrida* flowers. Physiol. Plant. 61: 643–648.

Wu, J.-Z., Y. Lin, X.-L. Zhang, D.-W. Pang and J. Zhao. 2008. IAA stimulates pollen tube growth and mediates the modification of its wall composition and structure in *Torenia fournieri*. J. Exp. Bot. 59: 2529–2543.

Yang, S.F. and N.E. Hoffman. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35: 155–189.

14

Prevalence of Self-Sterility and other Reproductive Traits in Angiosperm Families with High Diversification Rates

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ABSTRACT

We modeled variation in the absolute diversification rates using two calibrated trees of the angiosperm families and look for traits associated with families exhibiting high, expected or low species richness. Two shifts in the rate of origination of angiosperm families were identified with model fitting methods at 80 and 50 mya and at 65 and 35 mya according to the first and second calibration respectively. Each data set was divided into three separate geological intervals to obtain the maximum likelihood estimates of the rate of speciation, extinction, diversification and extinction fraction of angiosperm families. Diversification rates were significantly different among intervals and

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increased towards the present. 95% CI of the expected species richness of families over time were used to identify angiosperm families with high (only 21), expected (231) or low species richness (147) in both data sets. Six of the 13 traits analyzed with a stepwise logistic regression were significantly associated with high or expected species richness (polymorphic growth habit, presence of self-sterility, apomixis, CAM photosynthesis, dioecy, and zygomorphic flowers), while no trait was associated with low species richness.

Keywords: birth-death model, lineage through time plots, linear regression, stepwise logistic regression, birth and extinction rates, key traits

Introduction

Understanding why rates of speciation and extinction, and therefore net diversification rates, change over geological time and among lineages is one of the fundamental goals of evolutionary biology (Stanley 1979; Raup and Sepkoski 1984; Erwin and Anstey 1995; Heard and Hauser 1995; Nee 2006; Jablonski 2007). Furthermore, it has been suggested that rates speciation and extinction may be affected by long-term macroevolutionary changes such as plate tectonics, climate change and/or episodic catastrophic events (extrinsic factors), as well as by the presence of traits that affect the probabilities of speciation and extinction in species over micro and macroevolutionary time scales (intrinsic factors) (Slowinski and Guyer 1993; Purvis 1996). There is strong paleontological evidence that rates of speciation and extinction have changed over geological time (Stanley 1979; Raup and Sepkoski 1984; Erwin and Anstey 1995; Niklas 1997; Jablonski 2007), and molecular phylogenetic analyses of extant groups point to climate change and other physical (extrinsic) factors as being associated with shifts in diversification rates in the recent past (Erwin and Anstey 1995; Zink and Slowinski 1995; Kadereit et al. 2004).

In addition to the marked influence of extrinsic factors, studies primarily employing sister comparisons or relative rate tests have identified that biotic pollination (Eriksson and Bremer 1992; Dodd et al. 1999; Kay et al. 2008), biotic fruit dispersal (Ricklefs and Renner 1994; Wing and Boucher 1998), herbaceous growth habit (Dodd Silvertown and Chase 1999), polymorphic growth habit (Ricklefs and Renner 1994), floral nectar spurs (Hodges and Arnold 1995; Hodges 1997; Kay et al. 2008), self-sterility (Ferrer and Good 2012) and bilateral symmetry (Sargent 2004) have all being associated with high diversification rates in clades. Sister group comparisons use only topological information to compare differences in species richness between clades that have a common ancestor and cannot be used for comparing species richness between non-sister groups or to distinguish

whether heterogeneous rates are a reflection of a rate increase or decrease between pairs (Sanderson and Donoghue 1995). This problem is shared by all relative rate tests.

Another method used to detect differences in species richness among clades is to compare, based on clade age, the actual species richness of a clade with that expected under specific birth-death models. To take this approach, it is necessary to have both well-calibrated estimated ages of clades and null models describing the stochastic process of speciation (birth) and extinction (death). Extensive research has been conducted on the properties of stochastic birth-death models (Yule 1925; Kendall 1949; Moran 1951; Bailey 1964; Rohatgi 1976; Raup 1985; Foote 1994; Nee 2006). The most commonly used generalized birth-death model defines the probability that a clade that originated with a single species will reach size n after time t depending on the intrinsic per-lineage rate of speciation (λ) and extinction (μ), where the difference between them defines the net diversification rate, $r = \lambda - \mu$, and the relative extinction fraction is defined as $\varepsilon = \mu / \lambda$ (Kendall 1949; Moran 1951). Assuming that a clade survives and that rates of speciation and extinction are uniform across lineages, the number of species in a clade at time t assumes a nearly geometric distribution whose properties are well understood and can be used to devise statistics to test for evidence of non-uniform rates of diversification among clades (Bailey 1964; Magallón and Sanderson 2001; Nee 2001; Ricklefs 2003).

The critical parameter that must be estimated for the generalized birth-death model is the diversification rate, r. In the absence of extinction, the model reduces to the pure-birth model where $r = \lambda$ and then maximum likelihood (ML) estimators of r, such as the Kendall-Moran estimator (Baldwin and Sanderson 1998), depend only on estimates of n and t (Nee 2001). Estimating r is more difficult if extinction rates are not negligible because it is challenging to attain estimates of the extinction rate (μ) without fossil data. However, estimates of r, λ and/or μ can be derived either from species level phylogenies or species richness/clade age data. In the first case, Lineage Through Time (LTT) plots derived from calibrated molecular phylogenies are used to estimate r, λ and/or μ by curve fitting (Stanley 1979; Kubo and Iwasa 1995; Nee 2004) or by analyzing the distribution of waiting times between successive branching points on the tree (Nee May et al. 1994; Baldwin and Sanderson 1998; Rabosky 2006). In the second approach, data on the absolute age of clades and their species richness are used to estimate r assuming the pure-birth or the generalized birth-death process and , then r, λ and μ can all be estimated by ML iteration (Bokma 2003). Although the models used to estimate r are similar for both types of data, estimates of r based on LTT plots are usually made from species-level phylogenies, while those based on species richness/clade age data have been performed at various taxonomic hierarchical levels. A question

of particular interest for this research is the utility of estimating *r* from LTT plots based on higher-order rates of branching, such as the rate of origination of families or orders. Although such estimates of *r* do not reflect the rate of speciation, they have the potential to identify the temporal intervals in which widespread speciation or extinction occurred and, consequently, could estimate the extent to which extrinsic factors influence higher taxon macrophylogenetic processes.

There are several challenges involved in inferring diversification rates from either LTT plots or species richness/clade age data. First, because the branching process is inherently stochastic, the sizes, shapes and branch lengths of phylogenies resulting from the same net diversification rate can be very different (Raup et al. 1973), just as clades of the same age are not expected to have identical species richness (Bailey 1964). Consequently, a critical part of looking for differences in diversification rate or species richness among clades is to construct confidence intervals of their expected value. For species richness/clade age data this can be done by allowing the expected number of species over time to be defined by the mean diversification rate of the group and then calculatingthe upper and lower 2.5% of species expected at time *t* (Magallón and Sanderson 2001). This is the method utilized in this chapter to construct 95% confidence intervals of species richness based on the stochastic process. In addition, we employed ML methods to expand these confidence intervals in order to incorporate uncertainty in the estimate of *r* itself.

But what if diversification rates have shifted over time such that there are not one but multiple values of *r*? Identifying shifts in diversification rate is difficult because apparent shifts can result from "artificial" and/or real factors. As many authors have emphasized, as a lineage increases in age, the probability that all of its descendents go extinct also increases, leading to the observation that younger lineages are more influenced by the rate of speciation and older lineages more influenced by the rate of extinction even if diversification rates have been constant over time (Strathmann and Slatkin 1983; Raup and Sepkoski 1984; Nee May and Harvey 1994). In particular, high values of μ cause an increase in the estimate of *r* in younger compared to older lineages, a phenomenon called the "pull of the present" (Nee Holmes et al. 1994). Incomplete taxon sampling causes the reverse problem in LTT plots such that an apparent slowdown in the rate of origination of new lineages is observed (Nee Holmes et al. 1994). Diversification rates may also change over time because of real changes in intrinsic or extrinsic factors that have an influence on the probability of speciation or extinction. Various methods to identify these shifts based on inferences from LTT plots have been introduced, such as Pybus and Harvey's γ statistic (2000), the construction of confidence intervals via simulation of null phylogenetic trees (Good-Avila et al. 2006; McKenna and Farrell 2006),

and an ML method that can identify increases, decreases and shifts in r using LTT plots (Rabosky 2006).

In this chapter, we look for evidence of shifts in diversification rate inmost of the 454 families of angiosperms as recognized by the APG II, calculate confidence intervals to identify families with high, expected or low species richness in each time interval, and look for intrinsic traits associated with angiosperm family species richness. Previous work on diversification rates in angiosperms has estimated the mean rate of diversification primarily in orders of angiosperms (Magallón and Sanderson 2001; Magallón and Castillo 2009), as well as the mean rate of speciation and extinction in angiosperm orders (Bokma 2003); it has also looked for evidence of the heritability of diversification rates and for imbalances in the species richness of sister clades (Sims and McConway 2003; Davies et al. 2004). Here, we collect information on the species richness, phylogenetic relatedness and date of divergence of 415 and 425 of the 454 angiosperm families using two calibrated topologies, those presented by Wikström et al.(2001) and Davies et al. (2004). Based on this information, we first test for shifts in the rate of origination of angiosperm families using LTT plots to assess if there is evidence that extrinsic factors have influenced the broad patterns of angiosperm diversification. Secondly, using the species richness/family age data, we calculate ML estimates and confidence intervals for r assuming a pure-birth model, and for r, λ, μ, and ε assuming a birth-death model. Finally, after dividing angiosperm families into three intervals that have significantly different diversification rates, we use confidence intervals of the expected species richness to identify those families that have high, expected or low species richness and perform stepwise logistic regression to look for evidence that each of 13 intrinsic factors is associated with family species richness level. This work shows that diversification rates in angiosperm families have not been constant through time and that the timing of the broad shifts are associated with key geological and/or climatic shifts. Furthermore, we find support that 6 intrinsic factors are associated with high species richness.

Shifts in the Diversification Rates of Angiosperms

The topology of all 454 families as given by the Angiosperm Phylogeny Group II (Stevens 2008) was generated and then the age of divergence of the families based on the calibrations of the phylogeny given in Wikström et al. (2001) and Davies et al. (2004) were used to generate a calibrated topology. The Wikström et al. (2001) calibration, was obtained using nonparametric rate smoothing (NPRS Sanderson 2002) based on a topology generated from sequence data of three genes (two plastid, *rbc*L and *atp*B and one nuclear, 18S rRNA) for 560 taxa representing 316 families, while the Davies

et al. (2004) was constructed as a supertree to describe the relationship among 379 clades (mostly families) and then the tree was calibrated also using NPRS but in this case of rbcL sequences only. The former topology and calibration is known to underestimate the age of terminal branches (Wikström et al. 2003), while the latter provides maximum ages for the families (Davies et al. 2004). Because a goal of this analysis was also to examine the characteristics of families with high, expected and low species richness, we aimed to include as many families as possible in our calibrated tree. To obtain the ages of the families based on both calibrations, we started with the dates for the 316 families given by Wikström et al. (2001) or the 379 families given in Davies et al. (2004) and then added families for which there were sister groups present in the AGPII phylogeny. This resulted in a total of 425 and 416 families for the two phylogenies, respectively. The information concerning the names of each family, the orders to which they belong, and the estimated diversification rates of each family based on both calibrations is given in Table 1.

To determine whether the rate of origination of angiosperm families has been constant over geological time, the ultrametric trees were used to construct LTT plots of the rate of origination of all 425 (Wikström topology hereafter) or 416 (Davies topology hereafter) families of angiosperms. The vector of branching times was used to assess whether the rate of diversification of angiosperm families followed either a rate constant (pure-birth or birth-death) or variablerate (density dependent-exponential, density dependent-logistic, pure-birth with two shifts or pure birth with three shifts) model of diversification (Rabosky 2006). Shift points were allowed to occur at any of 150 intervals (~ every million years). To assess whether one of the variablerate models was preferred over the rate-constant models, 1000 random phylogenies were created that contained 454 taxa that diversified according to the birth-death model in which λ=1.0 and μ=0.931 (the MLE of λ and μ for all angiosperms as estimated below); from these trees, 425 or 416 taxa were sampled and the difference in AIC, ΔAIC, between the best rate constant and rate variable model was taken as the test statistic to choose the best model.

Tests aimed at identifying whether the rate of angiosperm diversification has been constant or variable over time revealed that the best model of diversification for both the Wikström and Davies calibrated trees was one that allowed two shifts in the rate of diversification. To determine this, we first simulated 1,000 null phylogenetic trees under a constant rate birth-death process and found that the largest difference in AIC, ΔAIC, that existed between any two topologies was 13.5 (not shown), indicating that 13.5 could be used as the critical value of ΔAIC to reject a constant rate in favour of a variable rate model. Indeed, the constant-rate modeld

Table 1. Maximum likelihood estimates for the diversification rates using Kendall Moran estimator for the families of angiosperms using Wikström et al. (2000) and Davies et al. (2004) estimates of the divergence ages. The order to which families belong, as well as the geological interval in which they appeared and the estimated number of species (according to Stevens 2008) are also presented. In the last two columns, families are classified according to their diversification rates as expected using a stochastic birth-death model, and are designated as having higher, lower or expected (exp.) diversification.Only families that showed a higher or lower number of species than in the analyses for the Wikström and Davies topologies described in the text are included.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Apiaceae	Apiales	3,780	0.1961	0.1326	PostKT	PreEocene	High	High
Apocynaceae	Gentianales	4,555	0.1589	0.1153	PostKT	PreEocene	High	High
Araceae	Alismatales	4,025	0.0748	0.0622	PreKT	Middle Cretaceous	High	High
Asteraceae	Asterales	23,600	0.2288	0.2216	PostKT	Eocene	High	High
Boraginaceae	Boraginales	2,740	0.1028	0.0845	PreKT	Middle Cretaceous	High	High
Brassicaceae	Brassicales	3,710	0.3736	0.1490	Oligocene	PreEocene	High	High
Ericaceae	Ericales	3,995	0.1481	0.0989	PostKT	PreEocene	High	High
Euphorbiaceae	Malpighiales	5,970	0.2229	0.1394	PostKT	PreEocene	High	High
Fabaceae	Fabales	19,400	0.1371	0.1439	PreKT	PreEocene	High	High
Gesneriaceae	Lamiales	3,200	0.1552	0.1292	PostKT	PreEocene	High	High
Lamiaceae	Lamiales	7,173	0.2114	0.2060	PostKT	Eocene	High	High
Lauraceae	Laurales	2,500	0.0860	0.0714	PreKT	Middle Cretaceous	High	High
Malvaceae	Malvales	4,225	0.1465	0.1163	PostKT	PreEocene	High	High
Melastomataceae	Myrtales	4,570	0.1154	0.1163	PreKT	PreEocene	High	High

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Myrtaceae	Myrtales	4,620	0.1259	0.0957	PreKT	Middle Cretaceous	High	High
Orchidaceae	Asparagales	20,250	0.1437	0.0956	PreKT	Middle Cretaceous	High	High
Poaceae	Poales	10,035	0.2710	0.2081	Oligocene	Eocene	High	High
Ranunculaceae	Ranunculales	2,525	0.0900	0.0818	PreKT	Middle Cretaceous	High	High
Rosaceae	Rosales	2,830	0.1046	0.0994	PreKT	PreEocene	High	High
Rubiaceae	Gentianales	10,000	0.1462	0.1113	PostKT	PreEocene	High	High
Solanaceae	Solanales	2,460	0.1201	0.1150	PreKT	PreEocene	High	High
Achatocarpaceae	Caryophyllales	7	0.0748	0.0317	Oligocene	Eocene	Low	Low
Acoraceae	Acorales	3	0.0087	0.0031	PreKT	Middle Cretaceous	Low	Low
Aextoxicaceae	Berberidopsidales	1	0.0000	–0.0074	PreKT	Middle Cretaceous	Low	Low
Agapanthaceae	Asparagales	9	0.0239	0.0386	PreKT	Eocene	Low	Low
Akaniaceae	Brassicales	2	0.0128	0.0000	PostKT	Eocene	Low	Low
Alseuosmiaceae	Asterales	10	0.0349	0.0262	PreKT	PreEocene	Low	Low
Alzateaceae	Myrtales	2	0.0098	0.0000	PreKT	PreEocene	Low	Low
Amborellaceae	Amborellales	1	0.0000	-0.0043	PreKT	Middle Cretaceous	Low	Low
Anarthriaceae	Poales	11	0.0533	0.0288	PostKT	PreEocene	Low	Low
Ancistrocladaceae	Caryophyllales	12	0.0654	0.0379	PostKT	Eocene	Low	Low
Aphanopetalaceae	Saxifragales	2	0.0117	0.0000	PostKT	PreEocene	Low	Low

Aphloiaceae	Crossosomatales	1	0.0000	–0.0064	PreKT	Middle Cretaceous	Low	Low
Aphyllanthaceae	Asparagales	1	0.0000	–0.0151	PostKT	Eocene	Low	Low
Asteropeiaceae	Caryophyllales	8	0.0359	0.0542	PostKT	Eocene	Low	Low
Austrobaileyaceae	Austrobaileyales	2	0.0052	0.0000	PreKT	Middle Cretaceous	Low	Low
Balanopaceae	Malpighiales	9	0.0372	0.0239	PostKT	PreEocene	Low	Low
Barbeuiaceae	Caryophyllales	1	0.0000	–0.0226	PostKT	Eocene	Low	Low
Barbeyaceae	Rosales	1	0.0000	–0.0126	PostKT	PreEocene	Low	Low
Bataceae	Brassicales	2	0.0169	0.0000	PostKT	Eocene	Low	Low
Berberidopsidaceae	Berberidopsidales	3	0.0122	0.0043	PreKT	Middle Cretaceous	Low	Low
Biebersteiniaceae	Sapindales	5	0.0282	0.0140	PostKT	PreEocene	Low	Low
Blandfordiaceae	Asparagales	4	0.0185	0.0093	PreKT	PreEocene	Low	Low
Butomaceae	Alismatales	1	0.0000	–0.0099	PreKT	PreEocene	Low	Low
Byblidaceae	Lamiales	6	0.0366	0.0211	PostKT	PreEocene	Low	Low
Cabombaceae	Nymphaeles	6	0.0299	0.0071	PostKT	Middle Cretaceous	Low	Low
Calycanthaceae	Laurales	11	0.0222	0.0143	PreKT	Middle Cretaceous	Low	Low
Campynemataceae	Liliales	4	0.0156	0.0067	PreKT	Middle Cretaceous	Low	Low
Carlemanniaceae	Lamiales	5	0.0255	0.0156	PostKT	PreEocene	Low	Low
Cephalotaceae	Oxalidales	1	0.0000	–0.0140	PostKT	Eocene	Low	Low
Ceratophyllaceae	Ceratophyllales	2	0.0029	–0.0020	PreKT	Middle Cretaceous	Low	Low

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Cercidiphyllaceae	Saxifragales	2	0.0076	0.0000	PreKT	Middle Cretaceous	Low	Low
Circaeasteraceae	Ranunculales	2	0.0064	0.0000	PreKT	Middle Cretaceous	Low	Low
Columelliaceae	unplaced near Dipsacales	4	0.0190	0.0078	PreKT	Middle Cretaceous	Low	Low
Coriariaceae	Cucurbitales	5	0.0310	0.0151	PostKT	PreEocene	Low	Low
Corynocarpaceae	Cucurbitales	6	0.0345	0.0181	PostKT	PreEocene	Low	Low
Crossosomataceae	Crossosomatales	12	0.0592	0.0562	PostKT	Eocene	Low	Low
Crypteroniaceae	Myrtales	10	0.0320	0.0261	PreKT	PreEocene	Low	Low
Ctenolophonaceae	Malpighiales	3	0.0169	0.0062	PreKT	PreEocene	Low	Low
Cyrillaceae	Ericales	2	0.0128	0.0000	PostKT	PreEocene	Low	Low
Daphniphyllaceae	Saxifragales	10	0.0262	0.0183	PreKT	Middle Cretaceous	Low	Low
Datiscaceae	Cucurbitales	2	0.0122	0.0000	PostKT	PreEocene	Low	Low
Degeneriaceae	Magnoliales	2	0.0098	0.0000	PreKT	Middle Cretaceous	Low	Low
Desfontainiaceae	unplaced near Dipsacales	1	0.0000	–0.0078	PreKT	Middle Cretaceous	Low	Low
Didymelaceae	Buxales	2	0.0061	0.0000	PreKT	Middle Cretaceous	Low	Low
Dioncophyllaceae	Caryophyllales	3	0.0289	0.0086	PostKT	Eocene	Low	Low
Dirachmaceae	Rosales	2	0.0122	0.0000	PostKT	PreEocene	Low	Low
Doryanthaceae	Asparagales	2	0.0224	0.0000	Oligocene	PreEocene	Low	Low

Drosophyllaceae	Caryophyllales	1	0.0000	–0.0108	PostKT	PreEocene	Low	Low
Ecdeiocoleaceae	Poales	2	0.0204	0.0000	Oligocene	Eocene	Low	Low
Emblingiaceae	Brassicales	1	0.0000	–0.0160	PostKT	Eocene	Low	Low
Eucommiaceae	Garryales	1	0.0000	–0.0077	PreKT	Middle Cretaceous	Low	Low
Euphroniaceae	Malpighiales	2	0.0104	–0.0046	PostKT	PreEocene	Low	Low
Eupomatiaceae	Magnoliales	3	0.0128	0.0051	PreKT	PreEocene	Low	Low
Eupteleaceae	Ranunculales	2	0.0057	0.0000	PreKT	Middle Cretaceous	Low	Low
Flagellariaceae	Poales	4	0.0283	0.0093	PostKT	PreEocene	Low	Low
Fouquieriaceae	Ericales	11	0.0292	0.0225	PreKT	PreEocene	Low	Low
Francoaceae	Geraniales	2	0.0117	0.0000	PostKT	Middle Cretaceous	Low	Low
Gelsemiaceae	Gentianales	12	0.0469	0.0279	PostKT	PreEocene	Low	Low
Gomortegaceae	Laurales	1	0.0000	–0.0071	PostKT	Middle Cretaceous	Low	Low
Goupiaceae	Malpighiales	2	0.0107	0.0000	PreKT	PreEocene	Low	Low
Griseliniaceae	Apiales	6	0.0284	0.0143	PostKT	PreEocene	Low	Low
Grubbiaceae	Cornales	3	0.0122	0.0042	PreKT	Middle Cretaceous	Low	Low
Halophytaceae	Caryophyllales	1	0.0000	–0.0247	Oligocene	Eocene	Low	Low
Hanguanaceae	Commelinales	6	0.0249	0.0121	PreKT	Middle Cretaceous	Low	Low
Helwingiaceae	Aquifoliales	3	0.0211	0.0053	PostKT	PreEocene	Low	Low
Himantandraceae	Magnoliales	2	0.0098	0.0000	PreKT	Middle Cretaceous	Low	Low
Huaceae	eurosid I	3	0.0123	0.0047	PreKT	Middle Cretaceous	Low	Low

