Pollination in *Ephedra* (Gnetales)

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Abstract

Pollination, i.e., the transport of male gametophytes to female gametophytes, can occur with biotic or abiotic vectors and is necessary for fertilization and completion of the lifecycle in all seed plants. Insect pollination and the co-evolution between angiosperms and insects have during the last century been discussed as one possible solution to Darwin’s abominable mystery and an important explanation for the relatively abrupt turn-over from a vegetation dominated by gymnosperms to a vegetation dominated by angiosperms in the Cretaceous. Insect pollination is, however, a much older phenomenon that can be traced back to the Devonian, but is it an ancestral trait that has been lost in many seed plant groups, or has it originated multiple times in parallel? These questions have to be addressed in a phylogenetic framework comprising extant and extinct seed plant groups. The Gnetales are constantly in focus in studies of seed plant phylogeny, probably because they have repeatedly been suggested, and refuted, to be the closest living relatives of angiosperms. The order consists of three genera, *Gnetum*, *Welwitschia* and *Ephedra*, of which the former two have long been known to be insect pollinated. Pollination biology in *Ephedra* has, however, been poorly studied and understood. In this thesis pollination mechanisms in *Ephedra* (Gnetales) are investigated by field experiments and observations (Paper I) and aerodynamic simulations and studies of pollen morphology (Paper II). The results show that there are multiple pollination mechanisms within this otherwise morphologically and ecologically uniform genus. Further, in contrast to what has often been assumed, insect pollination is shown to be ancestral in the Gnetales and not a derived feature that has evolved within the group. Using this new information on pollination biology in the Gnetales and data from the literature, I explore evolution of pollen morphology and pollination mode in seed plants.
List of Papers:

The following papers, referred to in the text by their roman numerals, are included in this thesis.


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Pollination in the Gnetales and implications for the evolution of insect pollination in seed plants

Introduction

Pollination involves the transportation of pollen (male gametophytes) to female gametophytes that leads to fertilization and reproduction in seed plants. The transportation can occur by means of abiotic vectors, for example wind or water, or by biotic vectors, for example insects or other animals. The relationship, through which plants rely on pollinators for their reproduction and pollinators rely on plants as a food source, was described already in 1793 by Sprengel and has fascinated scientist ever since (Sprengel 1793). The adaptation to insect pollination has been discussed as one of the explanations for the turn-over to an angiosperm dominated flora in the Cretaceous (for details on the geological time scale, see Appendix 1) (Hickey and Doyle 1977, Regal 1977, Crepet 1979, Burger 1981, Tiffney 1984, Bond 1989). However, insect pollination is probably a much older phenomenon.

Investigations of the evolution of insect pollination need to be conducted in a phylogenetic framework, which takes into consideration relationships among extinct and living seed plants. Based on morphological data (Crane 1985, Doyle and Donoghue 1986, Loconte and Stevenson 1990, Doyle and Donoghue 1992, Doyle and Donoghue 1993) and molecular data (Chaw, et al. 2000, Rydin and Källersjö 2002) the Gnetales are strongly supported as monophyletic but their relationship to the other five clades of seed plants has been very difficult to resolve (Chase, et al. 1993, Källersjö, et al. 1998, Chaw, et al. 2000, Rydin and Källersjö 2002, Schmidt and Schneider-Poetsch 2002, Doyle 2008, Mathews 2009, Rydin and Korall 2009, Mathews, et al. 2010). Also, or maybe therefore, to be able to understand the evolution of insect pollination in seed plants, the understanding of pollination mechanisms in the Gnetales is of great importance.

Pollination biology in the Gnetales has been studied for a few species, i.e., *Gnetum gnemon*, *Gnetum cuspidatum* (Kato, et al. 1995), Welwitschia (Wetschnig and Depish 1999), and a few species of *Ephedra* (see Paper I for references and details). Nevertheless, pollination biology in the Gnetales is considered poorly investigated and understood (Endress 1996, Gorelick 2001). Previous studies have, for example, come to deviating results on pollination in *Ephedra*. A trend towards entomophily in the genus has been suggested (Bino, et al. 1984b), but field observations of pollination in *Ephedra* has previously never been assessed in an evolutionary framework. Together with collaborators, I have investigated pollination mechanisms in *Ephedra*, using mechanistic field experimentation (Paper I), and aerodynamic simulations (Paper II). Here I put the resulting conclusions on pollination biology in the Gnetales in the perspective of evolution of insect pollination and pollen morphology in seed plants. When did insect pollination evolve? Is the presence of pollen sacs correlated with wind pollination and is a saccate pollen diagnostic of a clade of seed plants or has it evolved several times?
Material and methods

**Taxon sampling and character coding:** I selected a set of taxa that represent the major clades of seed plants, and for which pollination biology is known or assessed for at least some taxa. I chose four representative groups of extinct plants from the Paleozoic: Callistophytales, Cordaitales Lyginopteridales and Medullosales; four groups of extinct plants from the Mesozoic: Bennettitales, Caytoniales, Glossopteridales and Peltaspermales, and from the living clades: angiosperms, cycads, Ginkgo, conifers (several conifer genera to investigate the evolution of saccate pollen) and the Gnetales.

I have investigated pollen transfer in *Ephedra* (Gnetales) using field experiments; enclosing of female branches in insect nets to deny insect access to the cones, pollen traps for investigation of pollen dispersal, and diurnal and nocturnal insects observations (Paper I). Aerodynamic properties of selected species of *Ephedra* were assessed using computer simulations and studies of the ultrastructure of pollen grains of *Ephedra* (Paper II). Information on pollination mode and pollen morphology in other major groups of extant and extinct seed plants was taken from the literature.

