Pollen and pollination in *Ephedra* (Gnetales)

Kristina Bolinder

Abstract

Ephedra (Gnetales) is a gymnosperm genus with a long evolutionary history; the first dispersed pollen grains with affinity to the group are known already from the Permian. This thesis focuses on the evolutionary history of the group and different aspects of its pollination mechanisms. Despite the limited number of extant species of the genus (50-60), and a low morphological and genetic divergence among species, there is variation in pollination syndrome in the genus. The prevailing state in *Ephedra*, and most gymnosperms, is wind pollination. It is therefore surprising that one species, *E. foeminea*, is insect-pollinated. Together with co-workers I documented the pollination syndromes of *E. foeminea* and a sympatric species, *E. distachya*, based on long term field experiments in north-eastern Greece and aerodynamic investigations and calculations. Placing the results into an evolutionary framework reveals that the insect-pollinated species *E. foeminea* is sister to the remaining (mostly wind-pollinated) genus, and indicates that insect pollination is the ancestral state in the Gnetales. During the course of evolution of the group there has been a shift to wind pollination, which may have played a crucial role for the diversification of the crown group in the Paleogene. Pollination biology is often correlated with the morphology of the pollen such that pollen grains of anemophilous plants are small with a smooth surface, whereas pollen grains of entomophilous plants are larger with an ornamented surface and a covering of pollenkitt. The pollen morphology of *Ephedra* can be broadly divided into two types: an ancestral type with an unbranched pseudosulcus between each pair of plicae, and a derived type with a branched pseudosulcus between each pair of plicae. Further, the pollen morphology and ultrastructure of the pollen wall in *Ephedra* are to some degree correlated with the pollination syndrome and capability of long distance dispersal. Pollen of *E. foeminea* has a denser ultrastructure, as a result a higher settling velocity and is therefore capable of flying shorter distances than does pollen of the anemophilous *E. distachya*, and other investigated anemophilous species that show a more spacious ultrastructure of the pollen grain. These results can be useful in the reconstruction of the pollination mechanism of extinct taxa of the *Ephedra*-lineage in the future.

Keywords: *aerodynamics, evolution, moonlight, pollination, pollen morphology, ultrastructure.*

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"I have not failed. I've just found 10,000 ways that won't work."
― Thomas A. Edison

List of papers

This thesis is based on the following papers which are referred to in the text by their respective roman numerals:

I: **Bolinder, K.,** Humphreys, A.M., Ehrlén, J., Alexandersson, R., Ickert-Bond, S. & Rydin, C. 2016. From near extinction to diversification by means of a shift in pollination mechanism in the gymnosperm relict *Ephedra* (Ephedraceae, Gnetales). *The Botanical Journal of the Linnean Society* 180: 461-477. DOI: 10.1111/boj.12380

II: **Bolinder, K.,** Niklas, K.J. & Rydin, C. 2015. Aerodynamics and Ultrastructure of *Ephedra* (Gnetales). *American Journal of Botany* 102 (3): 457-470. DOI: 10.3732/ajb.1400517

III: **Bolinder, K.,** Norbäck Ivarsson, L., Humphreys, A.M., Ickert-Bond, S.M., Han, F., Hoorn, C. & Rydin, C. 2015. Pollen morphology of *Ephedra* (Gnetales) and its evolutionary implications. *Grana* 55(1): 24-51. DOI: 10.1080/00173134.2015.1066424

IV: Rydin, C. & **Bolinder, K.** 2015. Moonlight pollination in the gymnosperm *Ephedra* (Gnetales). *Biology Letters* 11: 20140993. DOI: 10.1098/rsbl.2014.0993