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Hydatellaceae	Poales	10	0.0480	0.0247	PostKT	PreEocene	Low	Low
Hydnoraceae	Piperales	7	0.0201	0.0104	PreKT	Middle Cretaceous	Low	Low
Hydroleaceae	Solanales	12	0.0377	0.0316	PreKT	PreEocene	Low	Low
Irvingiaceae	Malpighiales	10	0.0384	0.0219	PostKT	PreEocene	Low	Low
Ixerbaceae	Crossosomatales	1	0.0000	-0.0185	PreKT	Eocene	Low	Low
Ixioliriaceae	Asparagales	3	0.0146	0.0054	PreKT	PreEocene	Low	Low
Joinvilleaceae	Poales	2	0.0147	0.0000	PostKT	Eocene	Low	Low
Kirkiaceae	Sapindales	6	0.0373	0.0197	PostKT	PreEocene	Low	Low
Koeberliniaceae	Brassicales	1	0.0000	-0.0126	PostKT	PreEocene	Low	Low
Lactoridaceae	Piperales	1	0.0000	-0.0058	PreKT	Middle Cretaceous	Low	Low
Lanariaceae	Asparagales	1	0.0000	-0.0093	PreKT	PreEocene	Low	Low
Ledocarpaceae	Geraniales	12	0.0319	0.0177	PreKT	Middle Cretaceous	Low	Low
Lepidobotryaceae	Celastrales	3	0.0107	0.0034	PreKT	PreEocene	Low	Low
Limnanthaceae	Brassicales	8	0.0400	0.0212	PostKT	PreEocene	Low	Low
Limnocharitaceae	Alismatales	7	0.0299	0.0129	PreKT	Middle Cretaceous	Low	Low
Lophiocarpaceae	Caryophyllales	6	0.0943	0.0166	Oligocene	PreEocene	Low	Low
Luzuriagaceae	Liliales	5	0.0287	0.0172	PostKT	PreEocene	Low	Low
Mayacaceae	Poales	7	0.0282	0.0168	PreKT	PreEocene	Low	Low

Medusagynaceae	Malpighiales	2	0.0198	0.0000	Oligocene	PreEocene	Low	Low
Medusandraceae	unplaced eudicot	2	0.0100	0.0000	PreKT	PreEocene	Low	Low
Melianthaceae	Geraniales	11	0.0406	0.0168	PostKT	Middle Cretaceous	Low	Low
Montiniaceae	Solanales	5	0.0244	0.0135	PreKT	PreEocene	Low	Low
Moringaceae	Brassicales	12	0.0428	0.0268	PostKT	PreEocene	Low	Low
Muntingiaceae	Malvales	3	0.0203	0.0060	PostKT	PreEocene	Low	Low
Myrothamnaceae	Gunnerales	2	0.0064	0.0000	PreKT	Middle Cretaceous	Low	Low
Nelumbonaceae	Proteales	2	0.0032	-0.0023	PreKT	Middle Cretaceous	Low	Low
Neuradaceae	Malvales	10	0.0344	0.0218	PreKT	PreEocene	Low	Low
Oliniaceae	Myrtales	5	0.0233	0.0166	PreKT	PreEocene	Low	Low
Oncothecaceae	near Garryales euasterid I	2	0.0071	0.0000	PreKT	Middle Cretaceous	Low	Low
Paracryphiaceae	unplaced near Apiales	1	0.0000	-0.0100	PreKT	PreEocene	Low	Low
Paulowniaceae	Lamiales	1	0.0000	-0.0166	PostKT	Eocene	Low	Low
Pennantiaceae	Apiales	4	0.0217	0.0080	PostKT	Middle Cretaceous	Low	Low
Pentadiplandraceae	Brassicales	1	0.0000	-0.0141	Oligocene	Eocene	Low	Low
Penthoraceae	Saxifragales	2	0.0147	0.0000	PostKT	PreEocene	Low	Low
Peridiscaceae	Saxifragales	9	0.0241	0.0148	PreKT	Middle Cretaceous	Low	Low
Petrosaviaceae	Petrosaviales	4	0.0139	0.0058	PreKT	Middle Cretaceous	Low	Low
Phellinaceae	Asterales	12	0.0401	0.0326	PostKT	PreEocene	Low	Low

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Philydraceae	Commelinales	5	0.0227	0.0117	PreKT	PreEocene	Low	Low
Phyllonomaceae	Aquifoliales	4	0.0213	0.0085	PreKT	Middle Cretaceous	Low	Low
Physenaceae	Caryophyllales	2	0.0120	0.0000	PostKT	Eocene	Low	Low
Platanaceae	Proteales	10	0.0213	0.0128	PreKT	Middle Cretaceous	Low	Low
Plocospermataceae	Lamiales	1	0.0000	-0.0090	PreKT	PreEocene	Low	Low
Posidoniaceae	Alismatales	9	0.0244	0.0159	PreKT	Middle Cretaceous	Low	Low
Pterostemonaceae	Saxifragales	3	0.0239	0.0057	PostKT	PreEocene	Low	Low
Quillajaceae	Fabales	3	0.0155	0.0054	PreKT	PreEocene	Low	Low
Rhabdodendraceae	Caryophyllales	3	0.0132	0.0048	PreKT	Middle Cretaceous	Low	Low
Rhoipteleaceae	Fagales	1	0.0000	-0.0136	PostKT	PreEocene	Low	Low
Rhynchocalycaceae	Myrtales	1	0.0000	-0.0120	PreKT	PreEocene	Low	Low
Roridulaceae	Ericales	2	0.0075	0.0000	PreKT	PreEocene	Low	Low
Rousseaceae	Asterales	13	0.0337	0.0242	PreKT	PreEocene	Low	Low
Ruppiaceae	Alismatales	6	0.0262	0.0204	PreKT	Eocene	Low	Low
Salvadoraceae	Brassicales	11	0.0585	0.0506	PostKT	Eocene	Low	Low
Sarcobataceae	Caryophyllales	2	0.0277	0.0000	Oligocene	Eocene	Low	Low
Saururaceae	Piperales	6	0.0199	0.0137	PreKT	Middle Cretaceous	Low	Low
Scheuchzeriaceae	Alismatales	1	0.0000	-0.0067	PreKT	Middle Cretaceous	Low	Low

Setchellanthaceae	Brassicales	1	0.0000	-0.0104	PostKT	PreEocene	Low	Low
Simmondsiaceae	Caryophyllales	1	0.0000	-0.0100	PreKT	PreEocene	Low	Low
Sladeniaceae	Ericales	3	0.0164	0.0048	PreKT	Middle Cretaceous	Low	Low
Sphenocleaceae	Solanales	2	0.0105	0.0000	PreKT	PreEocene	Low	Low
Sphenostemonaceae	unplaced near Apiales	10	0.0281	0.0232	PreKT	PreEocene	Low	Low
Stachyuraceae	Crossosomatales	5	0.0383	0.0287	PostKT	Eocene	Low	Low
Stegnospermataceae	Caryophyllales	3	0.0262	0.0120	PostKT	Eocene	Low	Low
Strasburgeriaceae	Crossosomatales	1	0.0000	-0.0185	PreKT	Eocene	Low	Low
Strelitziaceae	Zingiberales	7	0.0671	0.0190	Oligocene	PreEocene	Low	Low
Surianaceae	Fabales	8	0.0310	0.0217	PreKT	PreEocene	Low	Low
Tapisciaceae	Huerteales	5	0.0183	0.0099	PreKT	Middle Cretaceous	Low	Low
Tetracarpaeaceae	Saxifragales	1	0.0000	-0.0107	PostKT	PreEocene	Low	Low
Tetrachondraceae	Lamiales	3	0.0200	0.0063	PostKT	PreEocene	Low	Low
Tetramelaceae	Cucurbitales	2	0.0136	0.0000	PostKT	PreEocene	Low	Low
Tetrameristaceae	Ericales	5	0.0402	0.0140	PostKT	PreEocene	Low	Low
Thurniaceae	Poales	4	0.0267	0.0121	PostKT	PreEocene	Low	Low
Ticodendraceae	Fagales	1	0.0000	-0.0145	PostKT	Eocene	Low	Low
Torricelliaceae	Apiales	10	0.0384	0.0254	PostKT	PreEocene	Low	Low
Tovariaceae	Brassicales	2	0.0239	0.0000	Oligocene	Eocene	Low	Low
Trimeniaceae	Austrobaileyales	6	0.0193	0.0081	PreKT	Middle Cretaceous	Low	Low

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Trochodendraceae	Trochodendrales	2	0.0056	0.0000	PreKT	Middle Cretaceous	Low	Low
Typhaceae	Poales	11	0.0373	0.0198	PostKT	Middle Cretaceous	Low	Low
Vahliaceae	unplaced euasterid I	8	0.0281	0.0162	PreKT	Middle Cretaceous	Low	Low
Vivianaceae	Geraniales	6	0.0230	0.0108	PreKT	Middle Cretaceous	Low	Low
Xeronemataceae	Asparagales	2	0.0100	0.0000	PreKT	PreEocene	Low	Low
Achariaceae	Malpighiales	150	0.1670	0.0563	Oligocene	PreEocene	Exp	Exp
Actinidiaceae	Ericales	355	0.1049	0.0801	PostKT	PreEocene	Exp	Exp
Adoxaceae	Dipsacales	200	0.0679	0.0534	PreKT	Middle Cretaceous	Exp	Exp
Agavaceae	Asparagales	637	0.1266	0.1252	PostKT	Eocene	Exp	Exp
Aizoaceae	Caryophyllales	2,020	0.3805	0.2258	Oligocene	Eocene	Exp	Exp
Alismataceae	Alismatales	81	0.0676	0.0380	PreKT	Middle Cretaceous	Exp	Exp
Alliaceae	Asparagales	795	0.1484	0.1165	PostKT	PreEocene	Exp	Exp
Alstroemeriaceae	Liliales	165	0.0912	0.0696	PostKT	PreEocene	Exp	Exp
Amaranthaceae	Caryophyllales	2,275	0.2973	0.1782	Oligocene	Eocene	Exp	Exp
Amaryllidaceae	Asparagales	800	0.1215	0.1536	PostKT	Eocene	Exp	Exp
Anacardiaceae	Sapindales	985	0.1467	0.1446	PostKT	Eocene	Exp	Exp
Anisophylleaceae	Cucurbitales	34	0.0526	0.0409	PreKT	PreEocene	Exp	Exp
Annonaceae	Magnoliales	2,220	0.2486	0.0889	Oligocene	PreEocene	Exp	Exp

Aponogetonaceae	Alismatales	43	0.0448	0.0275	PreKT	Middle Cretaceous	Exp	Exp
Aquifoliaceae	Aquifoliales	405	0.1155	0.0689	PostKT	PreEocene	Exp	Exp
Araliaceae	Apiales	1,450	0.1693	0.0948	PostKT	PreEocene	Exp	Exp
Arecaceae	Arecales	2,000	0.1188	0.0684	PostKT	Middle Cretaceous	Exp	Exp
Aristolochiaceae	Piperales	480	0.0556	0.0456	PreKT	Middle Cretaceous	Exp	Exp
Asparagaceae	Asparagales	230	0.1208	0.1083	PostKT	Eocene	Exp	Exp
Asphodelaceae	Asparagales	785	0.1282	0.1139	PostKT	PreEocene	Exp	Exp
Asteliaceae	Asparagales	36	0.0459	0.0386	PreKT	PreEocene	Exp	Exp
Balsaminaceae	Ericales	1,001	0.1502	0.0890	PostKT	PreEocene	Exp	Exp
Basellaceae	Caryophyllales	20	0.0966	0.0821	Oligocene	Eocene	Exp	Exp
Begoniaceae	Cucurbitales	1,401	0.1421	0.1362	PostKT	Eocene	Exp	Exp
Berberidaceae	Ranunculales	701	0.0753	0.0671	PreKT	Middle Cretaceous	Exp	Exp
Betulaceae	Fagales	110	0.1306	0.1206	Oligocene	Eocene	Exp	Exp
Bignoniaceae	Lamiales	800	0.1286	0.1324	PostKT	Eocene	Exp	Exp
Bixaceae	Malvales	21	0.0525	0.0318	PostKT	PreEocene	Exp	Exp
Bonnetiaceae	Malpighiales	35	0.0671	0.0498	PostKT	PreEocene	Exp	Exp
Bromeliaceae	Poales	1,400	0.1050	0.0881	PreKT	PreEocene	Exp	Exp
Brunelliaceae	Oxalidales	55	0.0716	0.0667	PostKT	Eocene	Exp	Exp
Bruniaceae	unplaced near Asterales	75	0.0514	0.0408	PreKT	Middle Cretaceous	Exp	Exp

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Burmanniaceae	Dioscoreales	95	0.0490	0.0383	PreKT	Middle Cretaceous	Exp	Exp
Burseraceae	Sapindales	550	0.1343	0.1310	PostKT	Eocene	Exp	Exp
Buxaceae	Buxales	70	0.0457	0.0286	PreKT	Middle Cretaceous	Exp	Exp
Cactaceae	Caryophyllales	1,500	0.4063	0.2359	Oligocene	Eocene	Exp	Exp
Calceolariaceae	Lamiales	200	0.1000	0.0806	PostKT	PreEocene	Exp	Exp
Calyceraceae	Asterales	60	0.1024	0.0804	PostKT	Eocene	Exp	Exp
Cannabaceae	Rosales	170	0.1116	0.1071	PostKT	Eocene	Exp	Exp
Cannaceae	Zingiberales	19	0.0685	0.0339	PostKT	PreEocene	Exp	Exp
Caprifoliaceae	Dipsacales	220	0.1586	0.0545	Oligocene	Middle Cretaceous	Exp	Exp
Cardiopteridaceae	Aquifoliales	43	0.0418	0.0340	PreKT	Middle Cretaceous	Exp	Exp
Caricaceae	Brassicales	34	0.0608	0.0424	PostKT	PreEocene	Exp	Exp
Caryocaraceae	Malpighiales	21	0.0507	0.0344	PostKT	PreEocene	Exp	Exp
Caryophyllaceae	Caryophyllales	2,200	0.2025	0.1729	PostKT	Eocene	Exp	Exp
Casuarinaceae	Fagales	95	0.1265	0.1162	Oligocene	Eocene	Exp	Exp
Celastraceae	Celastrales	1,300	0.1236	0.1543	PostKT	Eocene	Exp	Exp
Centrolepidaceae	Poales	35	0.0808	0.0484	PostKT	PreEocene	Exp	Exp
Chloranthaceae	Chlorantales	75	0.0306	0.0252	PreKT	Middle Cretaceous	Exp	Exp
Chrysobalanaceae	Malpighiales	460	0.1226	0.0863	PostKT	PreEocene	Exp	Exp

Cistaceae	Malvales	175	0.1435	0.0663	Oligocene	PreEocene	Exp	Exp
Clethraceae	Ericales	76	0.0802	0.0466	PostKT	PreEocene	Exp	Exp
Clusiaceae	Malpighiales	1,050	0.1288	0.1063	PostKT	PreEocene	Exp	Exp
Colchicaceae	Liliales	225	0.0967	0.0886	PostKT	PreEocene	Exp	Exp
Combretaceae	Myrtales	500	0.0956	0.0746	PreKT	PreEocene	Exp	Exp
Commelinaceae	Commelinales	652	0.1045	0.0877	PostKT	PreEocene	Exp	Exp
Connaraceae	Oxalidales	180	0.0731	0.0712	PreKT	PreEocene	Exp	Exp
Convolvulaceae	Solanales	1,601	0.1135	0.1080	PreKT	PreEocene	Exp	Exp
Cornaceae	Cornales	85	0.0488	0.0389	PreKT	Middle Cretaceous	Exp	Exp
Costaceae	Zingiberales	110	0.1068	0.0603	PostKT	PreEocene	Exp	Exp
Crassulaceae	Saxifragales	1,370	0.0938	0.0799	PreKT	Middle Cretaceous	Exp	Exp
Cucurbitaceae	Cucurbitales	845	0.1021	0.1257	PreKT	Eocene	Exp	Exp
Cunoniaceae	Oxalidales	280	0.0880	0.0930	PostKT	PreEocene	Exp	Exp
Cyclanthaceae	Pandanales	225	0.0808	0.0703	PreKT	PreEocene	Exp	Exp
Cymodoceaceae	Alismatales	16	0.0308	0.0219	PreKT	Middle Cretaceous	Exp	Exp
Dasypogonaceae	unplaced commelinid	16	0.0312	0.0195	PreKT	Middle Cretaceous	Exp	Exp
Diapensiaceae	Ericales	18	0.0526	0.0361	PostKT	PreEocene	Exp	Exp
Dichapetalaceae	Malpighiales	165	0.1309	0.0700	PostKT	PreEocene	Exp	Exp
Didiereaceae	Caryophyllales	16	0.0894	0.0741	Oligocene	Eocene	Exp	Exp
Diervillaceae	Dipsacales	16	0.0749	0.0241	PostKT	Middle Cretaceous	Exp	Exp

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Dilleniaceae	Dilleniales	300	0.0548	0.0438	PreKT	Middle Cretaceous	Exp	Exp
Dioscoreaceae	Dioscoreales	870	0.1026	0.0603	PreKT	Middle Cretaceous	Exp	Exp
Dipterocarpaceae	Malvales	680	0.2329	0.0864	Oligocene	PreEocene	Exp	Exp
Droseraceae	Caryophyllales	115	0.0765	0.0609	PostKT	PreEocene	Exp	Exp
Ebenaceae	Ericales	490	0.0826	0.0623	PreKT	Middle Cretaceous	Exp	Exp
Elaeagnaceae	Rosales	45	0.0656	0.0486	PostKT	PreEocene	Exp	Exp
Elaeocarpaceae	Oxalidales	605	0.1124	0.1075	PostKT	PreEocene	Exp	Exp
Elatinaceae	Malpighiales	35	0.0508	0.0499	PreKT	PreEocene	Exp	Exp
Eriocaulaceae	Poales	420	0.1258	0.0820	PostKT	PreEocene	Exp	Exp
Erythroxylaceae	Malpighiales	240	0.1015	0.0627	PostKT	PreEocene	Exp	Exp
Escalloniaceae	unplaced near Apiales	68	0.0570	0.0446	PreKT	PreEocene	Exp	Exp
Fagaceae	Fagales	670	0.1067	0.0911	PostKT	PreEocene	Exp	Exp
Frankeniaceae	Caryophyllales	90	0.1125	0.0850	PostKT	Eocene	Exp	Exp
Gentianaceae	Gentianales	1,655	0.1425	0.1093	PostKT	PreEocene	Exp	Exp
Geraniaceae	Geraniales	805	0.0847	0.0577	PreKT	Middle Cretaceous	Exp	Exp
Goodeniaceae	Asterales	400	0.1498	0.1252	PostKT	Eocene	Exp	Exp
Grossulariaceae	Saxifragales	150	0.0619	0.0609	PreKT	PreEocene	Exp	Exp

Gunneraceae	Gunnerales	45	0.0352	0.0260	PreKT	Middle Cretaceous	Exp	Exp
Gyrostemonaceae	Brassicales	18	0.0741	0.0435	PostKT	PreEocene	Exp	Exp
Haemodoraceae	Commelinales	116	0.0679	0.0522	PreKT	PreEocene	Exp	Exp
Haloragaceae	Saxifragales	145	0.1059	0.0662	PostKT	PreEocene	Exp	Exp
Hamamelidaceae	Saxifragales	82	0.0445	0.0365	PreKT	Middle Cretaceous	Exp	Exp
Heliconiaceae	Zingiberales	150	0.0945	0.0649	PostKT	PreEocene	Exp	Exp
Hemerocallidaceae	Asparagales	85	0.0478	0.0713	PreKT	PreEocene	Exp	Exp
Hernandiaceae	Laurales	55	0.0422	0.0284	PreKT	Middle Cretaceous	Exp	Exp
Humiriaceae	Malpighiales	50	0.0584	0.0454	PreKT	PreEocene	Exp	Exp
Hyacinthaceae	Asparagales	770	0.1477	0.1275	PostKT	Eocene	Exp	Exp
Hydrangeaceae	Cornales	190	0.0846	0.0581	PostKT	PreEocene	Exp	Exp
Hydrocharitaceae	Alismatales	116	0.0731	0.0578	PreKT	PreEocene	Exp	Exp
Hypericaceae	Malpighiales	560	0.1758	0.1279	Oligocene	Eocene	Exp	Exp
Hypoxidaceae	Asparagales	155	0.0731	0.0581	PreKT	PreEocene	Exp	Exp
Icacinaceae	Near Garryales	149	0.0527	0.0437	PreKT	Middle Cretaceous	Exp	Exp
Iridaceae	Asparagales	1,870	0.2511	0.0876	Oligocene	PreEocene	Exp	Exp
Iteaceae	Saxifragales	18	0.0628	0.0310	PostKT	PreEocene	Exp	Exp
Ixonanthaceae	Malpighiales	21	0.0417	0.0306	PreKT	PreEocene	Exp	Exp
Juglandaceae	Fagales	50	0.1029	0.0631	PostKT	PreEocene	Exp	Exp
Juncaceae	Poales	430	0.1555	0.1221	PostKT	Eocene	Exp	Exp