**Character evolution:** Evolution of pollination was estimated using parsimony optimization of two states: pollen transfer by biotic vector and pollen transfer by abiotic vector. Evolution of saccate pollen was estimated using the same approach but as a multistate character: number of pollen sacs 0, 1, 2 or 3. Ancestral character states were reconstructed, using accelerated transformation optimization as described by Farris (1970) and delayed transformation optimization (Swofford and Maddison 1987), on the most recent and most completely sampled phylogeny of living and fossil seed plants (Hilton and Bateman 2006). Relationships in the conifer crown group were modified according to most recent results (Leslie, et al. 2012). Further, three different hypotheses regarding the phylogenetic position of the Gnetales were considered; Gnetales member of an anthophyte clade (Crane 1985, Chase, et al. 1993), Gnetales sister to conifers (Rydin, et al. 2002) and the Gnetales nested among conifers, sister to the Pinaceae (Chaw, et al. 2000, Mathews, et al. 2010).

Results and Discussion

Seed plants can be traced back to the Devonian; the oldest known fossil remains are cupulated and integumented ovules/seeds, e.g., *Elkinsia* from the late Devonian, or possibly *Runcaria* from the middle Devonian (Taylor, et al. 2009). Many of the Paleozoic seed plants are referred to as “seed ferns”, which is the loosely defined collective term for extinct seed plants with “fern-like” leaves. Here, I avoid this term throughout. The male gametophyte of the earliest gymnosperms shows similarities to both the microspores of heterosporous free-sporing plants with trilete or monolete laesurae, and to true pollen of Mesozoic and Cenozoic seed plants. These grains are called prepollen (Chaloner 1970), and are ancestral in seed plants and characteristic of Late
Paleozoic seed plants. They lacked a pollen tube and germinated proximally though the laesurae, as do spores, but were multicellular as is pollen. Although, the insect fauna was diverse during the Carboniferous, pollen morphology and ovule reception methods suggest that anemophily was the dominant transfer mechanism during the Paleozoic. Also, the integument of early gymnosperms may have evolved as a response to its aerodynamic effect on successful pollination (Niklas 1981a, Niklas 1981b) but possibly both pollination and protection were important for the adaptive evolution of an integument (Rothwell and Scheckler 1988).

Pollen is likely to be the first reward for insects offered by plants, e.g., prepollen of *Lagenostoma* (Lyginoperidales) or the very large prepollen grains of Medullosans. If only pollen was the first important nutritional reward, a bisporangiate condition would be necessary for successful pollination in the early insect pollinated lineages. However, according to Crepet (1979) the mandibulate mouthparts suitable for chewing pollen were also suitable for predation of ovules and young seeds, and pollen and ovules were probably equally important reward sources to early pollinators (Crepet 1979). This could thus explain why insects would be motivated to visit both male and female organs that possibly were located on different plants (dioecious plants), or at least on different cones (monoecious plants). Beetles were the most diverse and abundant order of anthophilous insects during the time when insect pollination may have originated, and they were probably the first insect-pollinators (Crepet 1979). Still today are beetles extensive pollen-feeders, and the pollinators of some angiosperms and cycads.

**Paleozoic seed plants**

**Callistophytales**

*Callispermarion pusillum* from the middle Pennsylvanian is the earliest documented fossil evidence for a pollination drop mechanism (Rothwell 1977). The microgametophytes of Callistophytales, monosaccate true pollen, about 40µm in diameter (Taylor 1979) with an alveolate exine (Taylor and Zavada 1986) was surprisingly modern for a Paleozoic plant. These grains germinated from the distal pole, and there is even some evidence for the presence of a branched pollen tube (Taylor, et al. 2009). Ovules occurred on the lower surface of fernlike leaves and were facing downwards in relation to gravity (Rothwell 1981, Doyle 2010). The pollen probably floated upwards in the pollination drop and fertilized the ovule. This mechanism, which is similar to that of some conifers, was thus present already in the Carboniferous (Leslie 2008). Pollen transfer in Callistophytales is poorly understood. The pollen grains most likely had a low density and a low settling velocity, associated with an increased capability of wind dispersal (comparable to the saccate pollen of extant *Pinus* (Schwendemann, et al. 2007). Thus, there is some circumstantial evidence for wind pollination in the Callistophytales. Although the presence of capitate glands on the ovulate structures may represent attractive structures analogous to nectar bodies (Taylor 1979), most current evidence suggests that Callistophytales were predominantly anemophilous plants.
Cordaitales
The cordaites were Palaeozoic trees that could be nearly 30 m high with a diameter of 1 m in at the base of its trunks (Scott 1909), but could also be smaller trees with a height of 5 m and with stilt roots (Cridland 1964). Cordaites were probably monoecious (Bell and Hemsley 2000), and the compound fructifications consisted of a primary axis with monosporangiate cones in the axils of the bracts (Biswas and Johri 1997). The cones had spirally arranged scales with pollen sacs or ovules located close to the apex of elongated stalks (Florin 1951). The orientation of the ovules is not known for certain, but the micropyle was probably facing away and outwards from the cone axis at time of pollination (Scott 1909). The cordaites had true pollen with an alveolate pollen wall (Taylor and Zavada 1986), distal germination (Bell and Hemsley 2000) and one pollen sac (Florin 1951) of proportionately greater volumes than in extant conifers (Leslie 2008). The integument of cordaites most likely played an active role in sealing the pollen chamber after pollination had occurred (Rothwell 1988). The pollen morphology circumstantially supports the cordaites as wind-pollinated.

Lyginopteridales
The Lyginopteridales are a diverse and perhaps paraphyletic assemblage of Paleozoic seed plants, of which many are poorly documented and understood. Although many details are known from vegetative and (female) reproductive organs, the structures have not always been found in connection and possibilities for interpretation of whole plant concepts and the environmental preferences of the plants are limited. Most members of the Lyginopteridales had narrow stems and are thought to have had a scrambling or climbing habit (Masselter, et al. 2007, Taylor, et al. 2009). Pollen organs associated with the Lyginopteridales consist of clusters of fused or non-fused microsporangia. The prepollen was small with smooth surface and a trilete laesura. Pollen transfer is uncertain. It may have occurred with insects as vectors; their ovules were born single in tulip-shaped cupules, which had prominent glands on the abaxial sides of the cupule lobes (Oliver and Scott 1904). However, these glands may also have functioned for defense against predation, and the small and smooth prepollen may have been well-adapted for wind pollination. In conclusion, it is difficult to assess the mode of pollen transfer in this poorly understood order, and I have for this survey followed Taylor et al., (1988, 2009) who state that pollen of the Lyginopteridales were wind-borne.