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Contents

Introduction

Pollination is an essential part of the life cycle of all seed plants and involves the transportation of pollen, male gametophytes, to the ovule where fertilization can occur. The pollen can be transported by biotic and/or abiotic vectors, for example by insects, other animals, wind or water. Wind pollination may seem like a random and wasteful process, in which copious amounts of pollen are produced and released but only very few grains, by chance, end up at the right place to fertilize the ovule. However, although the pollen-to-ovule ratio often is larger for wind-pollinated plants than for insect-pollinated plants, wind pollination is actually a very efficient process not at all to be thought off as random (Niklas 1985b; Niklas 1992). A great example is the morphology of the wind-pollinated jojoba plant, *Simmondsia chinensis,* where a pair of leaves protruding above the base of an ovule-bearing flower leads the pollen trajectories downwards towards the flower and where the flower itself creates a turbulence increasing the pollen capture efficiency with 50% (Niklas 1985a).

Insect pollination can also be a very precise mechanism with a highly specialized insect pollinating only one single plant species; like the fascinating example of the Darwins orchid, *Angraecum sesquipedale*, that has an exceptionally long nectar spur and has coevolved with the hawk moth *Xanthopan morgani* that has an equally long proboscis. Insect pollination can, however, also be of a more generalist nature and actually quite random. As phenomenon, insect pollination was discovered already in 1793 by Sprengel (Sprengel 1793) and has fascinated scientist ever since. The earliest angiosperms are considered to have been pollinated by insects (Bernhardt & Thien 1987; Crepet & Friis 1987; Gottsberger 1988; Hu et al. 2008) and the remarkable species richness and diversity within the group is hypothesized as attributes of their floral adaptations to biotic pollinators (Crepet 1984; Dodd et al. 1999; Vamosi & Vamosi 2010). A shift towards insect pollination has also been thought of as one of the driving forces of the angiosperm radiation and the vegetation turnover from a gymnosperm dominated flora to an angiosperm dominated flora during the Late Cretaceous (Hickey & Doyle 1977; Regal 1977; Crepet 1979; Burger 1981; Tiffney 1984; Bond 1989), and thus a possible solution to Darwins abominable mystery. However, insect pollination is a very old phenomenon, perhaps as old as the Devonian and thus much older than the origin of the angiosperms (Crepet 1979). Insect pollina-

tion seems to have originated several times in distantly related groups (regardless of the position of the Gnetales in the seed plant phylogeny) (Bolinder 2014).

The Gnetales is strongly supported as a monophyletic group based on both morphology (Crane 1985; Doyle & Donoghue 1986; Loconte & Stevenson 1990; Doyle & Donoghue 1992, 1993) and molecular data (Chaw et al. 2000; Rydin et al. 2002) but the relationship between the Gnetales and other seed plant groups has repeatedly been shown extremely difficult to resolve (Chase et al. 1993; Källersjö et al. 1998; Chaw et al. 2000; Rydin & Källersjö 2002; Schmidt & Schneider-Poetsch 2002; Doyle 2008; Mathews et al. 2009; Rydin & Korall 2009; Mathews et al. 2010). To be able to understand the evolution of insect pollination, and perhaps also its role as driving force in the diversification among the angiosperms, the unresolved seed plant phylogeny is an important impediment but equally important is a better understanding of the pollination mechanisms within the Gnetales. The pollination biology of the Gnetales has been understudied and permeated with conflicting reports, especially in *Ephedra*, one of the three genera of extant Gnetales. This thesis focuses, therefore, on different aspects of the pollination biology and pollen morphology of *Ephedra* and puts the results in an evolutionary context.