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Juncaginaceae	Alismatales	15	0.0301	0.0332	PreKT	PreEocene	Exp	Exp
Krameriaceae	Zygophyllales	18	0.0452	0.0233	PostKT	Middle Cretaceous	Exp	Exp
Lardizabalaceae	Ranunculales	36	0.0629	0.0274	PostKT	Middle Cretaceous	Exp	Exp
Laxmanniaceae	Asparagales	178	0.0691	0.0958	PreKT	Eocene	Exp	Exp
Lecythidaceae	Ericales	310	0.0700	0.0557	PreKT	Middle Cretaceous	Exp	Exp
Lentibulariaceae	Lamiales	320	0.1109	0.0975	PostKT	PreEocene	Exp	Exp
Liliaceae	Liliales	635	0.1008	0.0910	PostKT	PreEocene	Exp	Exp
Linaceae	Malpighiales	300	0.0803	0.0653	PreKT	PreEocene	Exp	Exp
Linnaeaceae	Dipsacales	36	0.0969	0.0335	PostKT	Middle Cretaceous	Exp	Exp
Loasaceae	Cornales	265	0.0680	0.0623	PreKT	PreEocene	Exp	Exp
Loganiaceae	Gentianales	420	0.1162	0.0870	PostKT	PreEocene	Exp	Exp
Loranthaceae	Santalales	950	0.1071	0.0538	PostKT	Middle Cretaceous	Exp	Exp
Lythraceae	Myrtales	620	0.1021	0.0884	PostKT	PreEocene	Exp	Exp
Maesaceae	Ericales	150	0.1193	0.0738	PostKT	PreEocene	Exp	Exp
Magnoliaceae	Magnoliales	227	0.0583	0.0502	PreKT	Middle Cretaceous	Exp	Exp
Malpighiaceae	Malpighiales	1,250	0.1049	0.0876	PreKT	PreEocene	Exp	Exp
Marantaceae	Zingiberales	550	0.1434	0.0845	PostKT	PreEocene	Exp	Exp
Marcgraviaceae	Ericales	130	0.1217	0.0638	PostKT	PreEocene	Exp	Exp

Martyniaceae	Lamiales	16	0.0630	0.0391	PostKT	PreEocene	Exp	Exp
Melanthiaceae	Liliales	170	0.0597	0.0497	PreKT	Middle Cretaceous	Exp	Exp
Meliaceae	Sapindales	621	0.1786	0.1163	Oligocene	Eocene	Exp	Exp
Menispermaceae	Ranunculales	420	0.0586	0.0516	PreKT	Middle Cretaceous	Exp	Exp
Menyanthaceae	Asterales	40	0.0568	0.0523	PreKT	PreEocene	Exp	Exp
Molluginaceae	Caryophyllales	87	0.1314	0.1344	Oligocene	Eocene	Exp	Exp
Monimiaceae	Laurales	200	0.0582	0.0461	PreKT	Middle Cretaceous	Exp	Exp
Moraceae	Rosales	1,100	0.1522	0.2501	PostKT	Eocene	Exp	Exp
Musaceae	Zingiberales	35	0.0624	0.0430	PostKT	PreEocene	Exp	Exp
Myodocarpaceae	Apiales	19	0.0640	0.0396	PostKT	PreEocene	Exp	Exp
Myricaceae	Fagales	57	0.1064	0.0657	PostKT	PreEocene	Exp	Exp
Myristicaceae	Magnoliales	475	0.0571	0.0539	PreKT	Middle Cretaceous	Exp	Exp
Myrsinaceae	Ericales	1,435	0.2345	0.1792	Oligocene	Eocene	Exp	Exp
Nartheciaceae	Dioscoreales	31	0.0318	0.0240	PreKT	Middle Cretaceous	Exp	Exp
Nepenthaceae	Caryophyllales	90	0.0726	0.0572	PostKT	PreEocene	Exp	Exp
Nothofagaceae	Fagales	35	0.0573	0.0400	PostKT	PreEocene	Exp	Exp
Nyctaginaceae	Caryophyllales	395	0.2300	0.1725	Oligocene	Eocene	Exp	Exp
Nymphaeaceae	Nymphaeles	58	0.0366	0.0218	PreKT	Middle Cretaceous	Exp	Exp
Nyssaceae	Cornales	22	0.0336	0.0247	PreKT	Middle Cretaceous	Exp	Exp
Ochnaceae	Malpighiales	495	0.1773	0.0718	Oligocene	PreEocene	Exp	Exp

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Olacaceae	Santalales	103	0.0527	0.0344	PreKT	Middle Cretaceous	Exp	Exp
Oleaceae	Lamiales	615	0.1019	0.0973	PostKT	PreEocene	Exp	Exp
Onagraceae	Myrtales	650	0.1028	0.0891	PostKT	PreEocene	Exp	Exp
Orobanchaceae	Lamiales	2,061	0.1734	0.1664	PostKT	Eocene	Exp	Exp
Oxalidaceae	Oxalidales	770	0.0936	0.0943	PreKT	PreEocene	Exp	Exp
Paeoniaceae	Saxifragales	33	0.0397	0.0276	PreKT	Middle Cretaceous	Exp	Exp
Pandaceae	Malpighiales	15	0.0410	0.0305	PreKT	PreEocene	Exp	Exp
Pandanaceae	Pandanales	805	0.0999	0.0893	PreKT	PreEocene	Exp	Exp
Papaveraceae	Ranunculales	760	0.0526	0.0480	PreKT	Middle Cretaceous	Exp	Exp
Parnassiaceae	Celastrales	51	0.0678	0.0771	PostKT	Eocene	Exp	Exp
Passifloriaceae	Malpighiales	670	0.1859	0.0761	Oligocene	PreEocene	Exp	Exp
Pedaliaceae	Lamiales	70	0.0885	0.0786	PostKT	Eocene	Exp	Exp
Penaeaceae	Myrtales	20	0.0434	0.0418	PreKT	PreEocene	Exp	Exp
Pentaphragmataceae	Asterales	30	0.0453	0.0371	PreKT	PreEocene	Exp	Exp
Pentaphylacaceae	Ericales	337	0.0869	0.0648	PreKT	PreEocene	Exp	Exp
Phrymaceae	Lamiales	234	0.1299	0.0896	PostKT	PreEocene	Exp	Exp
Phyllanthaceae	Malpighiales	1,745	0.1098	0.0921	PreKT	PreEocene	Exp	Exp
Phytolaccaceae	Caryophyllales	65	0.2087	0.1136	Oligocene	Eocene	Exp	Exp

Picramniaceae	unplaced rosid	46	0.0430	0.0302	PreKT	Middle Cretaceous	Exp	Exp
Picrodendraceae	Malpighiales	85	0.0617	0.0522	PreKT	PreEocene	Exp	Exp
Piperaceae	Piperales	2,015	0.0845	0.0860	PreKT	Middle Cretaceous	Exp	Exp
Pittosporaceae	Apiales	200	0.1152	0.0663	PostKT	PreEocene	Exp	Exp
Plantaginaceae	Lamiales	1,700	0.1305	0.1181	PostKT	PreEocene	Exp	Exp
Plumbaginaceae	Caryophyllales	836	0.1602	0.1019	PostKT	PreEocene	Exp	Exp
Podostemaceae	Malpighiales	270	0.1555	0.1113	Oligocene	Eocene	Exp	Exp
Polemoniaceae	Ericales	350	0.0814	0.0682	PreKT	PreEocene	Exp	Exp
Polygalaceae	Fabales	1,045	0.1038	0.0866	PreKT	PreEocene	Exp	Exp
Polygonaceae	Caryophyllales	1,100	0.2334	0.1065	Oligocene	PreEocene	Exp	Exp
Polyosmaceae	unplaced near Apiales	60	0.0499	0.0430	PreKT	PreEocene	Exp	Exp
Pontederiaceae	Commelinales	33	0.0564	0.0425	PostKT	PreEocene	Exp	Exp
Portulacaceae	Caryophyllales	395	0.3322	0.1884	Oligocene	Eocene	Exp	Exp
Potamogetonaceae	Alismatales	102	0.0712	0.0791	PreKT	Eocene	Exp	Exp
Primulaceae	Ericales	900	0.2194	0.1665	Oligocene	Eocene	Exp	Exp
Proteaceae	Proteales	1,600	0.0683	0.0530	PreKT	Middle Cretaceous	Exp	Exp
Putranjivaceae	Malpighiales	210	0.0786	0.0632	PreKT	PreEocene	Exp	Exp
Quiinaceae	Malpighiales	55	0.1055	0.0432	PostKT	PreEocene	Exp	Exp
Rapateaceae	Poales	94	0.0640	0.0437	PreKT	Middle Cretaceous	Exp	Exp

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Resedacae	Brassicales	75	0.1107	0.0834	PostKT	Eocene	Exp	Exp
Restionaceae	Poales	520	0.1421	0.0939	PostKT	PreEocene	Exp	Exp
Rhamnaceae	Rosales	925	0.1067	0.0958	PostKT	PreEocene	Exp	Exp
Rhizophoraceae	Malpighiales	149	0.0927	0.0564	PostKT	PreEocene	Exp	Exp
Ruscaceae	Asparagales	475	0.1185	0.1249	PostKT	Eocene	Exp	Exp
Rutaceae	Sapindales	1,815	0.1668	0.1276	PostKT	PreEocene	Exp	Exp
Sabiaceae	basal eudicot	100	0.0360	0.0308	PreKT	Middle Cretaceous	Exp	Exp
Salicaceae	Malpighiales	1,010	0.1214	0.1072	PostKT	PreEocene	Exp	Exp
Santalaceae	Santalales	990	0.1061	0.0542	PreKT	Middle Cretaceous	Exp	Exp
Sapindaceae	Sapindales	1,580	0.1315	0.1136	PostKT	PreEocene	Exp	Exp
Sapotaceae	Ericales	1,100	0.1187	0.0677	PostKT	Middle Cretaceous	Exp	Exp
Sarcolaenaceae	Malvales	60	0.1462	0.0504	Oligocene	PreEocene	Exp	Exp
Saxifragaceae	Saxifragales	630	0.0848	0.0812	PreKT	PreEocene	Exp	Exp
Schisandraceae	Austrobaileyales	92	0.0486	0.0283	PreKT	Middle Cretaceous	Exp	Exp
Scrophulariaceae	Lamiales	1,700	0.1305	0.1181	PostKT	PreEocene	Exp	Exp
Simaroubaceae	Sapindales	95	0.1059	0.0782	PostKT	Eocene	Exp	Exp
Siparunaceae	Laurales	75	0.0830	0.0331	PostKT	Middle Cretaceous	Exp	Exp
Smilacaceae	Liliales	315	0.0899	0.0799	PostKT	PreEocene	Exp	Exp

Sphaerosepalaceae	Malvales	18	0.0507	0.0334	PostKT	PreEocene	Exp	Exp
Staphyleaceae	Crossosomatales	45	0.0680	0.0728	PostKT	Eocene	Exp	Exp
Stemonaceae	Pandanales	27	0.0445	0.0281	PreKT	Middle Cretaceous	Exp	Exp
Stemonuraceae	Aquifoliales	80	0.0487	0.0409	PreKT	Middle Cretaceous	Exp	Exp
Stilbaceae	Lamiales	39	0.0718	0.0520	PostKT	PreEocene	Exp	Exp
Stylidiaceae	Asterales	157	0.0722	0.0652	PreKT	PreEocene	Exp	Exp
Styracaceae	Ericales	160	0.0923	0.0721	PostKT	PreEocene	Exp	Exp
Symplocaceae	Ericales	320	0.0916	0.0618	PostKT	Middle Cretaceous	Exp	Exp
Tamaricaceae	Caryophyllales	90	0.1125	0.0850	PostKT	Eocene	Exp	Exp
Tecophilaeaceae	Asparagales	23	0.0418	0.0288	PreKT	Middle Cretaceous	Exp	Exp
Theaceae	Ericales	328	0.0982	0.0620	PostKT	Middle Cretaceous	Exp	Exp
Themidaceae	Asparagales	62	0.0917	0.0736	PostKT	Eocene	Exp	Exp
Theophrastaceae	Ericales	105	0.1135	0.0677	PostKT	PreEocene	Exp	Exp
Thymelaeaceae	Malvales	755	0.2285	0.0819	Oligocene	PreEocene	Exp	Exp
Tofieldiaceae	Alismatales	27	0.0439	0.0213	PreKT	Middle Cretaceous	Exp	Exp
Trigoniaceae	Malpighiales	28	0.0833	0.0419	PostKT	PreEocene	Exp	Exp
Triuridaceae	Pandanales	48	0.0461	0.0285	PreKT	Middle Cretaceous	Exp	Exp
Tropaeolaceae	Brassicales	95	0.0843	0.0691	PostKT	PreEocene	Exp	Exp
Ulmaceae	Rosales	35	0.0624	0.0546	PostKT	PreEocene	Exp	Exp
Urticaceae	Rosales	2,625	0.2715	0.2846	Oligocene	Eocene	Exp	Exp

Table 1. contd....

Table 1. contd.

			Diversification rates		Geological interval		Categories	
Family AGP	**Order**	**Species**	**Wikström**	**Davies**	**Wikström**	**Davies**	**Wikström**	**Davies**
Valerianaceae	Dipsacales	315	0.1085	0.0586	PostKT	Middle Cretaceous	Exp	Exp
Velloziaceae	Pandanales	240	0.0741	0.0517	PreKT	Middle Cretaceous	Exp	Exp
Verbenaceae	Lamiales	1,175	0.1334	0.1605	PostKT	Eocene	Exp	Exp
Violaceae	Malpighiales	800	0.1194	0.1021	PostKT	PreEocene	Exp	Exp
Vitaceae	Vitales	850	0.0625	0.0539	PreKT	Middle Cretaceous	Exp	Exp
Vochysiaceae	Myrtales	210	0.0732	0.0597	PreKT	PreEocene	Exp	Exp
Winteraceae	Canellales	75	0.0436	0.0339	PreKT	Middle Cretaceous	Exp	Exp
Xanthorrhoeaceae	Asparagales	30	0.0654	0.0516	PostKT	PreEocene	Exp	Exp
Xyridaceae	Poales	260	0.1069	0.0747	PostKT	PreEocene	Exp	Exp
Zingiberaceae	Zingiberales	1,188	0.1647	0.0961	PostKT	PreEocene	Exp	Exp
Zosteraceae	Alismatales	14	0.0400	0.0384	PreKT	PreEocene	Exp	Exp
Zygophyllaceae	Zygophyllales	285	0.0883	0.0526	PostKT	Middle Cretaceous	Exp	Exp
Campanulaceae	Asterales	2,300	0.1032	0.1088	PreKT	PreEocene	Exp	High
Schlegeliaceae	Lamiales	25	0.0732	0.0593	PostKT	Eocene	Exp	Low
Acanthaceae	Lamiales	3,500	0.1632	0.1752	PostKT	Eocene	High	Exp
Cyperaceae	Poales	4,350	0.2148	0.1747	PostKT	Eocene	High	Exp
Altingiaceae	Saxifragales	13	0.0262	0.0184	PreKT	Middle Cretaceous	Low	Exp

Argophyllaceae	Asterales	17	0.0457	0.0389	PostKT	PreEocene	Low	Exp
Atherospermataceae	Laurales	16	0.0544	0.0213	PostKT	Middle Cretaceous	Low	Exp
Boryaceae	Asparagales	12	0.0276	0.0207	PreKT	Middle Cretaceous	Low	Exp
Canellaceae	Canellales	13	0.0259	0.0175	PreKT	Middle Cretaceous	Low	Exp
Geissolomataceae	Crossosomatales	1	0.0000	0.0239	PreKT	Middle Cretaceous	Low	Exp
Lacistemataceae	Malpighiales	14	0.0463	0.0335	PostKT	PreEocene	Low	Exp
Lowiaceae	Zingiberales	15	0.0602	0.0306	PostKT	PreEocene	Low	Exp
Morinaceae	Dipsacales	13	0.0484	0.0217	PostKT	Middle Cretaceous	Low	Exp
Nitrariaceae	Sapindales	16	0.0486	0.0317	PostKT	PreEocene	Low	Exp
Sarraceniaceae	Ericales	15	0.0444	0.0276	PostKT	PreEocene	Low	Exp
Geissolomataceae	Crossosomatales	1		-0.0096		PreEocene	None	Low
Guamatelaceae	Crossosomatales	1	0.0000		PostKT		Low	--
Sparganiaceae	Poales	14	0.0419		PostKT		Low	--
Taccaceae	Dioscoreales	12	0.0382		PreKT		Low	--
Capparidaceae	Brassicales	480	0.2205		Oligocene		Exp	--
Dipsacaceae	Dipsacales	290	0.1668		Oligocene		Exp	--
Garryaceae	Garryales	17	0.0350		PreKT		Exp	--
Hydrostachyaceae	Cornales	20	0.0483		PostKT		Exp	--
Malesherbiaceae	Malpighiales	24	0.0963		Oligocene		Exp	--
Opiliaceae	Santalales	72	0.0563		PreKT		Exp	--
Turneraceae	Malpighiales	110	0.1424		Oligocene		Exp	--

always provide a much poorer fit to the data than the variable rate models (Table 2). According to the Wikström topology, the best fit model is a pure-birth model with two shifts in diversification rate occurring at 64 (Cretaceous) and 34 mya (Oligocene); the second best model is the pure-birth model with one shift point at 38 mya (Table 2). For the Davies topology, the best fit model is again the pure-birth model with two shifts points occurring at 80 (Middle Cretaceous) and 51 mya (Eocene), while the second best model is density-dependent with logistic growth in which the carrying capacity of angiosperm families is saturated at 370, but this model is significantly poorer than the two-shift model (Table 2). The difference between the temporal shift points in the two topologies probably reflects differences in their calibration: the Wikström calibration provides MLE of clade ages while those in Davies represent maximum family ages (Sanderson et al. 2004). The importance of these shifts points is graphical presented on the lineage through time plot of the rate of diversification of angiosperm families according to both divergence time estimates (Fig. 1).

The LTT analyses described above revealed that the rate of origination of angiosperm families has not been constant over time but exhibits two shifts in diversification rate based on both the Wikström and Davies

Table 2. Test of the fit of the rate of origination of angiosperm families to each of six stochastic diversification rate models for both the Wikström and Davies topologies. The likelihood of the fit of the branching times of angiosperm families according to the calibration of Wikström and Davies was used to compare the difference in AIC (ΔAIC) between the best constant rate model (Yule, Birth-death (BD), to the best variable rate models, density-dependent exponential growth (DDX), density-dependent logistic growth (DDX)) or pure-birth (Yule) with two or three diversification rates. Parameter estimates of each model are given.

	Wikström			*Davies*		
	Likelihood	**ΔAIC**	**Estimators**	**Likelihood**	**ΔAIC**	**Estimators**
Yule	–77.5	518.7	r = 0.014	–217.13	547.1	r = 0.011
BD	–77.5	520.7	r = 0.014, ε = 0	–217.1	549	r = 0.011, ε = 0
DDX	43.3	279.2	r = 0.67, X = 0.70	–59.7	234	r = 1.78, X = 0.95
DDL	10.7	344.5	r_1= 0.023, k = 869.9	40.9	39.5	r_1 = 0.061, k = 370.8
Yule2-rate	144.32	79.2	r_1=0.027, r_2 = 0.0016st = 38	1.2	115	r_1= 0.025, r_2 = 0.0017st = 58.2
Yule3-rate	185.9	0.00	r_1=0.04, r_2 = 0.016, r_3 = 0.001, st_1 = 64, st_2 = 34	57.7	0.00	r_1 = 0.04, r_2 = 0.011, r_3 = 8.4 x 10–4, st_1 = 80.1, st_2 = 50.3

r=diversification rate, ε =extinction fraction, k=carrying capacity, st=shift time, ΔAIC is the difference in AIC between the best variable rate and best constant rate model. The critical value for a significant difference between the best constant and variable rate models was estimated to be 13.5 via simulation (see text for details).

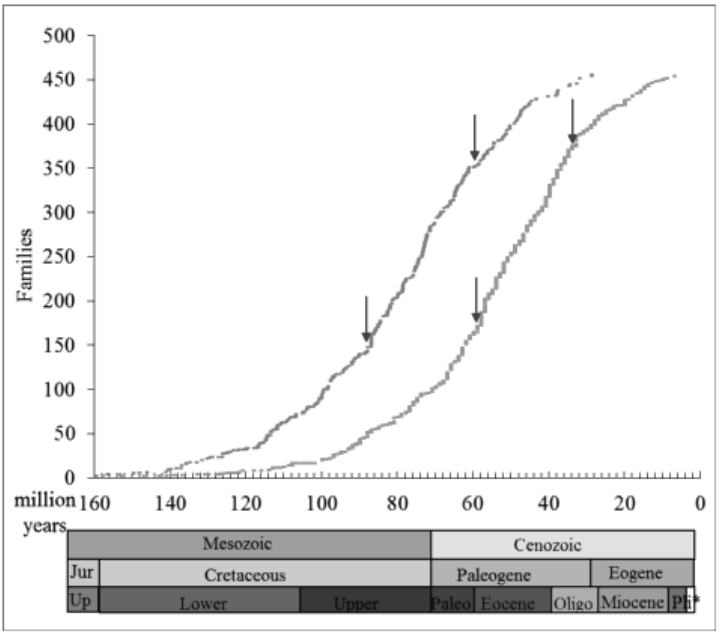

Figure 1. Cumulative number of angiosperm family lineages by divergence age from the modified topologies of Wikström et al. (2001) and Davies et al. (2004). The natural logarithm of the cumulative number of lineages against the ML estimateof the age of the family from Wikström et al. (2001) (solid line) or the maximum age of the family as estimated by Davies et al. (2004) (dashed line).

Color image of this figure appears in the color plate section at the end of the book.

topologies. Based on the Wikström topology, the percent of species (from all angiosperms species included in our analysis) in families that arose: a) prior to 64 mya was of 37.48%; b) between 64 and 34 mya was of 48.60%; and c) in the last 33 mya was of 13.91% (Table 3). The same figures based on the Davies topology were that families that originated: a) prior to 80 mya included 22.19% of the total number of species; b) between 80 and 51 mya harboured of 47.35%; and c) and those that originated during the last 50 mya included 30.46% of the species (Table 3). These differences in the number of species belonging to families originating in the three pediods suggests that average diversification rates between different geological periods should differ for all families included in each of the different periods in both topologies.

Table 3. Number of families and species per Geological intervals. The number of families included in the 95% confidence intervals describing the birth-death stochastic process of angiosperm families across all time periods or within the three time periods were found to have significantly different mean diversification rates. Additionally, the number and percent of families having lower or higher SR than expected is also given. Numbers in brackets refer to the minimum and maximum number of families that fall into each category (expected, low or high) taking into consideration uncertainty in the estimate of r, λ, μ.