Medullosales
The Medullosales were similar to tree-ferns, with fern-like leaves, but employed a typical gymnosperous mechanism of pollen capture using a pollination drop mechanism (Rothwell 1977). The monolete prepollen grains of Medullosa were extremely large, up to 0.6 mm, with a very thick sporoderm (Taylor 1979) most likely associated with a high terminal settling velocity and a reduced dispersal capability. Medullosans are found in coal swaps where they probably grew as a part of the understory vegetation (Wnuk and Pfefferkorn 1984). The reduced wind speed in understory vegetation (Whitehead 1983) in combination with the large, dense morphology of their prepollen makes wind pollination highly unfavorable (Taylor 1979). Further,
Medullosans are found scattered e.g., among the more dominant tree-like Marattialean fern *Psaronius* in floodplains (DiMichele and Phillips 1988). Wind-pollinated taxa typically grow in large monotypic stands and are rarely scattered within stands of other species (Whitehead 1983), as Medullosans appear to have been. Thus, circumstantial evidence clearly supports Medullosans as among the earliest known enthomophilous plants, most likely pollinated by pollen-feeding insects (Crepet 1979).

**Mesozoic seed plants**

**Bennettitales**

The Bennettitales are a group of extinct seed plants that were abundant and diverse during the Triassic, Jurassic and Cretaceous (Harris 1969, Watson and Sincock 1992). The reproductive organs of at least some bennettitalean taxa are somewhat similar to the angiosperm flower and morphologically diverse; both unisexual and bisexual structures have been described (Friis, *et al.* 2011). In the bennettitalean microsporangiate structure *Weltrichia* from the Jurassic, pollen sacs are placed inside bivalvate synangia (Harris 1969). The unisexual flower consists of a whorl of bracts that maybe free or fused to a cup that protected the developing pollen sacs, or functioned as attraction for insect visitors (Friis, *et al.* 2011). The internal surface of the bracts in *Weltrichia sol* is covered with small sacs that contain resinous bodies and are tentatively interpreted a remains of nectaries (Harris 1969). Pollen of the Bennettitales is relatively large, ovoid and psilate, with a longitudinally oriented sulcus (Osborn and Taylor 1995) and share a granular architecture of the infratectum with those of the Erdmanthecales, Gnetales and Pentoxyales (Osborn 2000). Pollen of *Cycadeoidea dacotensis* (Lower Cretaceous), found in situ, show a dense granular infratectum that is unlikely to be an artifact resulting from the fossilization process (Osborn and Taylor 1995). Consequently, this pollen probably had a high settling velocity and a poor flight capability, as is here demonstrated for the gnetalean *Ephedra foeminea* and inferred for *Welwitschia* (Paper II). Ovulate structures of the Bennettitales vary from large open and exposed radially symmetrical structures (*Williamsoniella*), to moderately sized structures with enclosed microsporophyll (*Cycadeoidea*) (Harris 1969, Crepet and Friis 1987, Friis, *et al.* 2011). The overall robust morphology of the bennettitalean reproductive structures, and the large pollen grains, suggest that most species of the known taxa were pollinated by beetles and possibly had a pollination syndrome similar to that of extant *Magnolia* (Crepet and Friis 1987, Friis, *et al.* 2011).

**Caytoniales**

*Caytonia* was first discovered by Thomas (1925). Based on the morphological similarities with several groups of so called “seed ferns”, and with angiosperms, he treated them as “intermediate” between gymnosperms and angiosperms (Thomas 1925). Also in some more recent phylogenetic studies based on both molecular and morphological data, *Caytonia* is sister to the angiosperms (Doyle 1996, Doyle 2008). The female reproductive structure of *Caytonia* consists of ovules, most likely with the micropylar opening facing downwards in relation to gravity. The ovules
were borne several together and surrounded by an enclosing cupule, with an opening at the end (Harris 1964). The number of ovules in each cupule varies, in *C. sewardi* there are up to ten ovules and in *C. thomasi* there are up to 30 ovules in each cupule (Harris 1933). The pollen grains were bisaccate (Harris 1964, Doyle 2010) and have been found in situ in the micropyle of the ovule (Harris 1951), which thus rejects ideas of angiospermy in *Caytonia*. The pollen sacs had a completely different ultrastructure than those of extant conifers (Osborn 2000), but functioned as a floating device for entering inverted ovules in a similar way as occurs in some extant conifers (Leslie 2008). The sacs are in addition likely to function as an aid for air dispersal, as do the sacs of pollen of the Pinaceae and the Podocarpaceae. The pollination syndrome and fertilization mechanism in *Caytonia* were probably similar to those of extant conifers (Harris 1933, Harris 1964) and *Caytonia* was thus most likely wind-pollinated.

**Glossopteridales**

Glossopterids were a highly successful group of gymnosperms that dominated the vegetation of many Late Carboniferous ecosystems. The diversity of Glossopterids started to decline in the Triassic and they went entirely extinct during the Jurassic (Stewart and Rothwell 1993). The ovulate organs consisted of a stalked fertile head often attached to a modified leaf-like organ (Gould and Delevoryas 1977). There is considerable variation in morphology and mode of attachment to the leaf-like structure and it is not clear whether or not the Glossopterids are a monophyletic group (Biswas and Johri 1997). There is a wide range of variation in number of heads attached to the leaf-like organ as well as number of ovules in each head. In *Scutum* there are up to 75 scattered ovules while in *Denkania* there is only one ovule in a cupule-like head (Surange and Chandra 1975). The pollen grains of Glossopterids are bisaccate with characteristic transverse striations on the corpus (Gould and Delevoryas 1977) and were, like most conifers, most likely wind-pollinated.