Extant *Ephedra* consists of about 50 xerophytic species in temperate regions of the world (Kubitzki 1990). They are dioecious, like all members of the Gnetales, have decussate phyllotaxis, reduced leaves and photosynthetic stems. At anthesis a sugary pollination drop is formed by the nucellus of the female cone and secreted through a micropylar tube to the outside of the cone (Rydin et al. 2010) (Figs 1A and 1C). The pollination drops function, like in most gymnosperms (Owens et al. 1998), as a receptive site and as transportation and germination media for pollen (Gelbart & von Aderkas 2002). The prevailing state in *Ephedra* is that only female cones produce pollination drops because the male cones do not have ovules (Fig. 1D). However, in *E. foeminea*, the sister species to all other *Ephedra* species (Rydin & Korall 2009; Thureborn & Rydin 2015), there are sterile female organs present in the male cones that produce sugary pollination drops even though they cannot produce viable seeds (Rydin & Endress in progress) (Fig. 1B). Both *Gnetum* and *Welwitschia,* the two other members of the Gnetales, are described as insect-pollinated (Hooker 1863; Baines 1864; van der Pijl 1953; Kato & Inoue 1994; Kato et al. 1995; Wetschnig & Depish 1999; Gong et al. 2015) and produce sugary pollination drops through sterile female organs in the male cone (Endress 1996). For successful pollination with insects as vector to occur there are three main aspects that needs to be fulfilled 1) attachment of the pollen at the vector 2) transportation to the receptive site and 3) detachment of the pollen at the receptive site. In dioecious

Figure 1: Reproductive organs of *Ephedra foeminea* and *E. distachya*. A: female cone of *E. foeminea*. B: male cone of *E. foeminea*; note the presence of two apical sterile female organs producing two pollination drops that unite into one drop. C: female cone of *E. distachya*. D: male cone of *E. distachya*; note the absence of sterile female organs.

species, the possibility to attract insects first to the male plant and then to the female plant is, thus, essential in order for successful insect pollination to occur. The pollination biology of *Ephedra foeminea*, and the pollination drop as possible attractant in both the male and female cone, were therefore subjects of Paper I and IV in this thesis.

Although there are examples of insect-pollinated gymnosperms also outside the Gnetales (Norstog et al. 1986; Norstog 1987; Labandeira et al. 2007) gymnosperms are generally considered to be solely wind-pollinated. As with insect pollination, there are three main aspects of wind pollination 1) the release of pollen into the air 2) the dispersal of pollen and 3) the capture at the receptive surface. After the pollen has been released into the air the movement of the pollen in the air, and thus the dispersal distance, is determined by the terminal settling velocity of the pollen and the ambient meteorological conditions (Niklas 1985c). The third aspect of wind pollination, the capture at the receptive surface, is dependent on the momentum and inertia of the pollen within the airflow vector patterns created by and around the receptive surface (Niklas 1985c). The pollen morphology is, thus, a very important aspect to consider when discussing reproductive plant biology. In Paper II we review and discuss the different aerodynamic aspects of pollination in several *Ephedra* species with different pollination syndrome and investigate what morphological characters of the pollen affect the terminal settling velocity, the forward momentum and the inertia important for the pollen dispersal and the capture efficiency at the female cone.

A pollen grain consists of a generative cell that will generate the male gametes, and one or several vegetative cell(s) of which one will generate a pollen tube, surrounded by a pollen wall (sporoderm). The pollen wall generally consists of two main layers, the outer exine and the inner intine. The exine can be divided into two layers, an inner endexine and an outer ektexine. The ektexine is further divided into a basal foot layer, an infratectum (sometimes called columella) and a tectum (Fig. 2A). There are many deviations from this general structure and layers can be thickened, structured in variable ways or absent. The *Ephedra* pollen is boat shaped and polyplicate (Figs 2B-C) and the pollen wall is composed of a compact tectum, a granular infratectum with a variable density of the granules, a thin adnate foot layer and a uniform lamellate endexine in the plicae region (Gullvåg 1966; Van Campo & Lugardon 1973; Hesse 1984; Zavada 1984; Kurmann 1992; Rowley 1995; El-Ghazaly et al. 1998; Tekleva & Krassilov 2009) (Figs 2D-E). Between plicae, the infratectum and sometimes also the foot layer is absent and the pollen tube breaks through the pollen wall in one of these weakness zones. The weakness zones extend along the whole length of the grain, and are called pseudosulci (see Paper II and III for details). The pseudosulci can be either branched or un-branched (Fig. 3). The pollen morphology in *Ephedra*