Geological intervals	Number of families	Minimum and maximum number of Families with expected SR	Minimum and maximum number of Families with low SR	Minimum and maximum number of Families with high SR	Number of species
Wikström					
All 159–0 my	425	229–232	164–170	26–29	261,977
PreKT 159–64 my	184	87–90	82–87	10–12	98,207
PostKT 64–34 my	197	107–115	72–79	11–12	127,305
Oligocene 34–0 my	44	27–29	10–13	2–7	36,465
Davies					
All 160–0	415	224–226	164–166	24–28	260,947
Middle Cretaceous 160–80	123	64–66	48–52	6–8	57,908
Pre Eocene 79–51	213	121–125	42–47	9–10	123,565
Eocene 50–0	80	46–48	28–30	3–5	79,474

Maximum Likelihood Estimators of the Diversification Rate using Species Richness/Clade Age Data

The results of the LTT analyses described above indicate that the rate of origination of angiosperm families has not been constant over time, but contain two shifts in diversification rate based on both the Wikström or Davies topologies. To test whether the shift points identified in the LTT analyses (based primarily on the diversification time of families) also improved the fit of the data based on the species richness/clade age data, we 1) calculated the ML of the fit of the species richness/clade age data to a model assuming either a constant rate or three rates (two-shift points) and 2) calculated confidence intervals for $\hat{r}_{\lambda-\mu}$, λ, and μ to assess whether diversification rates estimates were significantly different between the geological intervals suggested by the LTT analyses.

To perform these analyses, we first used the species richness/clade age data to estimate the average absolute diversification rates of angiosperm families in each time interval using both stochastic pure-birth and birth-death models of evolution. The average absolute diversification rate for families that originated in each geological period under the stochastic pure-birth model was estimated using the Kendall-Moran estimator denoted $\hat{r}_{KM}$ from equation 1 of Magallón and Sanderson (2001):

$$\hat{r}_{KM} = \frac{\ln(n)}{t}; \tag{1}$$

where the parameter t in $\hat{r}_{KM}$ was defined as the maximum age for that interval as given in Table 3.

The average absolute diversification rate for families considering the number of species that originited in all and each geological periods under the stochastic birth-death model was estimated using maximum likelihood estimates of $\hat{r}_{\lambda-\mu}$, λ, μ, and ε. For all families included in each topology and each geological period the ML estimates for the stochastic birth-death process are included in the function β (equation 2b from Magallón and Sanderson 2001):

$$\beta = \frac{\lambda\left[e^{(\lambda-\mu)t} - 1\right]}{\lambda e^{(\lambda-\mu)t} - \varepsilon} \tag{2}$$

that can be maximized using the likelihood given in Bokma (2003)

$$L(\lambda-\mu) = \ln l = \sum_{i}^{k} \ln(1-\beta_i) + \sum_{i}^{k}\left[(n_i - 1)\ln\beta_i\right]. \tag{3}$$

This equation was evaluated for different combinations of values for λ (ranging from 0 to 100, in intervals of 0.1 units) and r (ranging from –1

to 1, in intervals of 0.001 units) using the divergence age (t_i) and species richness of each family (n_i) that originated in each geological interval by ML inference (written in MAPLE, available upon request).

Plotting the average diversification rate of all angiosperm families over time emphasizes that there is an increase in diversification rate towards the present for both topologies (Fig. 2A Wikstrom, Fig. 2B Davies) such that using the average diversification rate of all angiosperm families based on either a pure-birth or birth-death diversification process, $\hat{r}_{KM}$ and $\hat{r}_{\lambda-\mu}$

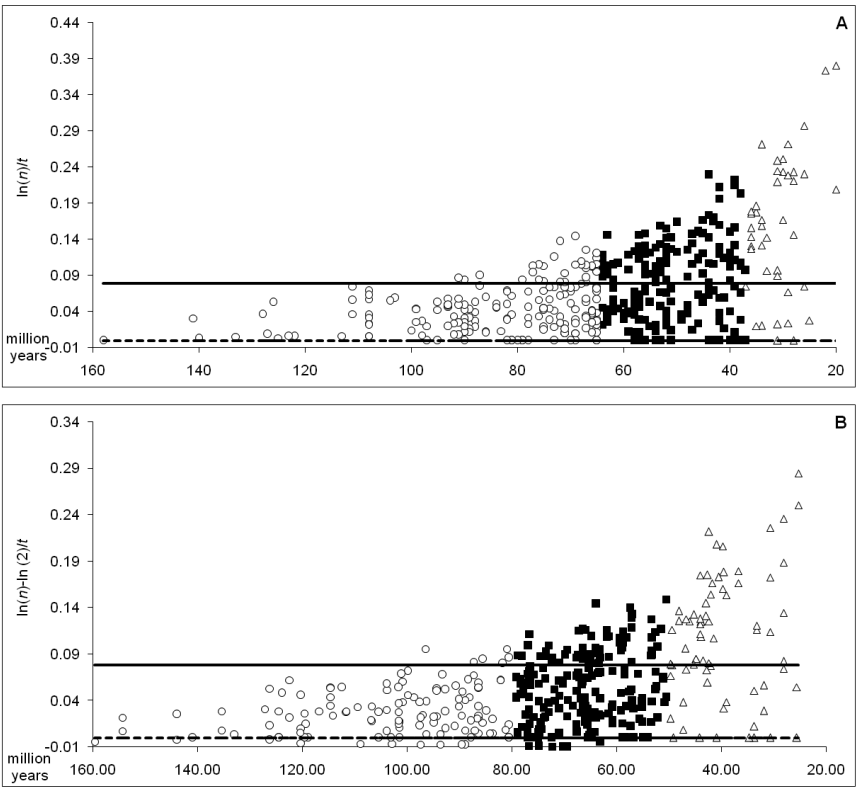

Figure 2. Graph of family age based on those given in A) Wikström et al. (2001) or B) Davies et al. (2004) against the diversification rate of 425 or 416 angiosperm families, respectively. Assuming a constant rate of diversification, the diversification rate of families would center around the mean diversification rate of all angiosperm families assuming a pure-birth (dashed line) or birth-death, process (solid lines). The apparent increase in diversification rate of angiosperm families towards the present led to the model prediction that families should be divided into those that a) originated during the PreKT (159 to 64 my, open circle), PostKT (64 to 34 my, closed square), and Oligocene (34 to 0 my open triangles) for the Wikström topology b) originated during the Middle Cretaceous (159–80 my, open circle), Pre-Eocene (80–50 my, closed square) and Eocene (50 my-present, open triangle) for the Davies topology (see text for details).

respectively, does not adequately capture the change in diversification rate of angiosperm families over time (Fig. 2A and 2B Davies).

For the species richness data, the ML estimates and confidence intervals of $\hat{r}$ show that $\hat{r}_{KM}$=0.079 and 0.069 for the pure-birth model (for Wikström and Davies topology respectively) and $\hat{r}_{\lambda-\mu}$= –0.080 (for both topologies) for the birth-death model when the data from all families are pooled together (Table 4). The estimate based on the pure-birth model is in close agreement with the value of 0.0767 calculated by Magallón and Sanderson (2001) based on their pure-birth model. However, our estimate of $\hat{r}_{\lambda-\mu}$ is lower than theirs for the stochastic birth-death model ($\hat{r}_{\lambda-\mu}$= 0.089). This can be attributed, in part, to our inclusion of almost all families of angiosperms and to our estimation of ε usingBokma's (2003) estimator as opposed to assuming values of 0.0 to 0.9 for the extinction rate as done by Magallón and Sanderson (2001) and Magallón and Castillo (2009). Additionally, our analyses show that the species richness of angiosperm families is better described by a birth-death than a pure-birth model and that the rates of speciation and extinction are more accurate when measured separately in three geological intervals.

In general, *r* was consistently significantly lower when based on the birth-death compared to the pure-birth model (Table 4). This may be caused, in part, by the high ML estimates of the extinction fraction, $\varepsilon = \mu / \lambda$ which is estimated to be >1 in all of the eight geological intervals. The values of $\hat{r}_{\lambda-\mu}$ increase over each geological interval for both topologies, however the confidence intervals for $\hat{r}_{\lambda-\mu}$ are overlapping for the three geological intervals identified as having significant shift points in the LTT analyses (Table 2 and 4).

Next, to assess whether angiosperm clades have diversified according to a single rate or a three rate (two shift) model of diversification, we calculated the likelihood of the models using equation (3) in which families were grouped together (one rate), as well as by taking the sum of the likelihoods for all families originating in each geological period supported by the LTT analyses was used (three rate). Models were then compared using Likelihood Ratio Tests and AIC criteria. Comparison of the likelihood of the data for the pure-birth and birth-death models of diversification with three rates using both the Wikström and Davies topologies shows that the birth-death model provides a better fit to the data based on AIC criterion (Table 5). Additionally, the three-rate birth-death model provides a better fit to the data than a one rate model for both topologies (Table 5), and for the Davies topology, a three rate, birth-death model, provides the best overall fit to the data (Table 5).

Table 4. Maximum likelihood estimators of $\hat{r}$ based on a pure-birth model ($\hat{r}_{KM}$) and a birth-death model ($\hat{r}_{\lambda-\mu}$) using the species richness of angiosperm families and their age of divergence based on Wikström et al. (2001) and Davies et al (2004) for all of the families grouped together, and by dividing the families into the geological intervals shown to have significantly different diversification rates. In addition, ML estimates of the rate of speciation (λ), extinction (μ), and the extinction fraction ($\varepsilon = \mu / \lambda$) for the birth-death model are presented using the methods described in the text. 95% Confidence intervals (CI) for the birth-death model were estimated by using the ML surface of the point estimates and by calculating the maximum and minimum values of $\hat{r}_{\lambda-\mu}$ and its paired values of (λ) and extinction (μ) which gave the maximum extinction fraction ($\varepsilon = \mu / \lambda$), laying within 4 natural logarithmic units of the maximum likelihood value (in brackets).

Geological intervals	$\hat{r}_{KM}$	$\hat{r}_{\lambda-\mu}$	λ	μ	ε
Wikström					
All 159-0 my	0.079	–0.080 (–0.070, –0.075)	50.00 (42.40, 50.00)	50.08 (42.47, 50.08)	1.002 (1.002, 1.002)
PreKT 159-64 my	0.073	–0.090 (–0.040, –0.080)	48.60 (21.40, 50.00)	48.69 (21.44, 50.08)	1.002 (1.002, 1.002)
PostKT 64-34 my	0.184	–0.080 (–0.030, –0.060)	49.80 (23.60, 50.00)	49.88 (23.63, 50.07)	1.002 (1.001, 1.001)
Oligocene 34-0 my	0.292	–0.090 (–0.040, 0.080)	48.60 (21.00, 50.00)	48.69 (20.98, 50.02)	1.002 (0.999, 1.000)
Davies					
All 160-0 my	0.069	–0.080 (–0.065, –0.075)	50.00 (40.00, 50.00)	50.08 (40.07, 50.08)	1.002 (1.002, 1.002)
Middle Cretaceous 160-80 my	0.079	–0.100 (–0.020, –0.090)	47.00 (10.20, 50.00)	47.10 (10.22, 50.09)	1.002 (1.002, 1.002)
PreEocene 79-51 my	0.147	–0.065 (–0.005, –0.095)	38.20 (9.80, 50.00)	38.27 (9.81, 50.10)	1.002 (1.001, 1.002)
Eocene 51-0 my	0.226	–0.010 (–0.030, 0.050)	30.40 (8.00, 50.00)	30.41 (7.95, 50.03)	1.000 (0.994, 1.001)

Table 5. Likelihoods (*ln* likelihood) and number of parameters (*n*) estimated for the models of diversification assuming birth (birth) and for birth-death stochastic model of divergence in the angiosperms assuming one constant diversification rates (one rate) and three constant rates (three rates) for each geological period in which families originated. Values of Δ AIC test to compare the model with lowest AIC vs. the remaining models The comparison among the best model for each topology is presented also in the last two rows of the table.

Models	**ln likelyhood**	***n***	**AIC**	**Δ AIC**
Davies				
Three rates	–3,084.61	6	6,181.22	
One rate	–3,098.915	2	6,201.83	41.22
Birth	–5,247.983	2	10,499.97	8,637.49
Wikström				
Three rates	–3,146.177	6	6,304.35	
One rate	–3,159.921	2	6,323.84	38.97
Birth	–5,694.65	2	11,393.30	10,177.89
Three rates				
Davies	–3,084.61	6	6,181.22	
Wikström	–3,146.177	6	6,304.35	246.26

Two sets of confidence intervals were estimated: one for the confidence associated with estimating $\hat{r}_{\lambda-\mu}$, λ, μ (error in the estimate itself) and the second to give the upper and lower confidence intervals for the number of species expected under the stochastic diversification process (error associated with the stochastic process of diversification). To incorporate error caused by estimating $\hat{r}_{\lambda-\mu}$, λ,μ and ε, we used the fact that when estimating three related parameters via ML (i.e., $\hat{r}_{\lambda-\mu}$=λ, μ), approximate confidence intervals are given by +/–2 units of the likelihood surface relating two of the parameters (Meeker and Escobar 1995). Using the ML surface and minimum and maximum 95% confidence interval, $\hat{r}_{\lambda-\mu}$ and ε were obtained and used for the following calculation of the confidence interval associated with the number of species per clade over time.

Confidence intervals associated with the number of species per clade expected under the stochastic diversification process were estimated separately for all families grouped together and for those originating in the three time intervals indicated by the Wikström or Davies topologies (see results). Confidence intervals for the stochastic process were estimated for both the pure-birth and birth-death models by obtaining the upper and lower 2.5% of species expected at time *t* assuming a birth model ($k_{(KM-upper)t}$ and $k_{(KM-lower)t}$) by solving the following equation:

$$k_{(KM-upper)t} = e^{\hat{r}_{KM-upper}t} + 2 \text{ and } k_{(KM-lower)t} = e^{\hat{r}_{KM-lower}t} + 2 \quad (4)$$

Where $\hat{r}_{KM-upper}$ and $\hat{r}_{KM-lower}$ are the upper and lower values for the $95\%CI(\hat{r}_{KM})$. For the birth-death model, the upper and lower number of species ($k_{(upper)t}$ and $k_{(lower)t}$) were estimated by solving equations 10a and 10b from Magallón and Sanderson (2001) using the following equations:

$$k_{(upper)t} = \frac{\ln(0.025)}{\ln(\beta)} + 1$$

$$k_{(lower)t} = \frac{\ln(0.975)}{\ln(\beta)} + 1 \quad (5)$$

where: α and β were obtained by solving α_t and β_t as given in Magallón and Sanderson (2001) from eq 8.47 of Bailey: (1964)

$$\alpha_t = \varepsilon\beta_t$$

$$\beta_t = \frac{e^{\hat{r}_{\lambda-\mu}t} - 1}{e^{\hat{r}_{\lambda-\mu}t} - \varepsilon} \quad (6)$$

in which $\hat{r}_{\lambda-\mu}$ and ε were taken as the value of the diversification rate and extinction fraction that maximized the likelihood given in equation 3, and t was the age of divergence of the family.

Dividing the data into three geological intervals and calculating confidence intervals for the expected number of species per family based on the birth-death model for both topologies improved the estimate of the number of families that have the expected species richness,but many families still remain outside the confidence limits (Fig. 3A–3C-Wikström topology-, and Fig. 3D–3CF -Davies topology-) (Table 3).

Using the species richness/clade age data, we generated a birth-death model of diversification that accounted for the identified shift points, and which caused a significant improvement in the data compared to models including no shift points or models based on the pure-birth process of diversification of angiosperms. The MLE estimates given in the Wikström topology indicated that the mass extinction at the K/T boundary influenced both the rate of origination of new families of angiosperms and the species richness of younger families. For both analyses (LTT and species richness/clade age), diversification rates were found to increase and extinction rates decrease towards the present: this is consistent with the observation that families that arose prior to 65 mya have undergone high levels of extinction.

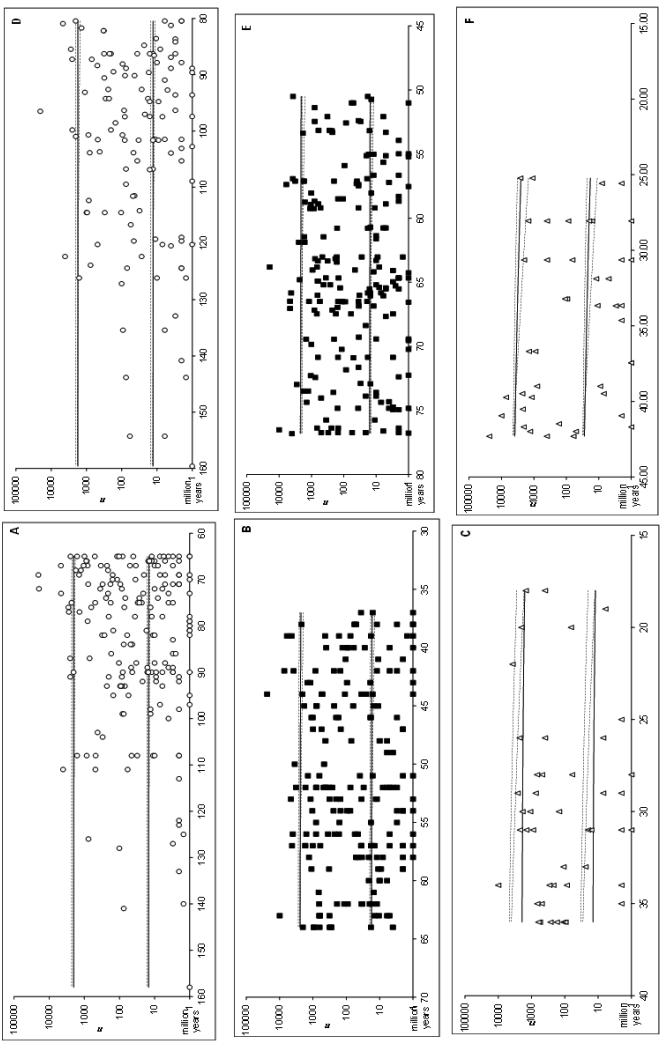

Figure 3. The number of extant species per family originating during the A) PreKT (open circle), B) PostKT (closed square) and C) Oligocene (open triangle) arranged by the age of divergence estimated by Wikström et al. (2001); and D) Middle Cretaceous (open circle), E) Pre Eocene (closed square) and F) Eocene (open triangle) arranged by the age of divergence estimated by Davies et al. (2004). 95% confidence intervals estimated for the expected number of species over time assuming a stochastic model of birth-death (solid lines) and the uncertainty in the MLE estimator (dashed lines) are indicated (see text for details).

Similarly, the species richness of families that originated less than 65 mya was equally pronounced: of the 454 families present today, 326 (72%) are less than 66 mya according to Wikström topology, and 198 (44%) are <66 mya according to Davies topology, with these families accounting for 90% and 57% of the species diversity, respectively. Other authors have noted that the most species rich families tend to be young (for example, Magallón et al. 1999): this analysis suggests that the increase in species richness of young families is partially attributable to the impact of the mass extinction at the K/T boundary. Paleontological data show that ~80% of plant species exhibited a sudden extinction at the K/T boundary which was paralleled by the extinction of many insect species (Johnson et al. 1989; Johnson 2002; Labandeira et al. 2002). Reconstruction of the fossil record history in Western and Central US indicates that plant diversity did not recover for at least 10 million years after the K/T event (Wing et al. 1995).

In addition to the importance of a shift at the K/T boundary, the analyses indicate that a second shift in the rate of angiosperm family origination and species richness occurred at 50 mya, according to the Davies topology or at 35 mya, according to the Wikström topology. This scenario closely parallels those observed in the fossil record: slow diversification during the early Cretaceous, a radiation in the Late Cretaceous and a brief but massive period of extinction at 65.5 mya; this was followed by a radiation of the group during the Paleocene and Eocene (Niklas 1997; Feild et al. 2004; Soltis et al. 2005). Cycles in which stasis and sporadic bursts of speciation intermingle are characteristic of angiosperm evolution and have been shown to be related to the presence of "Greenhouse" and "Icehouse" periods that characterized the earth's global climate before and after the Tertiary period (Willis and Niklas 2004). During the Paleocene-Eocene transition, high global temperatures reached a maximum at 55.8 mya (Wolfe 1978; Zachos et al. 2001) at the same time that an increase in low-latitude palynofloral diversity occurred (Jaramillo 2002), including the diversification and expansion of rainforests worldwide (Morley 2000). Immediately subsequent to this thermal maximum, global temperatures and CO_2 concentrations dropped and an extinction of ~20% of palynoflora occurred in the early Eocene (Harrington and Jaramillo 2007). This was followed by the prevalence of Icehouse conditions that lasted into the Oligocene. The range expansion that occurred during the warm period may have helped to cause an increase in rates of speciation during the icehouse conditions as populations became allopatrically separated (Willis and Niklas 2004). Thus, the increase in the rate of speciation of angiosperm families at the Paleocene-Eocene boundary may be associated with 1) a decrease in the number of angiosperm species and therefore an opening of niches after the K/T event and 2) climatic conditions favouring speciation in the early Tertiary to Oligocene.

In general, the percentage of families found to have the expected species richness ranges from about 47 to 66% depending on the geological interval considered. Furthermore, the number of families with expected, high or low species richness is similar for both topologies (Table 3). The percentage of families having lower species richness than expected varies from 20 to 47%, while the percentage of families with higher species richness is generally between 4 and 16% (Table 3). The more important results of this analysis is that, despite finding strong evidence for shifts in the mean rate of diversification of angiosperms over geological time, angiosperm species richness is not strongly correlated with clade age, and secondly that there are many species-poor families. This raises the question of whether families are a reliable taxonomic unit. Studies in molecular phylogenetics, however, indicate that the taxonomic limits of plant families generally hold up to molecular phylogenetic analyses (Soltis et al. 2005), whereas genera may not (Scotland and Sanderson 2004). In contrast, a correlation between clade age and species richness apparently does hold true in animals. A study by McPeek and Brown (2007) (found a positive relationship between clade age of animal phyla and species richness, suggesting that extrinsic and/or intrinsic factors influencing speciation and extinction rates in plants and animals may be very different.