**Peltaspermales**

Peltasperms were widespread on the Gondwana and the northern continents from the Pennsylvanian to the Triassic (Taylor, *et al.* 2009). They are associated with fern-like fronds referred to several form-genera, of which *Lepidopteris* is sometimes used for entire plants based on similarities in e.g., stoma structures (Stewart and Rothwell 1993). Reproductive morphology was probably diverse; ovules and microsporangia may be attached to fertile (parts of) fronds or to fan-shaped or peltate sporophylls (Taylor, *et al.* 2009). The early Permian peltasperm *Autunia* had bisaccate pollen (*Vesicaspora*) (Kerp 1988, Doyle 2010) and open seed-bearing structures (Kerp 1988, Naugolnykh and Kerp 1996) with one or two ovules attached to a fan shaped megasporophyll downwards oriented in relation to gravity (Kerp 1988). *Autunia* were most likely wind pollinated and is coded as such here. The late Permian peltasperm *Vittatina* lack pollen sacs on the pollen grains (Hart 1966, Meyen 1984). Furthermore, pollen grains of *Vittatina* have been found in the gut of the Permian insect *Idelopsocus* (Krassilov and Rasnitsyn 1996) circumstantially supporting *Vittatina* as insect pollinated.


**Ginkgo**

The earliest known ginkgoalean fossil is the *Trichopitys*, from the Early Permian, and the group was abundant in the northern hemisphere during the Mesozoic and early Cenozoic (Bell and Hemsley 2000). The only living species of this genus is *Ginkgo biloba* endemic to China. It is dioecious with male and female individuals occurring at a 1:1 ratio (Santamour Jr, *et al*. 1983). The pollen grains are non-saccate and boat-shaped with a large longitudinal aperture extending over the entire grain (Wodehouse 1935). They can be dispersed extremely long distances. In the Boston area, the more than 400 m distance between the closest male and female tree does not inhibit seed set (Del Tredici 1989). Thus, *Ginkgo biloba* is clearly wind-pollinated (Del Tredici 2007). The ovules are usually borne in pairs, symmetrically attached at the end of a stalk-like sporangiophore and facing upwards in relation to gravity (Bell and Hemsley 2000). At the time of pollination the small pollination droplet captures the air borne pollen (Jin, *et al*. 2012a). When conspecific pollen is captured, the secretion terminates and the pollen enters the pollen chamber as a result of evaporation and active withdrawal (Jin, *et al*. 2012b). The pollen grain germinates into a tube with haustorial function, which releases motile spermatozoids into the fluid located above the archegonia (Friedman 1987). This example of zooidogamous fertilization was discovered already in 1896 by Hirase (Hirase 1896).

**Conifers**

The oldest known conifers had saccate pollen grains (Florin 1951). Among extant conifers, saccate pollen is restricted to some members of the Podocarpaceae and the Pinaceae (Doyle 1945, Tomlinson 1994, Salter, *et al*. 2002, Leslie 2010). The number of pollen sacs varies from one sac in *Tsuga* (Pinaceae) to three pollen sacs in *Microstrobus* and *Dacrydcarpus* (Podocarpaceae) (Appendix 2) (Hesse, *et al*. 2008). The absence of pollen sacs in pteridophytes with wind-dispersed spores suggest that the primary function of the sacs is as a floating device for the pollen grain and or aid to float up the pollination drop and fertilize the ovule (Doyle 1945, Tomlinson 1994, Runions and Owens 1996, Leslie 2008). And extant conifers with saccate pollen also show erect stobili with ovules facing downwards in relation to gravity, and a pollination drop produced by the plant or by rainwater (Doyle 1945, Tomlinson 1994, Runions and Owens 1996, Salter, *et al*. 2002, Leslie 2010). However, pollen sacs is in addition interpreted as aid for pollen dispersal by decreasing the density and settling velocity of the pollen grain, and thus increase its dispersal capability (Proctor, *et al*. 1996, Schwendemann, *et al*. 2007). The Araucariaceae, *Saxegothaea* (Podocarpaceae), and some Pinaceae (*Abies* and *Tsuga*) have no pollination drop-mechanism (Eckenwalder 2009), and also (mostly) nonsaccate pollen (Eckenwalder 2009). In few other taxa, *Larix* and *Pseudotsuga* (Pinaceae), drop production is delayed until after pollen has landed on the dry micropylar surfaces (Gelbart and von Aderkas 2002). Also these two genera have nonsaccate pollen (Eckenwalder 2009). All extant conifers, even those without pollen sacs on the pollen grains, disperse their pollen grains by wind (Owens, *et al*. 1998). Wind pollination is also assumed for the extinct crown group conifer clade the Cheirolepidiaceae, based e.g., on the

**Gnetales**

The Gnetales consist of three distinct monophyletic genera, Ephedra L., Gnetum L. and Welwitschia Hook.f. Ephedra comprises about 40 xerophytic species in Eurasia and the Americas. Gnetum is restricted to tropical rain forests and consists of about 30 species. Welwitschia includes only one species endemic to the Namib Desert.

**Ephedra**

Ephedra has in general been referred to as wind-pollinated (Kubitzki 1990) and empirical studies have come to different conclusions. Jaccard (1894) proposed wind-pollination for E. helvetica based on field observations, confirmed by field experiments (Paper I), the pollen ultrastructure (Paper II) and the absence of pollination drop producing structures in the male cone. Aerodynamic experiments on the two North American species E. trifurca and E. nevadensis show that their cone and pollen morphology is compatible with wind pollination (Niklas, et al. 1986, Niklas and Kerchner 1986, Niklas and Buchmann 1987, Buchmann, et al. 1989). In contrast, E. aphylla has been described as partly entomophilous (Bino, et al. 1984a) and E. foeminea has been suggested to be exclusively entomophilous or entomophilous in combination with anemophily (Porsch 1910, Meewuse, et al. 1990). Bino, et al. (1984a) suggest an evolutionary trend towards entomophily within the genus. However, in Paper I, we conclude that the case is actually the opposite. Entomophily is ancestral in Ephedra, and in the Gnetales, and shift towards anemophily is possibly associated with the re-radiation of the genus (Paper I) 30 million year ago (Ickert-Bond, et al. 2009). The derived adaptations to anemophily extend to the microgametophytic level and pollen grains of wind-pollinated taxa have lower settling velocity associated with a more spacious exine (Paper II). Even though dispersal aid is suggested to be a secondary function the sacs of pollen of some conifers, it is nevertheless interesting to think about the functional similarities in pollen dispersal between the spacious infractectum in plicae in wind-pollinated Ephedra (Paper II), and the pollen sacs of members of the Pinaceae, Podocarpaceae and many Paleozoic seed ferns (see above).