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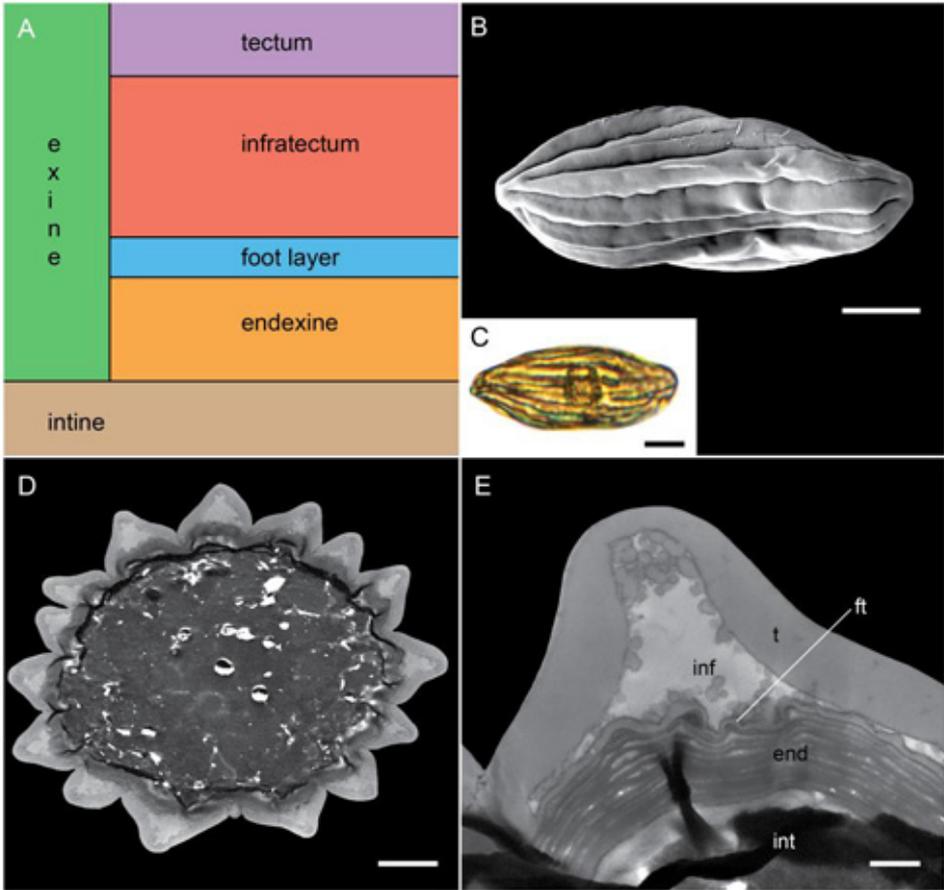
Figure 2: Pollen morphology and ultrastucture. A: schematic overview over the different layers in the pollenwall (redrawn from Hesse et al. 2009). B-E: pollen of *Ephedra likiangensis* J.F. Rock 2694 (NY). B: SEM photo of a pollen grain (scale bar 10µm). C: LM photo of a pollen grain (scale bar 10µm). D: TEM photo of the whole pollen grain in cross section (scale bar 3µm). E: TEM photo of the plicae region (scale bar 400 nm). Tectum= t, infratecrum =inf, foot layer=ft, endexine = end, intine=int.

is investigated for about 85% of the species (Paper III) using a combination of LM and SEM. The results are placed in an evolutionary context, using ancestral character state reconstruction.