The large discrepancy in species richness among angiosperm clades continues to be of major interest in plant systematics and evolution: approximately 8% of angiosperm families are monospecific and 12% have more than 1,000 species per family (Soltis et al. 2005; Stevens 2008). Variation in species richness among clades has been attributed to traits that promote adaptive radiation or differential extinction (Stebbins 1981; Ricklefs and Renner 1994; Sanderson and Donoghue 1994; Dodd Silvertown and Chase 1999; Sims and McConway 2003; Davies et al. 2004), bias in taxonomic classification (Scotland and Sanderson 2004), and to the presence of groups that serve as cradles versus museums of biodiversity (Stebbins 1974). One approach to identifying families with high or low species richness is to look for imbalance using the topological information of the phylogenetic relationship among clades (Slowinski and Guyer 1993; Davies et al. 2004; McConway and Sims 2004; McKenna and Farrell 2006). Another approach is to construct an explicit model of the diversification process, as performed here, and then to identify clades with higher or lower species richness than expected.

To test this approach, we sought to evaluate the influence of 13 intrinsic factors on determining whether a family was categorized as having higher than, expected or lower than expected species richness. Importantly, although the dates of divergence are generally older in the Davies topology, the families identified as having high, expected or low species richness

were similar between the two topologies: in total, 399 families had the same species richness category using both calibrations (Table 1). We performed the following analyses to look for traits associated with high and low DR of these 399 families (Table 1).

Traits Associated with Diversification Rates in Angiosperms

Using the broadest confidence intervals, the number and identity of families that had high, low, or expected species richness in each time interval based on the analyses of the Wikström and Davies topologies were tabulated (Table 1). Of the 399 families, 21 belonged to the high species richness category, 147 to the low species richness category and 231 had expected species richness (Table 1). From this data set, only those families that were found to have the same level of species richness (high, expected or low) according to the analyses performed on both the Wikström and Davies topologies were used (Table 1). Using this subset of the data, the information about whether a family had higher, expected or low species richness (given its geological interval) was used as the dependent variable in a stepwise logistic regression to look for possible correlates of DR in angiosperms using 13 intrinsic factors as independent variables.

The intrinsic factors were based on morphological, functional and ecological traits. The morphological traits were divided into those relating to 1) floral symmetry; 2) floral nectar spurs; 3) sexual system; and 4) growth habit. Floral symmetry data was gathered from several sources (Soltis et al. 2005; Kay et al. 2008; Watson and Dallwitz 2008) while that for the presence of nectar spurs was taken from (Soltis et al. 2005; Kay et al. 2008). Information on growth habit was collected mainly from the APG website (Stevens 2008), while that for sexual systems was retrieved primarily from Intkey (Watson and Dallwitz 2008) or from Kay et al. (2008). For floral symmetry, the presence of zygomorphic flowers (absence=0, presence=1) was used as an independent variable; floral nectar spurs were included as another variable (absence=0, presence=1). For sexual system, the presence of any form of dioecism in the family was scored (absence=0, presence=1). For growth habit, we included three variables:climbing habit (absent=0, present=1); polymorphic growth habit (absence=0, presence of three or more of herbs, vines, shrubs, trees =1); and herbaceous versus woody growth habit (herbaceous=0, woody=1, herbaceous and woody=2).

The functional traits were based on either 1) mating system or 2) mode of photosynthesis. For the mating system, prevalence of self-sterility, and presence of self-incompatibility and apomixis was scored (absence=0, presence=1, unknown=2 in the last two). For the prevalence of self-sterility, families were categorized on the following basis: all recorded species identified with self-compatibility (SC) =0; <50% recorded species with

self-sterility =1; >50% recorded species with self-sterility=2; or unknown=3. For the mode of photosynthesis, both the presence of C4 photosynthesis (absence=0, presence=1) and CAM photosynthesis (absence=0, presence=1) were considered. Information about mating systems was collected from Carman (1997) for the analysis of apomixis and from an updated Bertin and Newman (1993) data set for self-sterility (Ferrer and Good 2012). Information on photosynthesis was obtained from Lambers et al. (1998) and from Sage (2004).

Traits in the ecological category included evidence of biotic pollination (abiotic=0, biotic=1, abiotic and biotic=2), and the presence of fleshy fruits—assuming that species with fleshy fruits would have biotic dispersal (absence=0, presence of fleshy fruits=1). Pollination data were retrieved from Intkey (Watson and Dallwitz 2008) and Kay et al. (2008) and from some original sources. Information pertaining to the presence of fleshy fruits was obtained from IntKey (Watson and Dallwitz 2008) and the APG II website (Stevens 2008).

The prevalence of the observed traits among families was highly variable: C4 and CAM photosynthesis and floral nectar spurs were only observed in 10, 21 and 10 families (with high, expected, and lower than expected species richness) respectively, while some other traits such as fleshy fruits, dioecy and apomixis were present in 169, 119, and 117 of the 399 families respectively (Table 6). Despite this, some traits that were only poorly represented among angiosperms families were highly represented in families with high species richness: for example, of the 10 families observed to have C4 photosynthesis, 4 belonged to the high species richness category, and of the 21 families with CAM photosynthesis, 13 belonged to the high species richness category (Table 6). Not surprisingly, for the self-sterility and apomixi traits, the majority of families for which information was missing (state unknown) belonged to the low species richness category (Table 6).

A stepwise logistic regression was performed in SAS using PROC logistic. This included the explanatory variables in the following order:polymorphic growth habit (Wald χ^2=31.97, d.f. = 1, P< 0.0001), presence of self-sterility (Wald χ^2=18.00, d.f. = 2, P = 0.0001), apomixes (Wald χ^2=15.12, d.f. = 2 P = 0.0005), CAM photosynthesis (Wald χ^2= 13.02, d.f. = 1 P= 0.0003), dioecy (Wald χ^2=11.60, d.f. = 1 P = 0.0007), and zygomorphic flowers (Wald χ^2=10.33, d.f. = 1 P = 0.0013); each trait changed the odds of whether families had high versus expected or high versus low species richness. The positive association of each trait with high species richness is perhaps not unexpected, as many of the selected traits were previously identified as being associated with high diversification rates.

Families with polymorphic growth habit had an 86.6 higher odd of having high versus expected species richness and an 8.1 higher odd of belonging to families with high versus low species richness (Table 7).

Table 6. Number of families showing each state for 13 different traits used in the stepwise regression in the full data set (N=399) or in those families categorized as having high (N=21) or low (N=147) species richness.

Zygomorphic				Nectar Spurs				Dioecysm			
	Data	High	Low		Data	High	Low		Data	High	Low
Pres	62	7	13	Pres	10	1	0	Pres	119	10	24
Abs	279	13	110	Abs	331	19	123	Abs	332	10	99
Woody/herbaceous				Polymorphic Growth Habit				Climbing habit			
	Data	High	Low		Data	High	Low		Data	High	Low
Herb	88	1	39	Pres	85	17	23	Pres	73	11	12
Wood	164	4	81	Abs	256	3	121	Abs	268	9	111
Wood/herb	89	15	3								
Self-sterility				Self-incompatibility				Apomixis			
	Data	High	Low		Data	High	Low		Data	High	Low
SC	72	7	48	Pres	58	13	3	Pres	117	17	10
<50%SS	33	7	25	Abs	132	7	23	Abs	85	3	21
>50%SS	85	6	71	N/A	151	0	97	N/A	139	0	139
N/A	151	0	54								
C4 photosynthesis				CAM photosynthesis				Fleshy fruits			
	Data	High	Low		Data	High	Low		Data	High	Low
Pres	10	4	0	Pres	21	13	0	Pres	169	16	41
Abs	331	16	123	Abs	320	7	123	Abs	172	4	82
Pollination											
	Data	High	Low								
Abiotic	46	1	24								
Biotic	256	12	95								
Ab. /Biot	39	7	4								

Table 7. Odds ratio estimates for each effect considered in the stepwise regression for the comparisons of families classified as having expected versus high or high versus low species richness. For the odds ratio comparisons, the point estimate of the odds ratio is given as well as the 95% Wald confidence limits, only those confidence limits that do not include 1.0 are considered to be associated with a significant point estimate (significant estimates are given in bold).

Effect	Comparison	Division	Estimate	95% WCL*	Division	Estimate	95% WCL*
		Expected vs. High			*High* vs. *Low*		
<50% SI	1 vs. 0	**E**	**6.9**	**1.5**	H	1.9	0.29
<50% SI	1 vs. 0	**H**	**13.7**	**1.2**	L	0.145	0.03
>50% SI	2 vs. 0	**E**	**3.2**	**1.06**	H	0.774	0.09
>50%SI	2 vs. 0	H	2.5	0.24	L	0.313	0.10
Unknown SI	3 vs. 0	E	0.24	0.045	H	<0.001	<0.001
Unknown SI	3 vs. 0	H	<0.001	<0.001	L	4.2	0.78
Apomixis	1 vs. 0	**E**	**3.3**	**1.1**	**H**	**21.7**	**2.1**
Apomixis	1 vs. 0	**H**	**70.9**	**5.5**	L	0.31	0.1
Unknown Apomixis	2 vs. 0	E	5.4	0.8	H	101.8	<0.0001
Unknown Apomixis	2 vs. 0	H	605.7	<0.001	L	0.186	0.03
Dioecy	1 vs. 0	**E**	**2.7**	**1.2**	H	1.8	0.44
Dioecy	1 vs. 0	**H**	**5.1**	**1.009**	L	0.36	0.17
CAM	1 vs. 0	E	2.1	0.16	**H**	**34.9**	**5.5**
CAM	1 vs. 0	**H**	**72.6**	**3.3**	L	0.48	0.038
Growth habit	1 vs. 0	**E**	**10.6**	**3.2**	**H**	**8.2**	**1.7**
Growth habit	1 vs. 0	**H**	**86.6**	**12.1**	L	0.094	0.02
Zygomorphy	1 vs. 0	**E**	**3.1**	**1.3**	H	3.9	0.78
Zygomorphy	1 vs. 0	**H**	**12.1**	**1.9**	L	0.33	0.14
Fleshy fruits	1 vs. 0	**E**	**3.01**	**1.1**	H	3.5	0.7
Fleshy fruits	1 vs. 0	**H**	**10.7**	**1.7**	L	0.3	0.1

*Wald Confidence limits

The presence of CAM photosynthesis also greatly increased the odds of belonging to a high versus expected (72.6) and a high versus low (34.9) species-rich family (Table 7). The largest influence of the presence of self-sterility occurred was demonstrated when comparing families with high versus expected species richness:in families in which fewer than 50% (but more than 0%) of the species have been identified as being self-sterile, the odd of belonging to a family with high species richness was 13.7 higher, and 6.9 higher of having the expected species richness (Table 7). Families in which >50% of the species were self-sterile also showed significantly higher odds of having expected species richness (3.2), but there was only weak evidence for their having high species richness (the odds ratio estimate was 2.5 but the confidence interval included 1.0). When the presence of self-sterility was unknown (state 3), the odds of families having high species richness was essentially zero (<0.001), while the odds of belonging to a family with low compared to high species richness was 4.2 (although the confidence intervals included 1.0), showing that little is known about the breeding system of low species rich families.

The presence of apomixis also highly influenced species richness: comparing families with high to expected species richness, those presenting apomixis had a 70.9 higher odd of being highly diverse, and a 21.7 higher odd of belonging to high compared to low species rich families (Table 7). Families in which the presence of apomixis was unknown also had a much higher odd of belonging to high compared to expected or low species rich families, but the confidence intervals for both of the estimates were very broad, suggesting that the point estimate of the odds ratio is poor (Table 7). The presence of zygomorphic flowers increased the odds of belonging to high versus expected (12.1) but not to low species rich families (Table 7). The presence of fleshy fruits also increased the odds of belonging to families with high versus expected species richness, but there was no difference in families with high versus low species richness. Finally, the presence of dioecy affected the odds of belonging to families with high versus expected species richness, with high species rich families having a somewhat higher odd (5.0) of having dioecy than families with expected species richness (2.7 higher odd; Table 7).

The instrinsic factors that were shown to be most associated with high species richness were the presence of polymorphic growth habit, self-sterility, apomixes, CAM photosynthesis, dioecy sexual system and zygomorphic flowers. Previous studies have identified that growth habit has a significant influence of diversification rates; the shorter generation time of annual species should increase rates of speciation (Aarssen et al. 2006; Crepet and Niklas 2009), and many species-rich families are dominated by annuals (e.g., Asteraceae and Rubiaceae, as noted by Crepet and Niklas 2009). In

this study, we did not differentiate between annual and perennial herbs, but we re-confirm the conclusion of Ricklefs and Renner (1994) who found that families presenting both herbaceous and woody taxa (polymorphic growth habit for us) were 5.7 to 14 times more species rich than families presenting either growth form alone. Molecular work in *Arabidopsis* has shown that the genes coding for woody growth and cambium function are not unique to woody plants, which would suggest that the gain and loss of the woody habit may be more plastic than previously thought, facilitating speciation rates in such families (Petit and Hampe 2006). Nevertheless, although families presenting multiple growth forms were significantly associated with high species richness, an examination of the low species rich families reveals that they are dominated by woody families (Table 6). Thus, the relationship between polymorphic growth habit and species richness is complex, and higher rates of diversification are probably still found among predominantly herbaceous lineages (Wing and Boucher 1998).

Additionally, we find strong evidence that CAM, and to a lesser extent C4 photosynthesis has had a major impact on angiosperm family species richness. Recent studies have found similar results at lower macro-evolutionary levels, and suggest that the more arid conditions of the Eocene/Oligocene environment and even the occurrence of intervals with very low CO_2 concentration during the Eocene-Oligocene (Crepet 2009) selected for families that could use alternative modes of photosynthesis and that were adaptive in dry and/or low CO_2 conditions (Sage 2004). In confirmation of these results, this study determined that 13 of the 21 families identified as having high species richness utilize CAM photosynthesis to some degree.

Another poorly-studied trait that stands out as having a potentially important influence on angiosperm diversification rates is agamospermy. The stepwise regression indicated that families with apomixis were 70 or 22 times more likely to have high species richness compared to expected and low species rich families respectively, i.e., agamospermy is highly associated with high levels of species richness. Recently, Crepet and Niklas (2009) pointed out the potentially important role of agamospermy in angiosperm evolution, arguing that this unique angiosperm trait allows individuals to escape sterility as well as the cost of meiosis. Additionally, reproducing apomictically provides an important mechanism by which individuals can avoid the deleterious effects of inbreeding in hermaphroditic taxa, allowing individuals to propagate themselves while maintaining standing levels of heterozygosity. This could be important under a variety of evolutionary scenarios such as in a) hybrid populations that have become, perhaps temporarily, sterile, b) populations in which the cost of meiosis and/or inbreeding depression is high, and c) self-incompatible populations that are depauperate in *S*-alleles or in self-incompatible taxa

that undergo hybridization and polyploidization. Since self-incompatible lineages typically have high genetic loads, agamospermy can provide a mechanism to both restore fertility and avoid inbreeding depression in recent self-incompatible polyploids. It is interesting to note that in some families apomixis and self-incompatibility co-occur (Carman 1997), while in others, such as the Rosaceae family, there is a strong tendency for diploids to be self-incompatible and for polyploids to reproduce apomictically (Talent 2009). Based on recent attention to the hypothesis that polyploidization and hybridization may have been important generators of angiosperm diversity (Crepet and Niklas 2009), the results of this analyses suggest that agamospermy may also allow lineages to maintain high levels of genetic variation while avoiding sterility and thereby reducing the rate of extinction in apomictic lineages.

Additionally, we found evidence that self-sterility influenced family species richness. The results indicate that families which have some form of self-sterility tend to have greater species richness than those that do not. This is similar to the result obtained by Heilbuth (2000) and recently by Ferrer and Good (2012). Although most studies looking for traits that drive diversification rates focus on those that accelerate the rate of speciation, over long evolutionary periods, factors that decrease the rate of extinction may be equally or more important in determining standing levels of diversity (Ricklefs and Renner 1994; Crepet and Niklas 2009). Self-sterility may be precisely such a trait. The negative frequency dependent selection acting on *S*-phenotypes causes self-incompatible lineages to maintain higher levels of connectivity than self-compatible ones. Consequently, self-incompatible taxa have higher effective population sizes than self-compatible taxa (Richman 2000) which will theoretically cause a decrease in the rate of extinction. By the same argument, self-incompatible lineages are expected to have lower rates of speciation since population differentiation occurs more slowly. Since diversification rates are determined by the net difference of speciation versus extinction, it is not clear whether self-incompatible lineages would have higher or lower rates of diversification. The data presented here suggest that the effect of lowering the rate of extinction in self-esterile lineages may be greater than the effect of potentially higher rates of speciation in self-compatible lineages because a) families with at least some self-sterility dominated the high species richness category and b) proportionally more SC families belonged to the low species richness category (Table 5).

Furthermore, we found that dioecy, zygomorphy and the presence of fleshy fruits influenced the species richness category into which a family fell. Previous studies on the effect of dioecy have used sister comparisons to contrast the species richness of dioecious and non-dioecious clades and, in both cases, found that dioecious clades have lower species richness on average than non-dioceous clades (Heilbuth 2000; Kay et al. 2008). In both

studies, some dioecious families were found to have high species richness, but examination of all families reduced the effect. This suggests that dioecy per se may not be the generator of high species diversity, but rather that it may be correlated with other traits that drive high species richness. Indeed, Vamosi et al. (2003) found that dioecy was correlated with six other traits and Vamosi and Vamosi (2004) argue that it is the correlated evolution of dioecy with both tropical distribution and fleshy fruits that appears to drive higher rates of diversification in some dioecious families. We also found that the presence of fleshy fruits was associated with families that had both high and expected levels of species. A recent comparative analysis of 50 phylogenetically independent lineages revealed that fleshy fruit types have evolved independently multiple times in association with shifts from shaded to non-shaded habits, and are associated with an increase in species richness in woody but not herbaceous lineages (Bolmgren and Eriksson 2005). Seed and fruit sizes are known to have increased in the early Tertiary (Tiffney 1984; Wing and Boucher 1998), and have been associated with radiation in mammals and birds (fruit dispersers) as well as with climatic changes that permitted the spread of closed canopy forest (Eriksson et al. 2000). Crepet and Niklas (2009) suggest that the high species richness of clades with fleshy fruits may lead to higher dispersal rates which effectively reduce the extinction rate of such lineages.

Lastly, we find evidence that clades with zygomorphic flowers are more likely to occur in families with high or expected species richness. The effect of zygomorphy on flowering plant diversity has been appreciated for a long time (Stebbins 1974; Stebbins 1981; Ricklefs and Renner 1994; Sargent 2004): a phylogenetic examination of the gain and loss of zygomorphism in the Asteridae suggests that the trait has evolved multiple times independently, and that reversals to actinomorphy have probably also occurred (Donoghue et al. 1998); however, sister comparisons of zygomorphic and non-zygomorphic clades showed that some zygomorphic clades are significantly more species rich while others appear to be species poor compared to their sister clades (Kay et al. 2008). The results presented here support an important positive association between zygomorphic flowers and species richness; however, as is found for the effect of dioecy, it is probable that the influence of zygomorphy is correlated with other traits that together influence species richness. Indeed, the evolutionary mechanism driving plant-pollinator interactions and its influence on rates of diversification in angiosperm lineages is currently an area of considerable debate (Crepet and Niklas 2009).

To examine the effect of intrinsic factors associated with species richness in angiosperms, we performed a stepwise regression to eliminate the influence of correlated variables. The intent was to show that by making an explicit model of variation in the absolute rates of diversification among

families, traits (or their correlations) could be examined in a framework that allows for a temporal component and the possibility of differentiating between the factors influencing speciation and extinction rates. Interestingly, no trait was observed to be associated with low species richness in the angiosperms despite 147 of the 399 families included in the regression analyses belonging to the low species richness category. In addition to continuing to look for the traits and suites of correlated traits that explain the tremendous variation in angiosperm family species richness, more attention should be focused on why many angiosperm families exhibit very low species richness, seemingly independently of family age.

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References

Aarssen, L.W., B.S. Schamp and J. Pither. 2006. Why are there so many small plants? Implications for species coexistence. Journal of Ecology 94: 569–580.

Bailey, N.T.J. 1964. The Elements of Stochastic Processes with Applications to the Natural Sciences. Wiley, New York.

Baldwin, B.G. and M.J. Sanderson. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proceedings of the National Academy of Sciences of the United States of America 95: 9402–9406.

Bertin, R.I. and C.M. Newman. 1993. Dichogamy in angiosperms. The Botanical Review 59: 112–152.

Bokma, F. 2003. Testing for equal rates of cladogenesis in diverse taxa. Evolution 57: 2469–2474.

Bolmgren, K. and O. Eriksson. 2005. Fleshy fruits—origins, niche shifts, and diversification. Oikos 109: 255–272.

Carman, J.G. 1997. Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biological Journal of the Linnean Society 61: 51–94.

Crepet, W.L. and K.J. Niklas. 2009. Darwin's second 'abominable mystery': Why are there so many angiosperm species? American Journal of Botany 96: 366–381.

Davies, T.J., T.G. Barraclough, M.W. Chase, P.S. Soltis, D.E. Soltis and V. Savolainen. 2004. Darwin's abominable mystery: Insights from a supertree of the angiosperms. Proceedings of the National Academy of Sciences USA 101: 1904–1909.

Dodd, M.E., J. Silvertown and M.W. Chase. 1999. Phylogenetic analysis of trait evolution and species diversity variation among Angiosperm families. Evolution 53: 732–744.

Donoghue, M.J., R.H. Ree and D.A. Baum. 1998. Phylogeny and the evolution of flower symmetry in the Asteridae. Trends in Plant Science 3: 311–317.

Eriksson, O. and B. Bremer. 1992. Pollination systems, dispersal Modes, life forms, and diversification rates in Angiosperm families. Evolution 46: 258–266.

Eriksson, O., E.M. Friis and P. Lofgren. 2000. Seed Size, Fruit Size, and Dispersal Systems in Angiosperms from the Early Cretaceous to the Late Tertiary. The American Naturalist 156: 47–58.

Erwin, D. and R. Anstey. 1995. New approaches to speciation in the fossil record. Columbia University Press, New York.

Feild, T.S., N.C. Arens, J.A. Doyle, T.E. Dawson and M.J. Donoghue. 2004. Dark and disturbed: a new image of early angiosperm ecology. Paleobiology 30: 82–107.

Ferrer, M.M. and S.V. Good. 2012. Self-sterility in flowering plants: preventing self-fertilization increases family diversification rates. Annals of Botany 110: 535–553.

Foote, M. 1994. Temporal variation in extinction risk and temporal scaling of extinction metrics. Paleobiology 20: 424–444.

Good-Avila, S.V., V. Souza, B.S. Gaut and L.E. Eguiarte. 2006. Timing and rate of speciation in *Agave* (Agavaceae). Proceedings of the National Academy of Sciences 103: 9124–9129.