**Gnetum**

Most species of Gnetum have morphological bisexual, functionally unisexual, male reproductive structures, in which also male plants can produce sugary pollination drops and are emitting a strong scent (Endress 1996). Accordingly, entomophilous pollination syndrome is suggested for the genus by van der Pijl (1953), and Kato (1994, 1995). Gnetum gnemon is shown to be pollinated by nocturnal moths of Pyralidae and Geometridae whereas G. cuspidatum is pollinated by small flies of Lauxaniidae (Diptera) (Kato, et al. 1995). In both species, pollinators are attracted by strong scent (Kato and Inoue 1994, Kato, et al. 1995). Pollen grains of Gnetum is spheroidal, inaperturate, and Gnetum is the only gymnosperm with spinose to spinulose
ornamentation, although the surface sculpture varies among species (Gillespie and Nowicke 1994). The spinulose ornaments of *Gnetum* are homologous with the plica of *Ephedra* and *Welwitschia* (Osborn 2000, Yao, *et al.* 2004), and their ultrastructure is similar. The exine in *Gnetum* pollen has a dense granular infratectum in the spinulose regions and a thin tectum uniform throughout the grain (Yao, *et al.* 2004, Tekleva and Krassilov 2009). A spinulose ornamentation is common among entomophilous taxa, and maximizes the number of pollen grains that attach to the pollen vector (Wodehouse 1935, Faegri and van der Pijl 1979, Ackerman 2000, Culley, *et al.* 2002). In addition, pollen grains of *Gnetum* are sticky, even though they lack pollen kit (Hesse 1980, Hesse 1984), and adhere to the proboscides antennae of visiting moths or to the antennae and body of visiting flies (Kato, *et al.* 1995).

**Welwitschia**

Both male and female reproductive organs of *Welwitschia mirabilis* produce sugary pollination drops (Endress 1996) that are since long known to attract insects (Hooker 1863, Baines 1864, Pearson 1906). The species was through field experiments and field observations shown to be insect-pollinated, mainly by diurnal dipterans (Pearson 1909, Wetschnig and Depish 1999). However, as far as known, no nocturnal observations have been conducted on *Welwitschia* and since both *Gnetum* (Kato, *et al.* 1995) and *Ephedra foeminea* (Paper I) is pollinated by nocturnal moths it would be very interesting to see if there are any nocturnal visitors/pollinators of *Welwitschia mirabilis*. Pollen grains of *Welwitschia* are polypllicate and similar to those of *Ephedra*, but are monoaperturate; a broad sulcus extends over the entire grain (Wodehouse 1935). The pollen grains lack pollen kit but become sticky from tapetal debris (Hesse 1984) and are so adhesive that our attempts to investigate their terminal settling velocity failed (Paper II). However, previous authors state that the pollen grains of *Welwitschia* can travel only short distances (Wetschnig and Depish 1999), and a dense architecture of their exine has been observed (Paper II). These observations are consistent with a high settling velocity (i.e. similar to the case of *Ephedra foeminea*) (Paper II), which thus supports previous conclusions on entomophily in this monotypic genus.

**Cycadales**

The Cycadales are an ancient group that shares many presumably ancestral features with extinct Paleozoic seed plants, for example a zooidogamous fertilization mechanism (also present in *Ginkgo*), discovered already in 1896 (Ikeno 1896). The Cycadales appear for the first time in the fossil record during the Triassic (Crane 1986), and their diversity maximum was during the Jurassic-Cretaceous (Jones and Stevenson 1993, Watson and Cusack 2005). Extant clades (about 300 species in 11 genera, Hill, *et al.* 2004), are however thought to have radiated simultaneously, and as recent as the late Miocene (Nagalingum, *et al.* 2011). Aerodynamic experiments suggest that wind pollination alone may not be that efficient in cycads (Niklas and Norstog 1984), and Chamberlain (1935) observed a decrease in seed set with an increased distance from nearest microsporangiate plant. In most species of cycads, the ovules are enclosed by cone bracts at time
of pollination (Jones and Stevenson 1993). This obstructs pollen access to the ovules, and pollination requires two phases: first the transportation of pollen grains to the female megastrobilus and, in most cases, a second transportation from the megasporophylls to the ovules (Niklas and Norstog 1984). Cycad pollen is monosulcate and bilaterally symmetrical (Wodehouse 1935, Dehgan and Dehgan 1988). The exine is generally alveolate (Taylor and Zavada 1986) but the structure of the alveoli varies between the genera (Dehgan and Dehgan 1988) and so does the pollination syndrome.

**Cycas**

The megastrobili of *Cycas* deflect airflow passing over the cone toward leeward where pollen grain accumulates (Niklas and Norstog 1984). The loose aggregation of the sporophylls makes the ovules directly accessible to air-borne pollen, although some passive secondary transport of pollen by water is most likely required for successful fertilization (Niklas and Norstog 1984). Wind-pollinated plants generally produce dry, light pollen in large amounts (Faegri and van der Pijl 1979). Pollen grains of *Cycas* are subcircular in shape with a fossulate surface (Dehgan and Dehgan 1988) and are to a certain degree adapted to wind pollination (Faegri and van der Pijl 1979). The comparatively big male cones of *C. circinalis* shed large amounts of pollen at time of pollination (Norstog 1987), which further supports *Cycas* as wind-pollinated.