To explore all results in an evolutionary context is important and becomes particularly interesting in *Ephedra* considering the long evolutionary history of the group. The earliest dispersed *Ephedra*-like pollen are as old as from the Permian (Wilson 1962). But based on the megafossil record, ephedroids (*Ephedra*-like plants) did not diversify considerably until the Early Cretaceous; all megafossils assignable to the Gnetales are from a short period in the mid Early Cretaceous (Rydin & Hoorn 2016). During this time the ephedroids showed a great diversity (Krassilov 1986; Yang et al. 2005; Rydin et al. 2006b; Rydin et al. 2006a; Wang & Zheng 2010; Liu & Wang 2015; Yang & Ferguson 2015) with some fossils possessing unique characters shared with the genus *Ephedra* as we know it today (Rydin et al. 2004; Rydin et al. 2010). This increase in ephedroid diversity seems also reflected in the paleopalynological record, which shows a dramatic increase in abundance of ephedroid pollen during the Early Cretaceous (Crane & Lidgard 1989). Towards the end of the Mesozoic the diversity and abundance of gnetalean plants decrease (Crane & Lidgard 1989), and by the K-Pg boundary and the early Paleogene the group may have been on the border of extinction (Rydin et al. 2010). The crown group is currently estimated to be from the early Oligocene (Ickert-Bond et al. 2009) and to have radiated after a longer period of stagnation (Rydin et al. 2010). To further test the hypothesis about the evolutionary history of the group has been a main aim throughout this thesis work and in Paper I we evaluate the shift in pollination mechanism as a driving force of the Paleogene diversification.
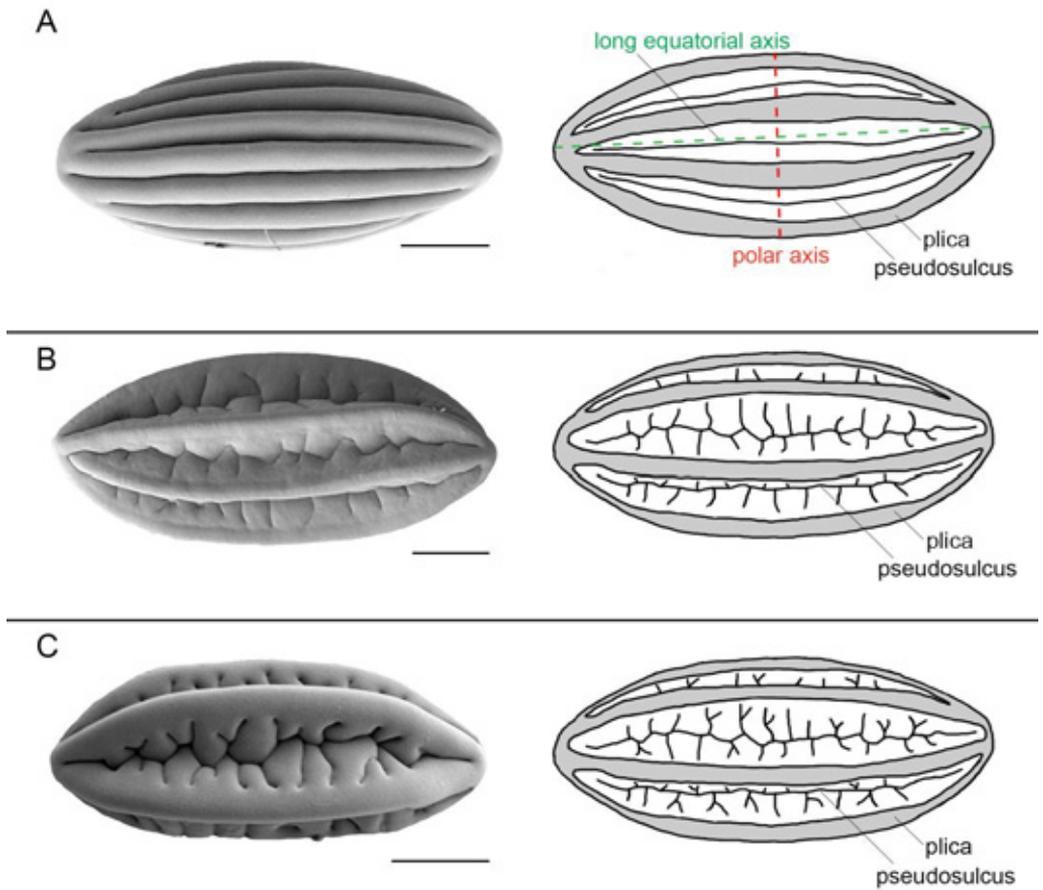
Figure 3: The three pollen types of *Ephedra*. A: pollen of the ancestral type with unbranched pseudosulci. B: pollen of the derived type with first order branches on the pseudosulci. C: pollen of the derived type with first and second order branches on the pseudosulci.