Harrington, G.J. and C.A. Jaramillo. 2007. Paratropical floral extinction in the Late Palaeocene-Early Eocene. Journal of the Geological Society 164: 323–332.

Heard, S.B. and D.L. Hauser. 1995. Key evolutionary innovations and their ecological mechanisms. Historical Biology 10: 151–173.

Heilbuth, J.C. 2000. Lower species richness in dioecious clades. The American Naturalist 156: 221–241.

Hodges, S.A. 1997. Floral nectar spurs and diversification. International Journal of Plant Science 158: S81–S88.

Hodges, S.A. and M.L. Arnold. 1995. Spurring plant diversification: are floral nectar spurs a key innovation? Proceedings of the Royal Society of London B 262: 343–348.

Jablonski, D. 2007. Scale and hierarchy in macroevolution. Palaentology 50: 87–109.

Jaramillo, C.A. 2002. Response of tropical vegetation to Paleogene warming. Paleobiology 28: 222–243.

Johnson, K. 2002. The megaflora of the Hell Creek and lower Fort Union formations in the western Dakotas: Vegetational response to climate change, the Cretaceous-Tertiary boundary event, and rapid marine transgression. *In:* J.H. Hartman, K.R. Johnson and D.J. Nichols (eds.). The Hell Creek Formation and the Cretaceous-Tertiary Boundary in the Northern Great Plains: An Integrated Continental Record of the End of the Cretaceous. Geological Society of America Special Paper.

Johnson, K.R., D.J. Nichols, M. Attrep and C.J. Orth. 1989. High-resolution leaf-fossil record spanning the Cretaceous/Tertiary boundary. Nature 340: 708–711.

Kadereit, J.W., E.M. Griebeler and H.P. Comes. 2004. Quaternary diversification in European alpine plants: pattern and process. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences 359: 265–274.

Kay, K.M., C. Voelckel, J.Y. Yang, K.M. Hufford, D.D. Kaska and S.A. Hodges. 2008. Floral characters and species diversification. *In:* L.D. Harder and S.C.H. Barrett (eds.). Ecology and Evolution of Flowers. Oxford University Press, Oxford.

Kendall, D.G. 1949. Stochastic processes and population growth Journal of the Royal Statistical Society. Series B (Methodological) 11: 230–264.

Kubo, T. and Y. Iwasa. 1995. Inferring the rates of branching and extinction from molecular phylogenies. Evolution 49: 694–704.

Labandeira, C.C., K.R. Johnson and P. Wilf. 2002. Impact of the terminal Cretaceous event on plant-insect associations. Proceedings of the National Academy of Sciences of the United States of America 99: 2061–2066.

Lambers, H., F.S. Chapin III and T.L. Pons. 1998. Plant Physiological Ecology. Springer-Verlag, New York.

Magallón, S. and A. Castillo. 2009. Angiosperm diversification through time. American Journal of Botany 96: 349–365.

Magallón, S., P.R. Crane and P.S. Herendenn. 1999. Phylogenetic pattern, diversity, and diversification of eudicots. Annals of the Missouri Botanical Garden 86: 297–372.

Magallón, S. and M.J. Sanderson. 2001. Absolute diversification rates in angiosperm clades. Evolution 55: 1762–1780.
McConway, K.J. and H.J. Sims. 2004. A likelihood-based method for testing for nonstochastic variation of diversification rates in phylogenies. Evolution 58: 12–23.
McKenna, D.D. and B.D. Farrell. 2006. Tropical forests are both evolutionary cradles and museums of leaf beetle diversity. Proceedings of the National Academy of Science of the United States of America 103: 10947–10951.
McPeek, M. and J. Brown. 2007. Clade age and not diversification rate explains species richness among animal taxa. The American Naturalist 169: E97–E106.
Meeker, W.Q. and L.A. Escobar. 1995. Teaching about Approximate Confidence Regions Based on Maximum Likelihood Estimation. The American Statistician 49: 48–53.
Moran, P.A.P. 1951. Estimation methods for evolutive processes. Journal of the Royal Statistical Society. Series B (Methodological) 13: 141–146.
Morley, R.J. 2000. Origin and evolution of tropical rain forests. John Wiley & Sons, Chichester.
Nee, S. 2001. Inferring speciation rates from phylogenies. Evolution 55: 661–668.
Nee, S. 2004. Extinct meets extant: simple models in paleontology and molecular phylogenetics. Paleobiology 30: 172–178.
Nee, S. 2006. Birth-death models in macroevolution. Annual Review of Ecology, Evolution, and Systematics 37: 1–17.
Nee, S., E.C. Holmes, R.M. May and P.H. Harvey. 1994. Extinction rates can be estimated from molecular phylogenies. Philosophical Transactions: Biological Sciences 344: 77–82.
Nee, S., R.M. May and P.H. Harvey. 1994. The reconstructed evolutionary process. Philosophical Transactions: Biological Sciences 344: 305–311.
Niklas, K.J. 1997. The evolutionary biology of plants. The Chicago University Press, Chicago.
Petit, R.J. and A. Hampe. 2006. Some Evolutionary Consequences of Being a Tree. Annual Review of Ecology, Evolution, and Systematics 37: 187–214.
Purvis, A. 1996. Using interspecies phylogenies to test macroevolutionary hypotheses. *In*: P.H. Harvey, A.J.L. Brown, J.M. Smith and S. Nee (eds.). New uses for new phylogenies. Oxford University Press, Oxford.
Pybus, O.G. and P.H. Harvey. 2000. Testing macro-evolutionary models using incomplete molecular phylogenies. Proceedings of the Royal Society of London Series B 167: 2267–2272.
Rabosky, D.L. 2006. Likelihoods methods for detecting temporal shifts in diversification rates. Evolution 60: 1152–1164.
Raup, D., S. Gould, T. Schopf and D. Simberloff. 1973. Stochastic models of phylogeny and the evolution of diversity. Journal of Geology 81: 525–542.
Raup, D.M. 1985. Mathematical models of cladogenesis. Paleobiology 11: 42–52.
Raup, D.M. and J.J. Sepkoski. 1984. Periodicity of extinctions in the geologic past. Proceedings of the National Academy of Sciences USA 81: 801–805.
Ricklefs, R.E. 2003. Global diversification rates of passerine birds. Proceedings: Biological Sciences 270: 2285–2291.
Ricklefs, R.E. and S.S. Renner. 1994. Species richness within families of flowering plants. Evolution 48: 1619–1636.
Richman, A.D. 2000. Evolution of balanced genetic polymorphisms. Molecular Ecology 9: 1953–1964.
Rohatgi, V.K. 1976. An introduction to probability theory and mathematical statistics. John Wiley & Sons Inc., New York.
Sage, R.F. 2004. The evolution of C4 photosynthesis. New Phytologist 161: 341–370.
Sanderson, M.J. 2002. Estimating absolute rates of molecular evolution and divergence times: A penalized likelihood approach. Molecular Biology and Evolution 19: 101–109.

Sanderson, M.J. and M.J. Donoghue. 1994. Shifts in diversification rate with the origin of angiosperms. Science 264: 1590–1593.
Sanderson, M.J. and M.J. Donoghue. 1995. Reconstructing shifts in diversification rates on phylogenetic trees. Trends in Ecology and Evolution 11: 15–20.
Sanderson, M.J., J.L. Thorne, N. Wikstrom and K. Bremer. 2004. Molecular evidence on plant divergence times. American Journal of Botany 91(10): 1656–1665.
Sargent, R. 2004. Floral symmetry affects speciation rates in angiosperms. Proceedings of the Royal Society B: Biological Sciences 271: 603–608.
Scotland, R.W. and M.J. Sanderson. 2004. The significance of few versus many in the tree of life. Science 303: 643–?.
Sims, H.J. and K.J. McConway. 2003. Nonstocahstic variation of species-level diversification rates within Angiosperms. Evolution 57: 460–479.
Slowinski, J.B. and C. Guyer. 1993. Testing whether certain traits have caused amplified diversification: An improved method based on a model of random speciation and extinction. The American Naturalist 142: 1019–1024.
Soltis, P.S., P.K. Endress, M.W. Chase and D.E. Soltis. 2005. Phylogeny and evolution of Angiosperms Sinauer Associates, Inc., Sunderland.
Stanley, S. 1979. Macroevolution, pattern and process. John Hopkins University Press, Baltimore.
Stebbins, G. 1974. Flowering plants: evolution above the species level. Belknap Press, Cambridge.
Stebbins, G.L. 1981. Why are there so many species of flowering plants? BioScience 31: 573–577.
Stevens, P.F. *Angiosperm phylogeny website* (Version 7), May 2006, 2008]. Available from http://www.mobot.org/MOBOT/research/APweb/.
Strathmann, R.R. and M. Slatkin. 1983. The improbability of animal phyla with few species. Paleobiology 9: 97–106.
Talent, N. 2009. Evolution of gametophytic apomixis in flowring plants: an alternative model from Maloid Rosaceae. Theory in Biosciences 128: 121–138.
Tiffney, B.H. 1984. Seed size, dispersal syndromes, and the rise of the angiosperms: Evidence and hypothesis. Annals of the Missouri Botanical Garden 71: 551–576.
Vamosi, J.C., S.P. Otto and S.C.H. Barrett. 2003. Phylogenetic analysis of the ecological correlates of dioecy in angiosperms. Journal of Evolutionary Biology 16: 1006–1018.
Vamosi, J.C. and S.M. Vamosi. 2004. The role of diversification in causing the correlates of dioecy. Evolution 58: 723–731.
Watson, L. and M.J. Dallwitz. 2008. The families of flowering plants: descriptions, illustrations, identification, and information retrieval.
Wikström, N., V. Savolainen and M.W. Chase. 2001. Evolution of the angiosperms: calibrating the family tree. Proceedings of the Royal Society of London Series B 268: 2211–2220.
Wikström, N., V. Savolainen and M.W. Chase. 2003. Angiosperm divergence times: congruence and incongruence between fossils and sequence divergence estimates. *In:* P.C.J. Donoghue and M.P. Smith (eds.). Telling the Evolutionary Time. Routledge, New York.
Willis, K. and K. Niklas. 2004. The role of Quaternary environmental change in plant macroevolution: the exception or the rule? Philosophical Transactions of the Royal Society B: Biological Sciences 359: 159–172.
Wing, S., J. Alroy and L. Hickey. 1995. Plant and mammal diversity in the Paleocene to early Eocene of the Bighorn Basin. Palaeogeography Palaeoclimatology Palaeoecology 115: 117–156.
Wing, S.L. and L.D. Boucher. 1998. Ecological aspectes of the Cretaceous flowering plant radiation. Annual Review of Earth and Planetary Sciences 26: 379–421.

Wolfe, J. 1978. A paleobotanical interpretation of Tertiary climates in the Northern Hemisphere. American Scientist 66: 694–703.

Yule, G.U. 1925. A mathematical theory of evolution, based on the conclusions of Dr. J.C. Willis, F.R.S. Philosophical Transactions of the Royal Society of London Series B Containing Papers of a Biological Character 213: 21–87.

Zachos, J., M. Pagani, L. Sloan, E. Thomas and K. Billups. 2001. Trends, rhythms, and aberrations in global climate 65 ma to present. Science 292: 686–693.

Zink, R.M. and J.B. Slowinski. 1995. Evidence from molecular systematics for decreased avian diversification in the pleistocene Epoch. Proceedings of the National Academy of Sciences of the United States of America 92: 5832–5835.

15

Polyembryony

Kundan Kishore

ABSTRACT

Polyembryony is a type of apomixis wherein autonomous development of supernumerary embryos takes place in the seed and consequently genetically similar progenies are developed. Supernumerary embryos are produced in different frequencies singlet, duplet, triplet, quadruplet, quintuplet, sextuplet and so on. Among different types of polyembryony, nucellar embryony (2n) is the most common. However formation of multiple embryos from cleavage of proembryos has also been observed in some plant species. The degree of polyembryony is influenced by pollen source and environmental factors; however it is controlled by a dominant gene having heterozygous allele (Pp). Morphologically zygotic embryos are usually larger than the other embryos; however morphological identification of nucellar seedlings is practically difficult due to the availability of very few morphological markers. On the other hand, biochemical and molecular markers are reliable tools to distinguish zygotic and nucellar seedlings. Nucellar and zygotic seedlings of citrus and mango have been identified through RAPD markers. In the recent development of molecular biology, genes like *msg-2* and *SERK* have been linked to somatic embryogenesis in many plant species. Polyembryony has great importance in generation of true-to-type quality plants; however it creates hurdle for hybridization programme as it hampers the production of zygotic seedling.

Keywords: Polyembryony, Apomixis, Nucellar, Zygotic embryos

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Introduction

Generally a seed contains a single embryo which geminates into a seedling, however, in some angiospermic family multiple embryos are developed in an individual seed and consequently multiple seedlings are produced which is known as polyembryony, whereas the term 'monoembryony' has been used to refer to a single seed that contains one embryo to describe strictly sexual seed parents. Thus polyembryony is the occurrence of two or more embryos in a developing ovule and the additional embryos result from the differentiation and development of various maternal and zygotic tissues associated with the ovule of the seed (Tisserat et al. 1979).

Polyembryony was first reported by Leeuwenhoek in citrus as early as 1719 and the different cases of polyembryony were studied by Braun in 1859. In 1878 Strasburger demonstrated the formation of plural embryos in many genera of angiosperms. In 1901, Earnst summarized the works done in polyembryony and classified the various means by which adventitious embryos are derived. Following Ernst's work, it soon became apparent that polyembryony is not an abnormal feature, which it was considered earlier, but rather a desirable character. About 255 genera belonging to 153 families are reported to exhibit polyembryony (Carman 1997).

In normal sexual cycle (amphimixis), diploid sporophytic cell (Megaspore Mother Cell) of ovule transforms to haploid gametophytic cells (embryo sac) through meiosis which contain egg cell that forms embryo after fertilization with male gamete (syngamy). But in some plants meiotic division and syngamy are eliminated and still a viable embryo is formed inside the ovule. The formation of embryo with asexual means is called apomixis (Apo=away from+ mixis=act of mixing) and seed is called apomictic seed. Thus apomixis refers to substitution of the usual sexual reproduction by a form of asexual reproduction which does not involve meiosis and syngamy for embryo formation (Bhojwani and Bhatnagar 1999). Polyembryony is a type of apomixes which initiates autonomous development of embryos through asexual mode and the resulting progeny are the genetic replicas of the mother plant. Polyembryony in plants occurs as facultative apomixis wherein simultaneous growth of multiple embryos of somatic origin co-exist in the same seed containing sexual embryo resulting from self or cross pollination. Polyembryony is exhibited by a number of plants, yet little is known about the origin of this reproductive phenomenon.

To understand the origin and formation of multiple ovules in angiospermic seed, the anatomical structure of ovule is imperative.

OVULE

The female reproductive apparatus in a flower is the gynoecium and its functional unit is carpel. A typical carpel is comprised of swollen ovary, style and stigma. The ovule forms inside an ovary which in turn forms seed. A well developed ovule consists of embryo sac, nucellus enclosed almost completely by one or two integuments leaving a small opening at the apical end and micropyle. The micropyle is the main passage for entry of pollen tubes in embryo sac. The base of the ovule is called funiculus which is attached with ovary wall through placenta. On the basis of the positioning of funiculus and micropyle ovule is divided into five types; anatropus, orthotropous, campylotropus, hemianatropus, amphitropus. Anatropous type of ovule is common in horticultural crops, in which micropyle lies close to funiculus and ovule looks inverted (Fig. 1). The central portion of the ovule is occupied by embryo sac which is surrounded by sporophytic nucellar cells. The nucellar region opposite to the micropyle is called chalaza. In horticultural crops, the embryo sac, nucellus and chalaza are surrounded by two integuments thus make the ovule bitegmic (Frost and Soost 1968; Bhojwani and Bhatnagar 2000).

The embryo sac, the female gametophyte, is the most important structure wherein fusion of male gamete takes place and in turn zygotic embryo and endosperm form. Embryo sac is a 7-celled structure containing two polar nuclei in the centre, egg apparatus at the micropyler region containing one egg cell and two synergids and three antipodal cells at the chalazal end (Fig. 2). Cells of egg apparatus and antipodal cells are uninucleate and

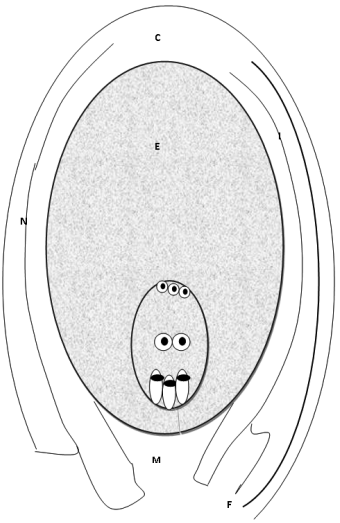

Figure 1. Anatropous ovule of citrus. E—Embryo sac, N—Nucellus, M—Micropyle, I—integument, C—Chalaza, F—Funiculus

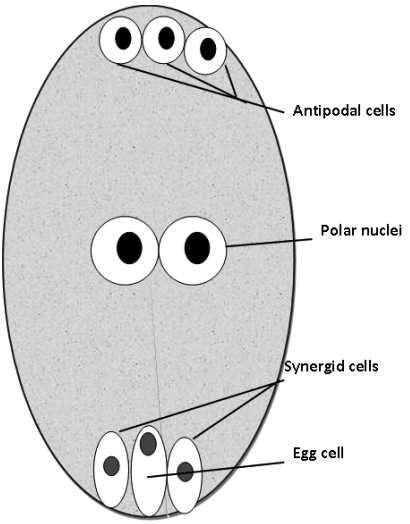

Figure 2. A typical embryo sac.

haploid whereas the central cell (polar nuclei) is binucleate or diploid. In angiosperm, double fertilization is the rule wherein one male gamete fuses with egg cell and forms embryo (2n) whereas other gamete fuses with the polar nuclei and forms endosperm (3n). In case of polyembryonate species, extra embryos are formed either by nucellar cells (2n), integuments (2n), synergids (n) or antipodal cells (n) (Pullaiah et al. 2001; Bhojwani and Bhatnagar 2000).

Different Ways of Polyembryony

The development of additional embryo may occur by different ways (Fig. 3). In plants polyembryonic cases arise from maternal tissue (adventitious polyembryony) is the most common followed by cleavage of the fertilized embryo (Bhatnagar and Bhojwani 1999; Ganeshaiah et al. 1991) whereas formation of additional embryo sac and participation of haploid cells in embryo formation is less common. The different ways of polyembryony are;

i. Formation of embryo by sporophytic/maternal tissue (2n) of ovule
ii. Formation of embryos by cells of embryo sac other than egg cell
iii. Development of more than one embryo sac within the same ovule
iv. Cleavage of proembryos

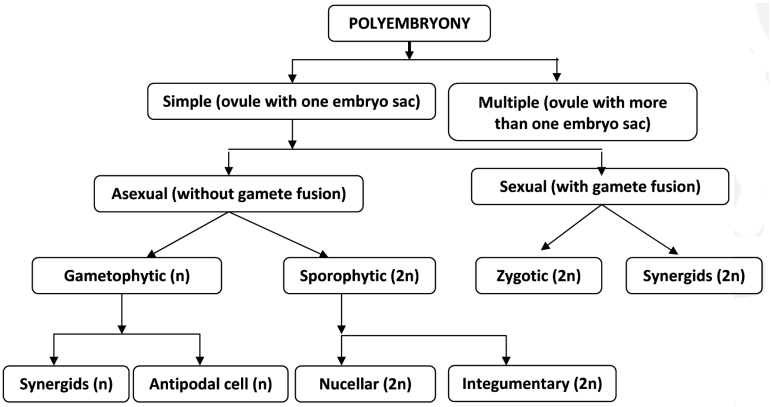

Figure 3. Schematic presentation of different ways of polyembryony.

Activation of Sporophytic Cells of the Ovule

The activation of sporophytic cells (nucellus and integument) of embryo sac is the most common force for polyembryony and the embryos arise from the sporophytic cells are called 'adventive embryos' and the phenomenon is called 'adventive embryony'. The maternal cells which involve in embryo development are primarily nucellus and integument. 'Nuceller embryony' (embryo develops from nucellus) is the most common feature in the families of horticultural importance (Kobayashi et al. 1979).

Cleavage of Proembryo

In this case the embryo that forms after normal fertilization divides irregularly and forms mass of cells that proliferate and develop into many embryos. The cleavage polyembryony is quite common in Orchidaceae, Poaceae and gymnosperm (Maheshwari 1950).

Embryos from other than the Egg Cell of Embryosac

In this category the most common source of additional embryo is the synergids (n). If embryo develops from unfertilized synergid the embryo will be haploid whereas fertilized synergids will give diploid embryo. The formation of diploid embryo from synergid is brought about by the entry of more than one pollen tube into the embryo sac or by the presence of additional male gamete in the same pollen tube. Embryos arising from unfertilized synergids are known in French bean (*Phaseolus vulgaris*). On the other hand, the development of embryo from antipodal cells and endosperm cells is rather rare.

Development of Additional Embryo Sac in the Same Ovule

Multiple embryo sac may arise in an ovule either from the same megaspore mother cell or from other megaspore mother cells or from any other sporophytic tissue of ovule.

Classification of Polyembryony

Polyembryony may be classified on the basis of source of origin, frequency of occurrence and ploidy level.

Camaron and Soost (1979) classified the polyembryony on the basis of frequency of polyembryony.

i. Strictly monoembryonic—Plant species in which the frequency of multiple embryos in less than 6% is described as strictly monoembryonic.
ii. Nearly monoembryonic—In case of nearly monoembryonic plant species the frequency of polyembryony varies between 6–10%.
iii. Polyembryonic—If the per cent of multiple embryo formation is more than 10% the condition is called polyembryony and plants are called polyembryonate.

On the other hand, Ernst (1918) divided polyembryony into two categories on the basis of embryogenesis.

i. True polyembryony—When two or more embryos arise in the same embryo sac from nucellus (citrus, mango, jamun), integument, synergid, etc.
ii. False polyembryony—In this case more than one embryo sac is formed in an ovule (*Fragaria* sp.) which is followed by the formation of multiple embryos.

Yakovlev (1967) proposed a genetic basis of classification of polyembryony.

i. Gametophytic—Multiple embryos arise from the gametic cells of the embryo sac (synergid, antipodal) after or without fertilization. In this case haploid/diploid embryos are formed.
ii. Saprophytic—When multiple embryos arise from sporophytic cells of the ovule (nucellus, integument) without fertilization and the resulting embryos are diploid and akin to parent.