**Zamia**

In contrast to *Cycas*, *Zamia* is most likely exclusively or partly insect-pollinated (Niklas and Norstog 1984, Norstog, et al. 1986, Tang 1987). Pollen of extant (Dehgan and Dehgan 1988) and fossil (Hill 1990) *Zamia* is broadly elliptic in shape and the alveolate exine is multilayered and similar throughout the genus (Dehgan and Dehgan 1988). Male cones of *Zamia* are comparatively small and produce far less amounts of pollen than do male cones of *Cycas* (Norstog 1987). Some pollen-transportation to the megastrobili occurs initially by wind and becomes secondarily transported to the ovules by insects (Niklas and Norstog 1984). In *Zamia furfuraceae* (Norstog, et al. 1986) and *Z. pumila* (Tang 1987), successful pollination may, however, occur in the absence of anemophilous pollen, by means of different species of the weevil *Rhopalotria*. Larvae of the weevil feed on tissue of the microsporangiate cone, pupate at inside the microsporangiate cone, and hatch as pollen-coated adults. Mature weevils visit the ovulate cones but are non-destructive to ovules, and thus effective pollinate the plant (Norstog, et al. 1986, Tang 1987).

**Angiosperms**

Numerous studies with the attempt to determine the age of the angiosperm crown group has been conducted during the last century (Ramshaw, et al. 1972, Magallón and Sanderson 2001, Wikström, et al. 2001, Bell, et al. 2010, Magallón 2010). Wikström et al. (2001) and Bell et al. (2010) estimate its age to the Early to Middle Jurassic, and Magallón (2010) to the Triassic. The earliest recognized angiosperm remains are younger, however, and consists of scattered
pollen. Synapomorpic for all angiosperm pollen is the columellate-reticulate architecture of the exine (Doyle 1978), and indisputable angiosperm pollen grains are known from the earliest Cretaceous (Valanginian-Hauterivian) (Friis, et al. 2011). Pollen grains that share some but not all their features with angiosperm pollen are reported from the Middle Triassic (Hochuli and Feist-Burkhardt 2013), and these grains were conceivably produced by extinct plants that represent stem groups along the lineage to the angiosperm crown group. The earliest angiosperms are repeatedly considered to have been insect-pollinated (Bernhardt and Thien 1987, Crepet and Friis 1987, Gottsberger 1988, Hu, et al. 2008) and possibly pollinated e.g., by beetles, flies (Empididae), micropterigid moths, sawflies and sphecid wasps. All these insect groups are known from the Early Cretaceous (Crepet and Friis 1987), and co-evolution with pollinators and a pollinator driven speciation are thought to be one of the mechanisms of angiosperm diversification in the Cretaceous (Hickey and Doyle 1977, Regal 1977, Crepet 1979, Burger 1981, Tiffney 1984, Bond 1989). A consequence of this hypothesis is that wind pollination is a derived state in angiosperms, thought to have evolved repeatedly in response to ecological conditions that make animal pollination less favorable (Linder 2000, Friedman and Barrett 2008). However, based on my brief survey I find it clear that pollination biology and pollen morphology varies considerably among and within early diverging angiosperm clades, even to the extent that it was difficult or impossible to score the ancestral states of angiosperms in my data matrix (see further below).

Conclusions

Pollen morphology in an evolutionary perspective

Evolution of three different pollen traits (Appendix 2) was assessed under three topological frameworks that differ (only) regarding the position of the Gnetales: sister to Pinaceae (Figs 1a-c), sister to conifers (not shown) and sister to angiosperms and allied extinct taxa (not shown). Regardless of the relationship of the Gnetales to other seed plant groups a rounded shape of pollen grains is the ancestral state in conifers and in the anthophyte clade (equivocal in anthophytes if Gnetales are included in anthophytes) (Fig. 1a). Transitions to boat-shaped pollen have occurred repeatedly, for example within the Gnetales and Bennettitales. Among earlier diverging clades accelerated and delayed transformations to boat-shaped pollen produce, under my topological framework, equally parsimonious results. Aperture (regardless of direction or shape) evolved early in the history of seed plants (Fig. 1b), but is however missing in several clades within the Gnetales. Repeated losses of the aperture within the Gnetales (Ephedra and Gnetum), or one loss and regains in Welwitschia, are equally parsimonious (Fig. 1b).

Many Paleozoic groups with true pollen had one or several pollen sacs (Millay and Taylor 1974), a feature that typically is correlated with downward oriented ovules (Leslie 2008, Hernandez-Castillo, et al. 2009). The flotation hypothesis, described by Doyle (1945) and Tomlinson (1994), and experimentally tested by Leslie (2010), according to which pollen sacs mainly functions as a
flotation device and pollen grains float upwards into the ovule, is well-known and widely accepted for conifers. The same mechanism was however most likely established also among other seed plants, such as Callistophytes, *Caytonia* and Glossopterids, and was thus present already during the Paleozoic (Leslie 2008, Doyle 2010, Leslie 2010). Callistophytes are sometimes discussed as possibly related to conifers and cordaites, but the presence of pollen sacs (and perhaps an associated floatation function) in pollen of Glossopterids and *Caytonia* appears to be independently evolved. In extant plants saccate pollen is restricted to some genera of the Pinaceae and Podocarpaceae. Monosaccate pollen present in *Tsuga* (Pinaceae) but in general rare among living plants (Appendix 2). From my survey it is not possible to assess whether saccate pollen is ancestral within conifers, or has originated several times, and twice within extant conifers (Fig. 1c). Evolution of trisaccate pollen in the Podocarpaceae is also unclear (Fig. 1c). Character evolution of pollen sacs shows a comparatively complicated pattern, which would be interesting to model across a sample of trees. Regarding pollination, I conduct these analyses using a relatively small set of terminals, and optimizing a single binary state character, for which I suggest multiple transformations along branches are unlikely. In such cases, parsimony has been shown to be more appropriate for ancestral state reconstruction than methods that employ more parameter rich models (Pirie, et al. 2012). A major drawback of my approach is however that I optimize the character on only three alternative topologies, the reliability of which can be questioned. In that respect, optimization over a sample of Bayesian trees could have been advantageous. The relationship among major clades of seed plants is a very difficult phylogenetic question, far beyond the scope of this study. In my opinion, it is nevertheless interesting to investigate the evolution of pollination biology and pollen morphology, using the currently best estimate of phylogeny of seed plant as a framework.