Material and Methods

Paper I: Pollination biology in *Ephedra*

The pollination biology of two distantly related species, *Ephedra foeminea* and *E. distachya*, was evaluated through extensive field studies over four years in north-eastern Greece where they grow in sympatry. Branches of female cones of both species were enclosed in bags of two different fabrics (mesh pore sizes 1x1 mm and 0.3x0.3 mm, respectively) to test the effect of seed set when insects are denied access to the cones compared to completely naked branches. To account for apomixis additional branches were enclosed in bags of a completely closed fabric. At the end of the season all ovules were checked for embryo presence and the groups compared statistically. To explore the dispersal behavior of pollen of the two species pollen traps were put up at distances of 0, 1, 2, 4, 6, 10 and 16 meters away from solely growing male plants at anthesis. The number of pollen in the traps was counted under microscope and the results compared statistically. Additional traps were put up close to a solely growing female plant, at least 30 m away from a male plant, to investigate how many pollen could transport themselves there by wind. Observations of insect visits to male and female cones were performed in 10 minutes interval spread over all hours of the day and night. Potential pollinators were photographed on site and collected for further study and identification. All captured insects were screened for pollen presence at the body using a stereo microscope and microscope with long working distance. Since several species of ants were frequently observed visiting male and female cones of *E. foeminea* and since ants are generally considered rare as pollinator due to the reduction of pollen viability in the presence of formicid acid secreted by the ants (Beattie 1982; Beattie et al. 1984; Beattie 2006), we decided to test the effect of metapleural secretions on pollen germination. Therefore, male cones were put in closed containers in the presence of ants, one container per ant species, during 24 h followed by a germination experiment of pollen in growth medium (Brewbaker & Kwack 1963).

Paper II: Aerodynamics and pollen ultrastructure

The aerodynamics of two wind-pollinated *Ephedra* species; *E. trifurca* and *E. nevadensis*, had previously been carefully studied through wind-tunnel experiments, computer modeling and field observations (Niklas et al. 1986; Niklas & Kerchner 1986; Niklas & Buchmann 1987; Buchmann et al. 1989; Niklas 2015). In Paper I we concluded that *E. foeminea* is insect-pollinated and in Paper II we investigate the aerodynamic differences between previously studied wind-pollinated species and the insect-pollinated *E. foeminea* and *Welwitschia mirabilis.* Pollen of *E. foeminea* was collected at a field locality in Croatia and pollen of *Welwitschia* at the Bergius Botanical garden, Stockholm. The terminal settling velocity was determined by stroboscopic photography of pollen grains at free fall in a glass cylinder and compared to the already studied species of *Ephedra* (Niklas et al. 1986; Niklas & Kerchner 1986; Niklas & Buchmann 1987; Buchmann et al. 1989).