Nucellar Embryogenesis

Nucellar embryony is the most common phenomenon of polyembryony in plants. It is an adventitious form of apomictic reproduction wherein the

somatic cells of the nucellus tissue are initialized to enter into an embryonic pathway of development Koltunow et al. (1995). In polyembryonic seed, many nonzygotic nuceller embryos are initiated directly from the maternal cells surrounding the embryo sac containing a developing zygotic embryo. During embryo sac expansion, embryogenic nuceller cells obtain access to endosperm and develop into embryo along side the zygotic embryo that may or may not develop completely. Nuceller embryo gives rise to seedlings that are of the same genotype as the female parent.

The adventive embryogenesis is completed in four steps: (I) formation of adventive Embryo Initial Cells (AEICs), (II) differentiation of AEICs, (III) division of AEICs and (IV) development of adventive embryos (Wakana and Uemoto 1988). AEICs generally appear before anthesis and are characterized by their homogenous cytoplasm, a large nucleus and irregular plastids. After pollination the cell composition is changed and starts synthesizing more energy. In fertilized seeds the division of the AEICs generally occurs before the division of zygote but after endosperm division (Kobayashi et al. 1979; Koltunow et al. 1995). The recent studies show that the initiation of adventive embryos in Citrus occurs autonomously and not affected by pollination, fertilization or the development of zygotic embryo or endosperm (Wilms et al. 1983). However the development of adventive embryos is greatly influenced by endosperm development. Lack or poor development of endosperm results in poor development of adventitve embryos. Therefore, in unfertilized seeds the nucellar embryos fail to develop beyond certain stage and are incapable of normal germination (Wakana and Uemeto 1987). The presence of endosperm promotes the development of adventive embryos at the micropyler end, but suppresses their development towards chalazal end. The degree of suppression is directly related to the distance of embryo from the micropylar end. In normal seeds the AEICs at the chalazal end generally do not develop beyond the initial celled stage.

In a genotype that produces polyembryonic seeds by nucellar embryony, normal zygotic embryo is also formed and such a genotype can produce different type of seeds (Wakana and Uemoto 1988): i) seeds with one mature zygotic embryo developed by sexual reproduction and in this case seed becomes monoembryonic: ii) seeds with one mature nucellar embryo only and in this case also seed shows monoembryony: iii) seeds with multiple mature nucellar embryos resulting in polyembryonic seed: iv) seeds with one mature zygotic embryo and one or more mature nucellar embryo and in this case seed becomes polyembryonic.

In citrus, nucellar embryony does not prevent normal sexual reproduction and zygotic embryo is also formed (Esen and Soost 1977; Wilms et al. 1983). Thus citrus seed produces both type of seedling having zygotic and nucellar origin. The number of nucellar embryo varies with

seed to seed and species to species. So a seed may contain two or more seedlings but not every seed produced by the plant with nucellar embryony has multiple mature embryos.

Regarding the location of embryos in the seed, the positioning of nucellar embryos is not fixed and they may be arranged near micropyle or away from micropyle. The zygotic embryos are generally present at the micropylar end of embryo (Thakur and Bajwa 1971).

Carimi et al. (1998) reported that zygotic embryo developed at faster rate and was at more advanced stage at the micropyler region of the embryo after 105 days of pollination (DAP), while the growth of nucellar embryos was relatively slower and they attained the developmental stage similar to zygotic embryo after 150–200 days of pollination.

Polyembryonate Crops

In fruit tree crops, polyembryony is also common and occurs in many crops; citrus (Frost 1938), mango (Sachar and Chopra 1957), *Syzigium* sp. (Narayanswamy and Roy 1960), kiwi (Crete 1944), almond (Kester and Gradziel 1996), *Fragaria* sp. (Lebegue 1952) and peach (Toyama 1974). The occurrence of polyembryony depends up on species and varieties, in other words not all species of a genus exhibit polyembryony and not all varieties of a species show polyembryony and the reason could be genetic. Most of the citrus species show polyembryony, while *C. medica* (citron), *C. grandis* (pummel) *C. latifolia* (Tahiti lime) and *C. nobilis* (King mandarin) are monoembryonic. In mango, most of the varieties grown in coastal area show polyembryony. A list of polyembryonic species and varieties are given below.

Crop	**Reported by**
Citrus sp.	Cameron and Soost 1973; Kultnow et al. 1996
Mangifera sp.	Ravishankar et al. 2004
Jamun	Van der Pijl (1974)
Kiwi fruit	Crete 1944
Prunus sp.	Toyama 1974
Almond	Kester and Gradziel 1996
Fragaria sp.	Lebegue 1952
Maize	Erdelska and Vidovencova 1992

Factors Affecting Polyembryony

The number and type of embryo produced may vary from tree to tree and also at different positions on a single tree (Parlevliet and Cameron 1959). The variation has been suggested to be controlled by minor genes,

pollen sources and environmental conditions (Khan and Roose 1988). If the zygotic embryo does not survive, the development of nucellar embryo is dependent on the initial development of zygotic embryo or on the process of fertilization of ovule (Tisserat et al. 1979) or on pollination. Thus nucellar embryo apparently may not develop independently of the process of sexual reproduction and in most of the cases it requires the development of zygotic embryo. In citrus, neither the seed setting nor the formation of the nucellar embryos takes place without pollination of flower. The development of nucellar embryos is induced by fertilization (Koltunow 1993). It has been established that pollen plays a definite role in the formation of additional embryos.

Polyembryony is also affected by the type of pollinator (Soares Filho et al. 1995), pollen viability, plant nutrition, temperature, environmental and soil humidity. The development of endosperm is required for the growth of embryos since endosperm supplies food to developing embryos. The fertilization is prerequisite for the production of matured embryo. The growth of nucellar embryo is arrested at later stage of development if endosperm is not formed (Koltunow et al. 1996). Therefore any factor that affects pollination, fertilization or seed development will also affect the percentage of polyembryony and embryo number per seed.

Causes of Polyembryony

Many theories have been proposed to explain the occurrence of polyembryony but most of them are not sufficiently validated. Haberlandt (1921, 1922) proposed the 'necrohormone theory'. The theory advocated that the degenerating cell of the nucellus acts as a source of stimulus for the adjacent cells to divide and form adventives embryo. But the theory could not be validated as adventives embryo could not be induced by damaging the nucellar cells. The monoembryonate conditions in some species of citrus have been ascribed to the synthesis and release of certain volatile and non-volatile embryogenic inhibitors in their ovules which do not occur in the ovules of polyembryonate species. Ethanol and ethylene are important volatile inhibitors produced by the ovule of monoembryonate species of citrus (*Citrus medica*). The ethylene among other substances could be repressing the development of nucellar embryos through its union with an ethylene receptor protein of nucellar cells. The fewer number of adventives embryos in south side of tree than that of north side, might be due to the more synthesis of ethylene, ethanol and abcisic acid due to variation in sunlight and temperature (Garcia et al. 1999). The non-volatile component of the inhibitors was auxin, ABA and GA_3. But genetic theory is the most accepted theory as the presence of polyembryony is determined by the gene.

Inheritance of Nuceller Embryony

Polyembryony in citrus and mango is generally controlled by a dominant gene having heterozygous allele (Pp) while homozygous recessive gene (pp) is present in monoembryonic citrus species (Parlevliet and Cameron 1959; Aron et al. 1998). In monoembryonic species, these recessive genes may synthesize a potent inhibitor of embryogenesis (Esen and Soost 1977). A variable degree of polyembryony was recorded in polyembryonate offspring obtained by crossing monoembryonic and polyembryonic and between polyembryonic parents that implied the presence of minor genes affecting degree of polyembryony. Moreover, the presence of modifier or duplicate genes should also be considered as in some crosses between monoembryonic and polyembryonic parents, and between polyembryonic parents, the progeny ratio varied greatly from 1:1 to 3:1 ratios. It may be concluded that mostly polyembryony is controlled by heterozygous dominant gene and the absence of dominant allele leads to monoembryony. But in some of the cases modifier genes and minor genes are also present that tinker with the ratios and degree of polyembryony respectively.

Degree of Polyembryony

The degree of polyembryony is the frequency of occurance in multiple seedlings in a seed. It varies with species and varieties and the variation also occurs with location, environment and position of fruits on tree. On the basis of degree of polyembryony species/varieties can be divided

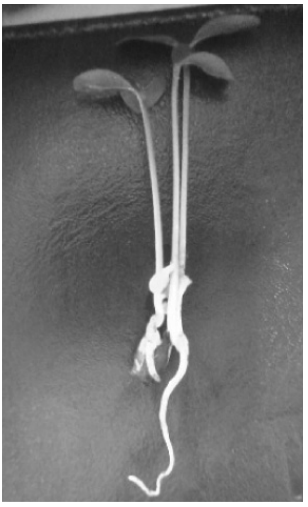

Figure 4. Polyembryony in *Citrus jambhiri*.

Color image of this figure appears in the color plate section at the end of the book.

in to three categories; slightly polyembryonic (polyembryony up to 25%), moderately polyembryonic (polyembryony up to 50%) and highly polyembryonic (polyembryony > 50%). On the basis of number of embryos (1, 2, 3, . .), embryony may be divided into different morphotypes: singlet (one embryo/seed), duplet (two embryos/seed), triplet (three embryos/seed), quadruplet (four embryos/seed), quintuplet (five embryos/seed), sextuplet (six embryo/seed), heptuplet (seven embryos/seed), octuplets (eight embryos/seed) and so on. In apomictic *Citrus* sp. quadruplet and triplet embryos are more frequent regardless the genotypes (Kishore et al. 2012).

Identification of Nucellar Embryo/Seedlings

Morphological identification of nucellar and zygotic seedlings at juvenile stage is practically difficult, moreover it is not an effective method, as the chance of selecting wrong seedling is more. Although, visual recognition is the simplest method and is effective when the male and female parents differ significantly in their growth habit but when the two parents are similar, as is the case with most plant species, separation is more difficult. The difference in zygotic and nucellar seedlings can be made only after fruiting and the plant possessing the characters of mother pant will be nucellar in origin while the plant that shows variation in characters is zygotic in origin. There is considerable variation among zygotic seedlings and visual recognition on the basis of height, leaf size, thorn length, petiole length and stem diameter is especially difficult in non-hybrid cultivars. In citrus the zygotic seedling can be identified morphologically if one variety is crossed with trifoliate orange (*Poncirus trifoliate*) as the resultant zygotic seedlings will have trifoliate leaves as this character is controlled by dominant gene (Khan and Roose 1988).

Cytologically the nucellar cells destined to form adventives embryos can be distinguished from other cells of the nucellus by their dense cytoplasm and starchy contents (Wilms et al. 1983). Zygotic embryo was usually larger than the other embryos and also took the most space in a seed, while, nuclear embryos were tiny, heart shaped and green and were crowded at the micropyler region and most of these could be easily separable (Das et al. 2005). Generally, the most vigorous and first germinating seedling of citrus is considered to be zygotic as is has largest cotyledons and occupies most of the space of seed.

Similarly, in mango, the earlier concept was that the zygotic plantlets are the weakest in polyembryonic mango seed because it probably degenerates due to competition with nucellar plantlets (Sachar and Chopra 1957). The recent findings proved that zygotic plantlets are vigorous and positioned near micropyle and possessed big cotyledons. The vigorocity of zygotic

plants can be explained by a heterotic effect of cross between the female plant and unidentified male plant (Maria et al. 2006).

The biochemical techniques are effective and expeditious tools to identify zygotic and nucellar seedlings at an early stage. Among biochemical techniques, isozyme markers are commonly used to distinguish nucellar (true-to-type) and zygotic (off-type) citrus seedlings (Moore and Castle 1988; Anderson et al. 1991). Gill et al. (2002) reported that the nucellar seedlings could be distinguished from the zygotic seedlings on the basis of banding pattern of different isozymes.

Molecular techniques by using molecular markers (RAPD, RFLP, SSR, ISSR, QTL, etc.) are the recent, advanced, effective, expeditious and reliable tools to study gene expression and to identify zygotic and nucellar seedlings at an early stage as results are not influenced by external factors. Genes show conspicuous difference in expression between polyembryonic (apomictic) and monoembryonic (nonapomictic) genotypes. The *msg-2* gene was highly expressed in the late stage of somatic embryogenesis in monoembryonic cultivars of *Citrus*, whereas, *msg-2* was not expressed in the initiation stage of embryogenesis in polyembryonic cultivar suggesting the suppressing role in initial cell formation of somatic embryos (Nakano 2013). Similarly, genes like *Ig1* in maize (Evans 2007), *OsCem* (Yang and Hwa 2008) and *OsPE* in rice (Puri et al. 2009) associated to polyembryony have been reported. Somatic Embryogenesis Receptor Kinase (SERK*)* gene, a leucine-rich repeat trans-membrane protein kinase, enhances the ability of the apical meristem to form somatic embryos. SERK genes have been linked to somatic embryogenesis (SE) in a number of species including *Dactylis glomerata* (Somleva et al. 2000), *Arabidopsis thaliana* (Hecht et al. 2001), *Medicago truncatula* (Nolan et al. 2003), *Helianthus annuus* (Thomas et al. 2004), *Ocotea catharinensis* (Santa-Catarina et al. 2004), *Citrus unshiu* (Shimada et al. 2005), and *Theobroma cacao* (de Oliveira Santos et al. 2005). SERK genes have also been described in relation to apomixis in *Hieracium* (Tucker et al. 2003) and *Poa pratensis* (Albertini et al. 2005) as well as zygotic embryogenesis in carrot, *Arabidopsis*, and wheat (Schmidt et al. 1997; Hecht et al. 2001; Singla et al. 2008). The best defined SERK gene in relation to SE is the Arabidopsis SERK1 (AtSERK1) and over expression of this SERK was shown to enhance embryogenic competence in *Arabidopsis* cultures (Hecht et al. 2001). Das et al. (2007) identified the zygotic seedlings through RAPD markers and reported that zygotic seedlings (twin or triplets) usually had one or more extra band than those of the nucellar seedlings they also observed the variability in mandarins of north east by RAPD profiling. Maria et al. (2006) identified the nucellar seedlings of mango through RAPD and also determined the position of zygotic and nucellar embryos in an ovule.

Significance of Polyembryony

Polyembryony or nucellar embryony plays an important role in horticulture, cytogenetics and plant breeding.

a. Nucellar embryony helps in producing genetically uniform seedlings of the parental type for better clones of scion and rootstock.
b. Polyembryony helps in the large scale propagation of desired genotype.
c. The nucellar seedlings show a restoration of the vigour lost after repeat vegetative propagation.
d. The nucellar embryos are free from diseases as *in vitro* nucellar embryony is the only practical approach to raise virus free clones of polyembryonate citrus varieties in nature.
e. Haploids can be used for cytogenetic studies.
f. Homozygous diploids can be raised from haploids by cochicine treatment.

References

Albertini, E., G. Marconi, L. Reale, G. Barcaccia, A. Porceddu, F. Ferranti and M. Falcinelli. 2005. SERK and APOSTART. Candidate genes for apomixis in *Poa pratensis*. Plant Physiology 138: 2185–2199.

Anderson, C.M., W.S. Castle and G.A. Moore. 1991. Isozymic identification of zygotic seedlings in Swingle *Citrus paradisi* x *Poncirus trifoliata* nursery and field population. J. American Soc. Hort. Sci. 116: 322–6.

Aron, Y., H. Czosnek, S. Gazit and C. Degani. 1998. Polyembryony in mango (*Mangifera indica* L.) is controlled by a single dominant gene. Hort science, Alexandria 33(7): 1241–2.

Bhojwani, S.S. and S.P. Bhatnagar. 1999. Polyembryony. *In*: The Embryology of Angiosperms. Vikas Publishing House Pvt. Ltd., New Delhi, pp. 236–253.

Bhojwani, S.S. and S.P. Bhatnagar. 2000. Polyembryony. *In:* The Embryology of Angiosperms. Vikas Publishing House Pvt. Ltd., New Delhi.

Camaron, J.W. and R.K. Soost. 1979. Sexual and nucellar embryony in F1 hybrid and advanced crosses in *Citrus* with *Poncirus*. J. American Soc. Hort. Sci. 104(3): 408–10.

Carimi, F., M.C. Tortorici, F.de. Pasquale and F.G. Crescimanno. 1998. Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explants of sweet orange naval group (*Citrus sinensis* (L.) Osb.). Plant Cell, Tissue Org. Cult. 54(3): 183–189.

Carman, J.G. 1997. Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biological J. Linn. Soc. 61: 51–94.

Crete, P. 1944. Polyembryony in *Actinidia chinensis.* Bull. Soc. Botany France 91: 89–92.

Das, A., B. Mandal, J. Sarkar and S. Chaudhuri. 2005. Variability in multiple embryo formation and seedling development of mandarin orange (*Citrus reticulata* Blanco) of north-eastern Himalayan region of India. PGR News Letter 143: 35–9.

Das, A., B. Mandal, J. Sarkar and S. Chaudhuri. 2007. Occurrence of zygotic twins seedlings in mandarin orange plants of northeastern Himalayan Region. Curr. Sci. 92(11): 1488–9.

de Oliveira, S.M., E. Romano, K.S.C. Yotoko, M.L.P. Tinoco, B.B.A. Dias and F.J.L. Aragao. 2005. Characterization of the cacao somatic embryogenesis receptor-like kinase (SERK) gene expressed during somatic embryogenesis. Plant Science 168: 723–729.

Erdelska, O. and Z. Vidovencova. 1992. Cleavage polyembryony in maize. Sex. Pl. Repr. 5: 224–226.
Ernst, A. 1918. Bastardierung als Ursache der Apogamie im Pflanzenreich: Ein Hypothese zu experimentellen Vererbungs- und Abstammungslehre. Fischer, Jena.
Esen, A. and K. Soost. 1977. Adventitve embryogenesis in Citrus and its relation to pollination and fertilization. Am. J. Bot. 64: 607–14.
Evans, M.M.S. 2007. The indeterminate *gametophyte 1* gene of maize encodes a LOB Domain protein required for embryo sac and leaf development. The Plant Cell 19: 46–62.
Frost, H.B. 1938. Nucellar embryony and juvenile characters in clonal varieties of citrus. J. Heredity 29: 423–32.
Frost, H.B. and R.K. Soost. 1968. Seed Production: Development of gametes and embryos. *In*: The Citrus Industry Vol. II. W. Reuther, L.D. Batchelor and H.J. Webber (eds.). Division of Agricultural Science, University of California, USA, pp. 292–320.
Ganeshaiah, K.N., R. Uma Shaanker and N.V. Joshi. 1991. Evolution of polyembryony: consequences to the fitness of mother and offspring. J. Gen. 70: 103–127.
Garcia, R., M.J. Asin, J. Forner and E.A. Carbonell. 1999. Genetic analysis of the apomixis in *Citrus* and *Poncirus* by molecular markers. Theor. Appl. Gene. 99: 511–18.
Gill, M.I.S., N. Chawla and H.R. Chopra. 2002. Isozyme characterization and indexing of *in vitro* raised nucellars in citrus rootstocks. Ind. J. Hort. 59: 122–126.
Haberlandt, G. 1921. Wundhormone als Erreger von Zellteilungen. Berlin *In:* Beiträge zur Allgemeinen Botanik 2: S.1–53.
Haberlandt, G. 1922. Über die Ursache des Ausbleibens de Reduktionsteilung in den Samenanlagen einiger parthenogeneti scher Angiospermen. – Sitzungsber. Preuss. Akad. Wiss., Phys.-Math. Kl. 283–294.
Hecht, V., J.P. Vielle-Calzada, M.V. Hartog, E.D.L. Schmidt, K. Boutilier, U. Grossniklaus and S.C. de Vries. 2001. The Arabidopsis Somatic Embryogenesis Receptor Kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiology 127: 803–816.
Kester, D.E. and T.M. Gradzeil. 1996. Almond. *In*: Fruit Breeding. J. Janik and J.D. Moore (eds.). John Wiley and Sons, New York, pp. 1–97.
Khan, I.A. and M.L. Roose. 1988. Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. J. American Soc. Hort. Sci. 113: 105–10.
Kishore, K., N. Monika, D. Rinchen, B. Lepcha and B. Pandey. 2012. Polyembryony and seedling emergence traits in apomictic citrus. Sci. Hort. 138: 101–107.
Kobayashi, S., I. Ieda and M. Nakantani. 1979. Studies on the nucellar embryogenesis in citrus. Formation of the primordial cells of the nucellar embryony on the ovule of the flower bud, and its meristematic activity. J. Japanese Soc. Hort. Sci. 48: 179–85.
Koltunow, A.M., T. Hidaka and S.P. Robinson. 1996. Polyembryony in *Citrus*: Accumulation of seed storage proteins in seeds and in embryos cultured *in vitro*. Plant Physiology 110(2): 599–609.
Koltunow, A.M., K. Soltys, N. Nito and S. Mcclure. 1995. Anther, ovule, seed and nucellar embryo development in *Citrus sinensis* cv. Valencia. Can. J. Bot. 73: 1567–82.
Koltunow, A.M., T. Hidaka and S.P. Robinson. 1996. Polyembryony in Citrus. Plant Physiology 110: 599–609.
Koltunow, A.M. 1993. Apomixis: Embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell 5: 1425–37.
Lebegue, A. 1952. Polyembryony in Angiosperm. Bull. Soc. Bot. France 99: 329–69.
Maheshwari, P. 1950. An introduction to the embryology of angiosperm. MC-Graw-Hills, New York.
Maria Cristina Rocha, Cordiero Alberto Carlos, Queiroz Pinto, Victor Hugo Vargas, Ramos Fabio Galop Falaro and Lilia Marta Santo Fraga. 2006. Identification of plantlets genetic

origin in polyembryonic mango (*Mangifera indica* L.) Rosinha seeds using RAPD markers. Revista Brasileira de Fruiticulture 28(3): 454–7.

Moore, G.A. and W.S. Castle. 1988. Morphological and isozymic analysis of open pollinated citrus-rootstock population. J. Heredity. 79: 59–63.

Nakano, M., K. Kogoshi, T. Shimizu, T. Endo, T. Shimada, H. Fujii and M. Omura. 2013. Characterization of genes associated with polyembryony and *in vitro* somatic embryogenesis in *Citrus*. Tree genetics and Genome 9: 795–803.

Narayanswamy, S. and S.K. Roy. 1960. Embryo sac development and polyembryony in *Syzigium cumini*. Botanical Note 3: 273–84.