**Evolution of insect pollination**

Mutualistic relationships between insects and plants in the form of pollination were established well before the origin of the angiosperms, and co-evolution between angiosperms and insects cannot solely explain the turn-over from a gymnosperm dominated to an angiosperm dominated vegetation (Crepet and Niklas 2009). There are several early examples of entomophilous seed plants, for example the Carboniferous Medullosans and Mesozoic groups such as some Peltasperms and the Bennettitales. My character optimization unequivocally resolves insect pollination as independently evolved in Medullosans, Peltasperms and cycads (Figs 2a–c). This is consistent with indications based on literature information on these respective clades. For example, circumstantial evidence (most importantly a transition from open seed-bearing structures to ovules enclosed in cupules, and a transition from saccate to non-saccate pollen) supports a shift to insect pollination within Peltasperms (Meyen 1984) (see also above). Similarly, although insect pollination has been suggested for many extant cycads, the alveolate pollen exine of extant (Dehgan and Dehgan 1988) and fossil (Hill 1990) species is most likely associated with a low settling velocity. This indicates, in accordance with my optimization, that anemophily is the ancestral pollination syndrome in the Cycadales.
It is, however, unclear from my optimization whether or not insect pollination evolved independently in the Gnetales, the Bennettitales and the angiosperms (Figs 2a-c). As before, this is true under all three topological frameworks used here, i.e., the Gnetales nested within the conifer (Fig. 2a), sister to the Pinaceae (Fig. 2b), or members of an anthophyte clade (Fig. 2c) (although an association between conifers and the Gnetales clearly means an independent origin of insect pollination in the Gnetales). Furthermore, it appears questionable if insect pollination should be considered ancestral in angiosperms, and I scored several characters as question marks for angiosperms (Table 1). Recent character optimization by Hu, et al. (2008) resolves insect pollination as ancestral in angiosperms, in line with ideas in many previous studies (references above). However, this interpretation may be an oversimplification. My literature survey clearly shows that many of the early diverging angiosperm clades, for example Amborella, Nymphaeales and Trimeniaceae, are diverse in terms of pollen morphology (Endress and Honegger 1980, Sampson and Endress 1984, Osborn, et al. 1991, Sampson 1993, Hesse 2001, Remizowa, et al. 2008), and probably also in pollination mode. Conceivably, early angiosperms were generalists regarding pollination, utilizing several mechanisms simultaneously, and with morphological and functional variation even within species and individuals.

Studies of the evolution of pollination require knowledge of pollination mode in extinct seed plants, but such information related to function is not always readily available for fossils. Indirect inference can sometimes be made, based for example on pollen morphology, ultrastructure of pollen, the abundance of a certain pollen type as dispersed grains, and discoveries of structures for pollinator reward. It is also possible to simulate aerodynamic properties of pollen grains and female structures, and thereby assess how far pollen can travel by air and how efficiently ovules can trap air-borne pollen. For the Gnetales, all available information points towards insect pollination as the ancestral and prevailing state. My studies indicate that wind pollination evolved relatively late in the evolutionary history of the Gnetales, i.e., in the current core Ephedra clade, which is estimated to have originated only about 30 million years ago. In contrast to these conclusions is the presence of a relatively abundant and widespread fossil record of dispersed “ephedroid” pollen in the Cretaceous as well as in the Cenozoic, which as such may indicate wind pollination.

**Future perspective**

I will continue my ongoing studies of morphology and ultrastructure of living and fossil “ephedroid” pollen. The aim is that the results will prove useful for 1) evaluations of the gnetalean affinity of different palynomorphs, 2) to infer the pollination biology of the plants that produced the pollen, and 3) to assess the paleoenvironment, in which they lived in order to assess the relevance of “ephedroids” as climatic indicators.
Pollination, det vill säga transport av pollen från pollenproducerande organ till fröämnen, förekommer med biotiska eller abiotiska vektorer och är i princip nödvändigt för befruktning och fullbordandet av livscykeln hos alla fröväxter. Ett ömsesidigt förhållande mellan insekter och växter där båda är beroende av varandra, insekter för föda och växter för dess förökning, har varit känt ända sedan 1700-talet. Denna samevolution mellan insekter och växter har också diskuterats som en möjlig förklaring till den, med geologiska mått, plötsliga övergången från en gymnospermdominerad vegetation till en blomväxtdominerad vegetation under slutet av krita-perioden. Insektspollination är dock ett mycket äldre fenomen som går tillbaka ända till devon-perioden, men är det en ursprunglig karakter som sedan försvunnit i många fröväxtgrupper, eller har insektspollination uppkommit parallellt hos avlägsnat besläktade grupper?


I den här avhandlingen presenteras resultat av studier av pollinationsmekanismer inom *Ephedra* (Gnetales) genomförda som fältexperiment och observationer (kapitel I) och genom aerodynamiska simuleringar och studier av pollenmorfologen (kapitel II). Resultaten visar att det finns flera pollinationssätt inom detta annars morfologiskt och ekologiskt homogena släkte. Vidare, i motsats till vad som ofta antagits, är insektspollination ursprungligt inom släktet och inom hela Gnetales, och inte en egenskap som utvecklats inom gruppen. Med hjälp av denna nya kunskap om Gnetales pollinationsbiologi och litteraturuppgifter diskuteras här evolution av pollenmorfologi och pollinationssyndrom inom fröväxter mer generellt.
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Figure 1: Character evolution of three pollen traits, mapped onto a phylogeny of extant and extinct seed plants estimated by Hilton and Bateman (2006) but with relationships among conifers constrained according to most recent results (Leslie et al. 2012) and the Gnetales constrained as sister to the Pinaceae. Ancestral state reconstruction was in addition performed on a topology in which the Gnetales are sister to a) all conifers, and b) to angiosperms and allied extinct taxa (not shown, see text for details).