Nolan, K.E., R.R. Irwanto and R.J. Rose. 2003. Auxin up-regulates MtSERK1 expression in both Medicago truncatula root-forming and embryogenic cultures. Plant Physiology 133: 218–230.

Parlevliet, J.E. and J.E. Camaron. 1959. Evidence of the inheritance of nucellar embryony in citrus. Proc. American Hort. Soc. 74: 252–60.

Pullaiah, T., K. Lakshminarayan and B. Hanumanth Rao. 2001. Polyembryony. *In*: Textbook of Embryology of Angiosperm. Regency Publication, New Delhi, pp. 164–77.

Puri, A., P.O. Basha, M. Kumar, D. Rajpurohit, G.S. Randhawa, S.F. Kianian, A. Rishi and H.S. Dehaliwai. 2009. The polyembryo gene (*OsPE*) in rice. Functional & Integrative Genomics 10: 359–366.

Ravishankar, K.V., P. Chandrashekara, S.A. Sreedhara, M.R. Dinesh, A. Lalitha and G.V.S. Saiprasad. 2004. Diverse genetic bases of Indian polyembryonic and monoembryonic mango (*Mangifera indica* L.) cultivars. Curr. Sci. 87: 870–871.

Sachar, R.C. and R.N. Chopra. 1957. A study of endosperm and embryo in *Mangifera*. Indian J. Agric. Sci. 27(1): 219–38.

Santa-Catarina, C., L.R. Hanai, M.C. Dornelas, A.M. Viana and E.I.S. Floh. 2004. SERK gene homolog expression, polyamines and amino acids associated with somatic embryogenic competence of *Ocotea catharinensis* Mez. (Lauraceae). Plant Cell Tiss. Org. Cult. 79: 53–61.

Schmidt, E.D.L., F.Guzzo, M.A.J. Toonen and S.C. de Vries. 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124: 2049–2062.

Shimada, T., T. Hirabayashi, T. Endo, H. Fujii, M. Kita and M. Omura. 2005. Isolation and characterization of the somatic embryogenesis receptor-like kinase gene homologue (CitSERK1) from *Citrus unshiu* Marc. Sci. Hort. 103: 233–238.

Singla, B., J.P. Khurana and P. Khurana. 2008. Characterization of three somatic embryogenesis receptor kinase genes from wheat, *Triticum aestivum*. Plant Cell Reports 27: 833–843.

Soares, F.W.S., L.M. Lee and A.P. Cunha Sobrinho. 1995. Influence of pollinators on polyembryony in citrus. Acta Horticulture 403: 256–265.

Somleva, M.N., E.D.L. Schmidt and S.C. de Vries. 2000. Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by SERK expression. Plant Cell Reports 19: 718–726.

Thakur, D.R. and B.S. Bajwa. 1971. Extent of polyembryony in some species and varieties of citrus. Indian J. Horticulture 2: 25–28.

Thomas, C., D. Meyer, C. Himber and A. Steinmetz. 2004. Spatial expression of a sunflower SERK gene during induction of somatic embryogenesis and shoot organogenesis. Plant Physiol. Biochem. 42: 35–42.

Tisserat, B., E.B. Esan and T. Murrashige. 1979. Somatic embryogenesis in angiosperm. Horticultural Review 1: 1–78.

Toyama, T.K. 1974. Haploidy in peach. Horticulture Science 9: 187–8.

Tucker, M.R., A.-C.G. Araujo, N.A. Paech, V. Hecht, E.D.L. et al. 2003. Sexual and apomictic reproduction in *Hieracium* subgenus *Pilosella* are closely interrelated developmental pathways. The Plant Cell 15: 1524–1537.

Wakana, A. and S. Uemoto. 1987. Adventive embryogenesis in citrus. I. The occurrence of adventives embryo without pollination or fertilization. Am. J. Bot. 74: 517–30.

Wakana, A. and S. Uemoto. 1988. Adventive embryogenesis in citrus (Rutaceae). II. Post fertilization development. Am. J. Bot. 75: 1033–47.

Wilms, H.J., J.L. Van Went, M. Cresti and F. Ciampolini. 1983. Adventive embryogenesis in Citrus. Caryologia 36: 65–78.

Yakovlev, M.S. 1967. Polyembryony in higher plants and principles of its classification. Phytomorph. 17(1–4): 278–82.

Yang, X.C. and C.M. Hwa. 2008. Genetic and physiological characterization of the *OsCem* mutant in rice: formation of connected embryos with multiple plumules or multiple radicles. Heredity 101: 239–246.

Color Plate Section

Chapter 1

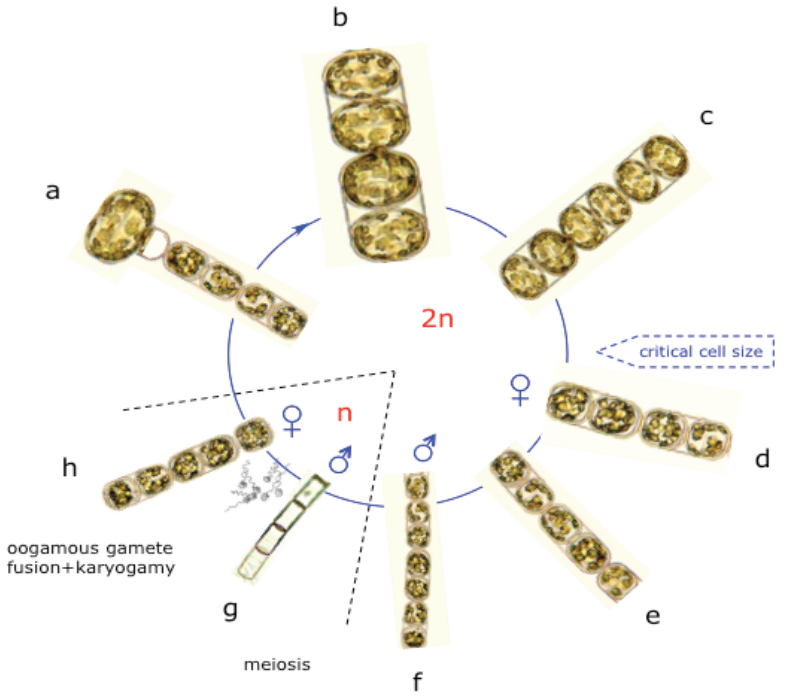

Figure 2. Diagrammatic Representation of the Life Cycle in a Centric Diatom *Melosira* sp. (a) an initial cell formed in a mature auxospore, (b-f), because of specific construction of the frustule the cell size decreases while cells pass through mitotic cycles, (g) male and (h) female gametogenesis; n and 2n, haplontic and diplontic phases (cell diameter range: ca. 20–80 µm).

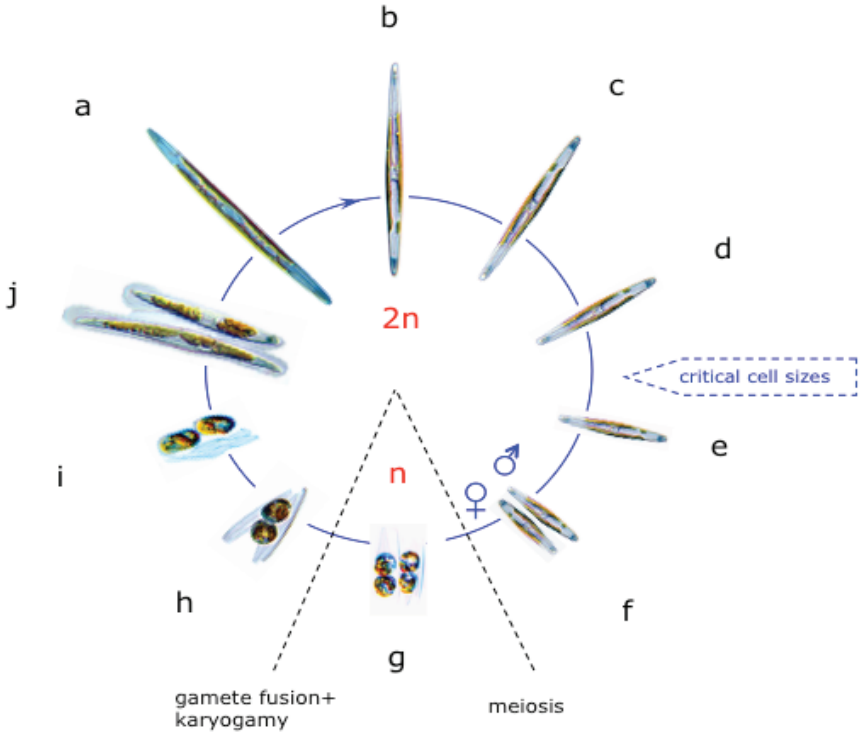

Figure 3. Diagrammatic Representation of the Life Cycle in the Pennate Diatom *Haslea karadagensis.* (a) an initial cell, (b-e) vegetative cells passing through mitotic cycles, (f) pairing of gametangia, (g) gametogenesis, (h) zygotes, (i-j) auxospore formation; n and 2n, haplontic and diplontic phases (cell length range: *ca.* 20–95 µm).

Chapter 3

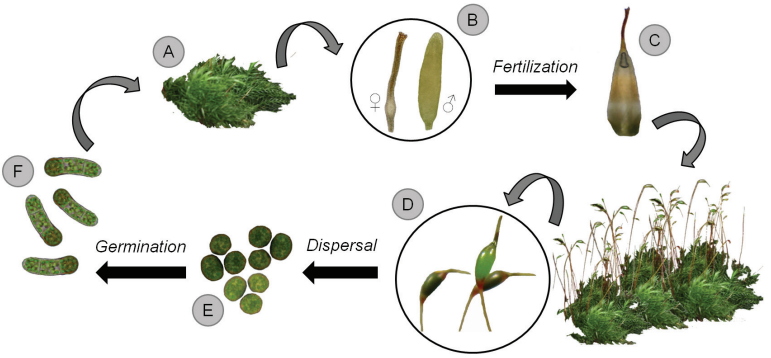

Figure 1A–F. Life cycle of a monoicous moss (*Pyrrhobryum spiniforme*). A. Leafy gametophytes; B. Female (archegonium) and male (antheridium) gametangia; C. Fertilized archegonium; D. Sporophytes attached to gametophytes; E. Spores; F. Protonemata (chloronema phase).

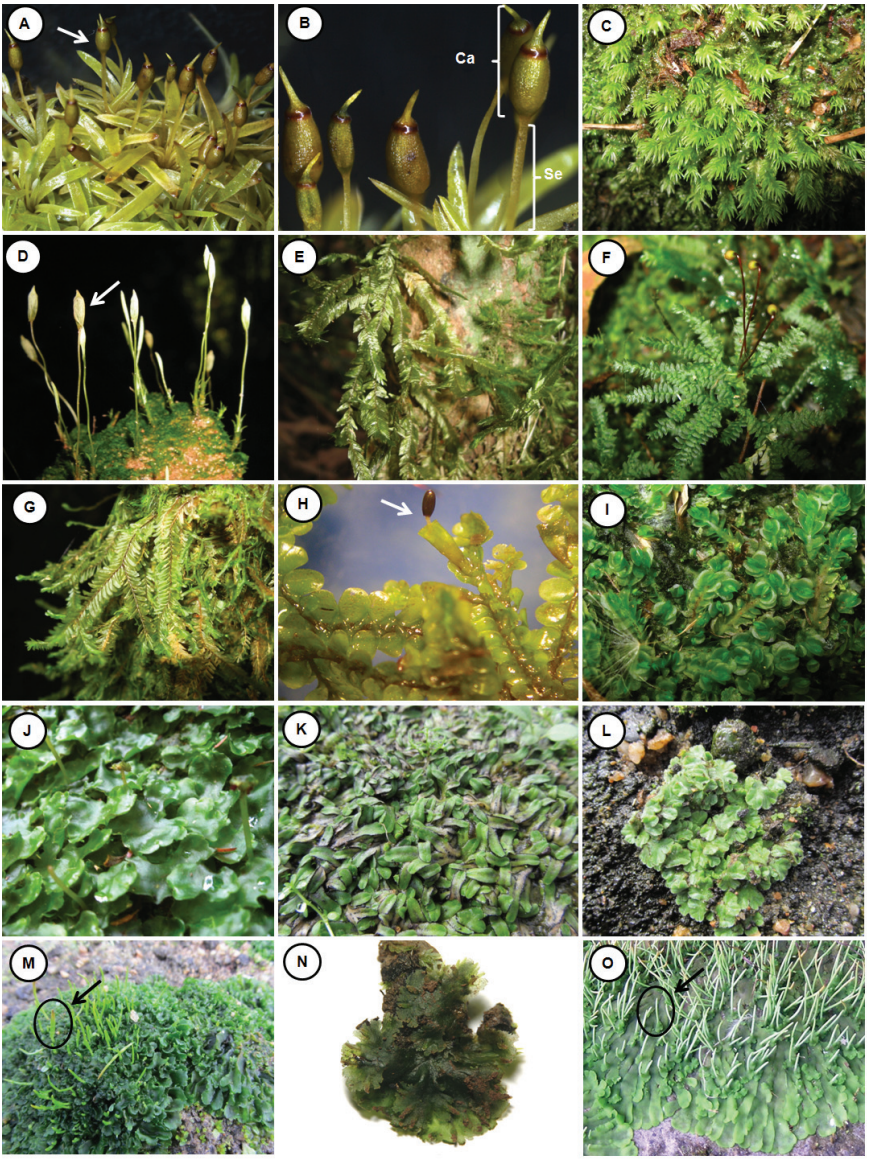

Figure 2 A–N. Diversity of bryophytes. A-D. Acrocarpous mosses; B. Detail of the sporophytes. E-F. Pleurocarpous mosses; G-I. Leafy liverworts; J-L. Thallose liverworts. M-O. Hornworts. Ca. Capsule; Se. Seta; Arrows indicate sporophytes. Pictures K-M by Nivea D. Santos and pictures N-O by Juan Vilarreal.

Chapter 5

Figure 1. Three ovules of *Ginkgo biloba* at time of pollination. Fully exuded drop at top, partially exuded drop at bottom, and ovule with no drop at left. Scale bar = 1 mm.

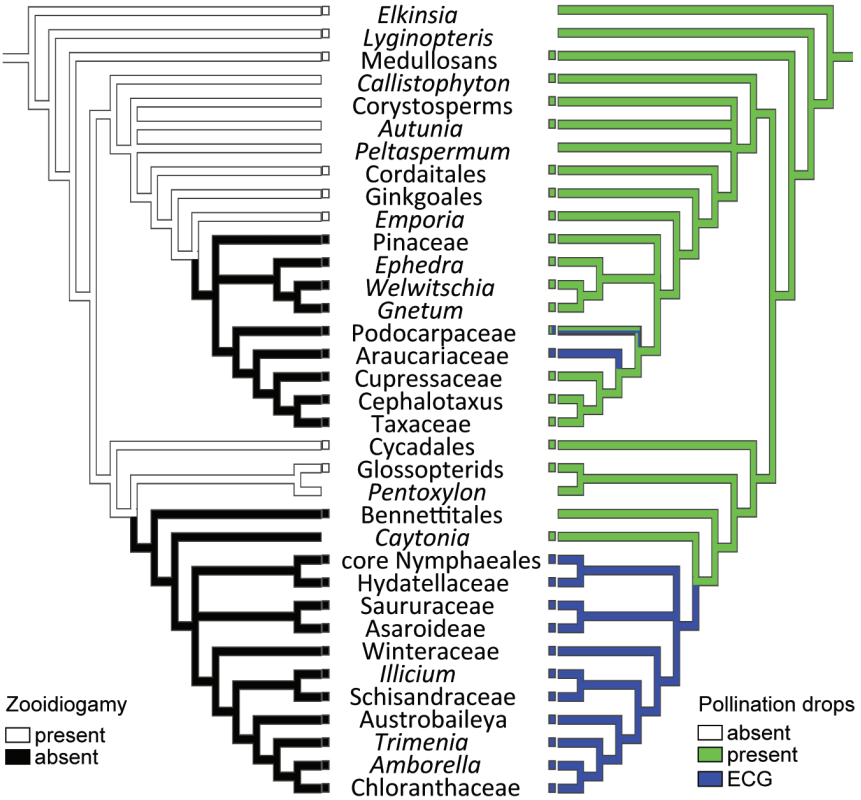

Figure 2. Mirror trees showing the parsimony based character mapping on the strict consensus of 10 trees; parsimony model for characters is unordered. Left, mapping of zoodiogamy presence and absence; fossil taxa with prepollen scored as present. Right, mapping of pollination drop absence, presence, or occurrence of ECG (extra-ovular capture and germination); fossil taxa with saccate pollen scored as present.

Chapter 6

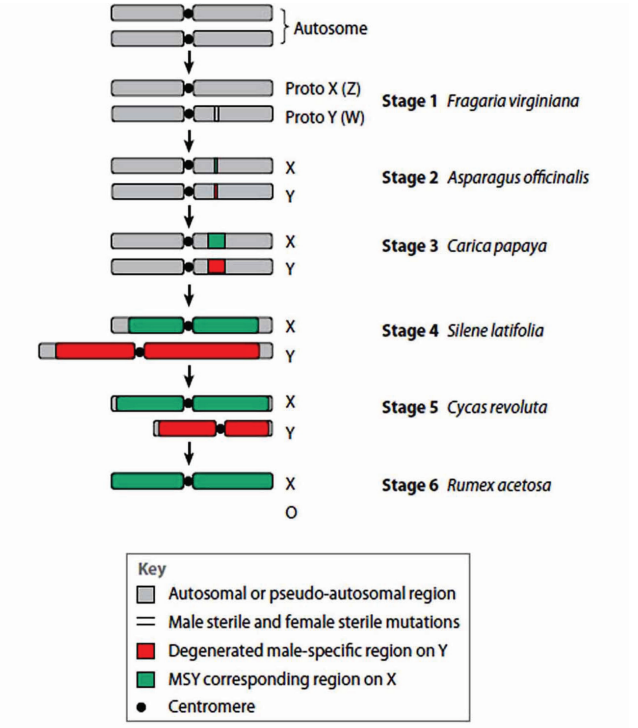

Figure 1

The six stages of sex chromosome evolution. Stage 1: Unisexual mutation of two sex determination genes with complementary dominance. Stage 2: Suppression of recombination between the two sex determination genes and YY genotype is viable. Stage 3: Suppression of recombination spread to neighboring regions and a small male-specific region of the Y chromosome (MSY) region evolved. YY genotype is not viable. Stage 4: The MSY expands in size and degenerates in gene content via accumulation of transposable element insertions and intrachromosomal rearrangements. The X and Y chromosomes become heteromorphic. Stage 5: Severe degeneration of the Y chromosome. Deletion of nonfunctional DNA sequences results in reduction of Y-chromosome size. Stage 6: Suppression of recombination spreads to the entire Y chromosome. The Y chromosome is lost, and X-to-autosome ratio sex determination system has evolved (Ming et al. 2011).

Chapter 8

Figure 1. Left: *Fritillaria meleagris* in flower with both purple- and white-flowered individuals. Photo by M. Zych. Right: *Fritillaria meleagris* L. (Liliaceae). Photograph by M. Zych, with kind permission from Springer Sciences and Business Media.

Chapter 10

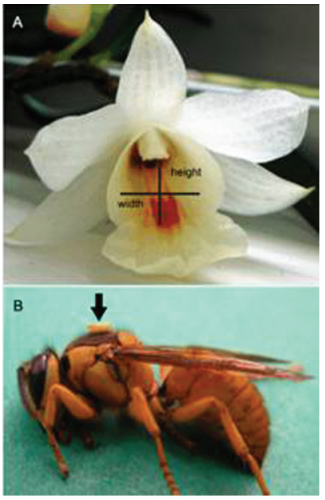

Figure 1. *D. sinense* flower and *Vespa bicolor* forager. *D. sinense* flower (A) and *V. bicolor* forager with pollinia stuck onto the thorax (B) (Brodmann et al. 2009).

Figure 2. *D. jiajiangense* and its pollinator. (A). *Andrena parvula* pollination method, the black arrow expresses the direction of the anther cap pushed by *A. parvula*. (B). *A. parvula* carrying pollinia (Pang et al. 2012).

Chapter 14

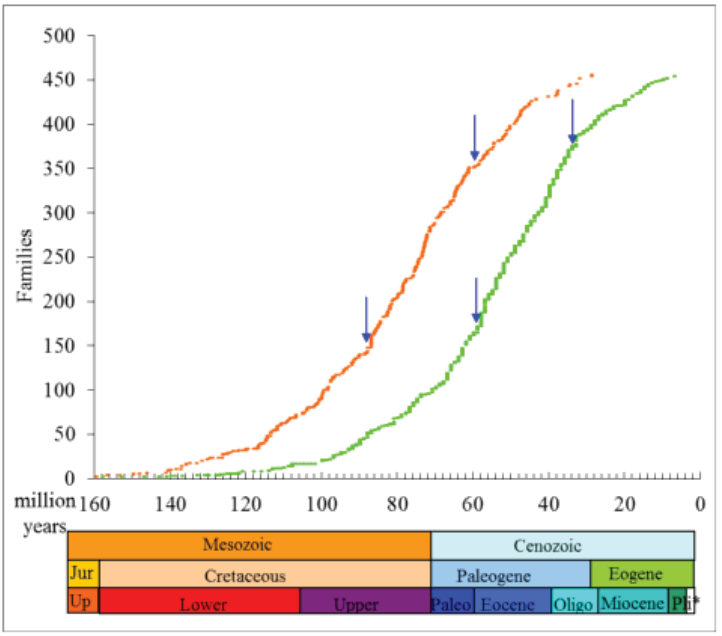

Figure 1. Cumulative number of angiosperm family lineages by divergence age from the modified topologies of Wikström et al. (2001) and Davies et al. (2004). The natural logarithm of the cumulative number of lineages against the ML estimateof the age of the family from Wikström et al. (2001) (solid line) or the maximum age of the family as estimated by Davies et al. (2004) (dashed line).

Chapter 15

Figure 4. Polyembryony in *Citrus jambhiri*.

Reproductive biology is the basis of species improvement and a thorough understanding of this is needed for plant improvement, whether by conventional or biotechnological methods. This book presents an up to date and comprehensive description of reproduction in lower plants, gymnosperms and higher plants. It covers general plant biology, pollination, pollen-pistil interaction, post-fertilization changes, and seed dormancy.

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