(a) Pollen shape. Rounded pollen grains is the ancestral state in conifers and the anthophyte clade (equivocal in anthophytes if the Gnetales are included in anthophytes). Transitions to boat-shaped pollen grains have occurred within several clades, e.g., the Gnetales and the Bennettitales. (b) Presence or absence of aperture (regardless of its shape and direction). An aperture evolved early in the history of seed plants but is missing in several clades among the Gnetales. Repeated losses, or one loss and regain in Welwitschia, are equally parsimonious. (c) Number of pollen sacci. In extant plants saccate pollen is restricted to some genera of the Pinaceae and Podocarpaceae. Evolution of trisaccate pollen in the Podocarpaceae is unclear.
Figure 2: Character evolution of pollination syndrome in seed plants. According to the results, insect pollination has evolved independently in Medullosans, Peltasperms and cycads. From this survey it is not clear whether insect pollination has evolved independently in the Gnetales, the Bennettitales and the angiosperms, although an association between conifers and the Gnetales clearly means independent evolution of insect pollination in the Gnetales. (a) Pollination syndrome optimized on a phylogeny, in which the Gnetales are sister to conifers (b) sister to the Pinaceae, and (c) members of an anthophyte clade.
Appendix 1
Geological time scale. Redrawn from Gradstein et al. 2012
<table>
<thead>
<tr>
<th>Group</th>
<th>Pollination</th>
<th>Prepollen/ pollen</th>
<th>Aperture</th>
<th>Saccus/sacci</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyginopteridales</td>
<td>wind</td>
<td>pre-pollen</td>
<td>No, proximal germination; laesura(e)</td>
<td>0</td>
<td>rounded?</td>
</tr>
<tr>
<td>Medullosales</td>
<td>insekt</td>
<td>pre-pollen</td>
<td>No, proximal germination; laesura(e)</td>
<td>0</td>
<td>boatshaped?</td>
</tr>
<tr>
<td>Bennettitales</td>
<td>insect</td>
<td>pollen</td>
<td>Yes, sulcus</td>
<td>0</td>
<td>boatshaped</td>
</tr>
<tr>
<td><em>Caytonia</em> (Caytoniales)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes?</td>
<td>2</td>
<td>rounded?</td>
</tr>
<tr>
<td>Glossopteridales</td>
<td>wind</td>
<td>pollen</td>
<td>Yes?</td>
<td>2</td>
<td>rounded?</td>
</tr>
<tr>
<td><em>Autania</em> (Peltaspermales)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes?</td>
<td>2</td>
<td>rounded?</td>
</tr>
<tr>
<td><em>Vittatina</em> (Peltaspermales)</td>
<td>insect</td>
<td>pollen</td>
<td>No, inaperturate</td>
<td>0</td>
<td>boatshaped?</td>
</tr>
<tr>
<td><em>Ginkgo</em> (Ginkgoales)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, sulcus</td>
<td>0</td>
<td>boatshaped</td>
</tr>
<tr>
<td>Cordaitales</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, distal pole</td>
<td>1</td>
<td>rounded</td>
</tr>
<tr>
<td>Callistophytales</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, distal pole</td>
<td>1</td>
<td>rounded?</td>
</tr>
<tr>
<td>Cheirolepidiaceae</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, distal circle</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td>Araucariaceae</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td>Cupressaceae s.l.</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, distal circle</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Tsuga</em> (Pinaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct</td>
<td>1</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Pinus and Picea</em> (Pinaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>2</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Larix</em> (Pinaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct?</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Pseudotsuga</em> (Pinaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Abies</em> (Pinaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>2</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Cedrus</em> (Pinaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>2</td>
<td>rounded</td>
</tr>
<tr>
<td>Phyllocladaceae</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>2</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Podocarpus</em> (Podocarpaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>3</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Microstrobus</em> (Podocarpaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>3</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Dacrydium</em> (Podocarpaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct between sacci</td>
<td>2</td>
<td>rounded</td>
</tr>
<tr>
<td>Saxegothaæa (Podocarpaceae)</td>
<td>wind</td>
<td>pollen</td>
<td>Yes, indistinct</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td>Ephedra (Gnetales)</td>
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<td>pollen</td>
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<td>0</td>
<td>boatshaped</td>
</tr>
<tr>
<td><em>Gnetum</em> (Gnetales)</td>
<td>insect</td>
<td>pollen</td>
<td>No, inaperturate</td>
<td>0</td>
<td>rounded</td>
</tr>
<tr>
<td><em>Welwitschia</em> (Gnetales)</td>
<td>Insect</td>
<td>pollen</td>
<td>Yes, distal sulcus</td>
<td>0</td>
<td>boatshaped</td>
</tr>
<tr>
<td><em>Cycas</em></td>
<td>wind</td>
<td>pollen</td>
<td>Yes, distal sulcus</td>
<td>0</td>
<td>boatshaped</td>
</tr>
<tr>
<td><em>Zamia</em></td>
<td>insect</td>
<td>pollen</td>
<td>Yes, distal sulcus</td>
<td>0</td>
<td>boatshaped</td>
</tr>
<tr>
<td>Angiosperms (summary)</td>
<td>generalized</td>
<td>pollen</td>
<td>Yes, variable number and shape</td>
<td>0</td>
<td>rounded?</td>
</tr>
</tbody>
</table>

1 Cupressaceae s.l. refers to a clade comprising Cupressaceae, “Taxodiaceae”, Taxaceae, Cephalotaxaceae, and Sciadopitys