Important factors for the pollen dispersal and the capture efficiency at the female cone are the settling velocity, the forward momentum and the inertia that is controlled by the density and the morphological characters of the pollen grain (Niklas 1985c). The density of the protoplasm in the pollen does not vary between closely related species (Niklas 1985c). We therefore decided to investigate the morphology and ultrastructure of the pollen wall of key species using SEM and TEM. Size (length of the equatorial axis), shape (as interpret by the P/E ratio) and number of plicae were documented for six species representing all major clades of *Ephedra* (Rydin & Korall 2009). Results were evaluated statistically and in a phylogenetic framework.

The capture efficiency of the female cones of *E. foeminea* and the pollen behavior around the ovulate organ were simulated in a computer program devised by Karl J. Niklas and Vincent Kerschner (unpublished) at an average wind speed during the week of anthesis measured at the weather station closest to the field locality. The results were compared to those of preciously studied species (Niklas et al. 1986; Niklas & Kerchner 1986; Niklas & Buchmann 1987; Buchmann et al. 1989).

Paper III: Pollen morphology

First, we established the best way of studying natural variation in *Ephedra* pollen by comparing results obtained from a) freshly collected pollen, b) pollen extracted directly from a herbarium sheet, c) pollen pre-treated with ethanol, and d) pollen treated as above in addition to treatment with a PBS

buffer. After establishing that pollen extracted directly from the herbarium sheets without further pre-treatment best represents the natural variation in *Ephedra* pollen, 45 *Ephedra* species, representing 85% of currently recognized species, were selected for the study, covering the phylogenetic and geographical diversity of the genus. A minimum of 20 pollen grains from 1-3 specimens per species were extracted and put directly onto aluminum stubs using double-sided tape, and sputter coated with gold. The lengths of polar and equatorial axes were measured and a shape estimate (P/E ratio) was obtained. The number of plicae was counted on the visible side of the grain and multiplied by two to obtain the total number of plicae. Presence or absence of first and second order branches on the pseudosulci was scored for each pollen grain. In addition, 15 pollen grains from one representative specimen per species were extracted and mounted in glycerine and studied under LM using the x40 objective for comparison. All characters were investigated for phylogenetic autocorrelation and compared statistically within and between species. Parsimony reconstruction of the ancestral state of all investigated pollen characters, continuous and discrete, was estimated using a pruned version of the to date most complete estimate of the *Ephedra* phylogeny (Rydin & Korall 2009).

Paper IV: Moonlight pollination

While working with Paper I, we needed to predict when *E. distachya* and *E. foeminea* were at anthesis to be able to travel to the field locality in Greece at the right time. After succeeding to predict exactly when *E. distachya* was producing pollination drops each year but failing to predict when *E. foeminea* did the same we started to wonder why. Phenology is in angiosperms often determined by weather conditions or light. Therefore, we downloaded weather data for all the years, for which we had phenology data, and compared mean temperature and precipitation using Pearson's correlation test in R (R Development Core Team 2014). After going through photos taken at the field locality during 2011 and 2012, where a full moon was clearly visible (Fig. 4), we also matched the phenology data with the lunar calendar using the same approach as above.

Figure 4: Full moon at one of the field localities 3rd of July 2012. Asprovalta, Greece.

Results and Discussion

Paper I – Pollination mechanisms

In the introduction of Paper I we argue that the divergent and conflicting information in the literature regarding the pollination syndrome in *Ephedra* indicate, either misconceptions, or the existence of several pollination mechanisms in this small genus, where divergence among species is otherwise sparse. Our results showed that both are true; there are misconceptions in the literature and there are at least two different pollination mechanisms in the genus: *Ephedra foeminea* is insect-pollinated and *E. distachya* is wind-pollinated. The differences in features related to reproductive biology between these two sympatric species are surprisingly many and concern pollen morphology and flight capability, the nature and frequency of insect visitation and reproductive success in the absence of insects (see Paper I for more details).

Pollen of *E. foeminea* can travel only very short distances compared to pollen of *E. distachya* that travel significantly longer distances. Furthermore, only very few pollen of *E. foeminea* reaches the female plant whereas the opposite is true for *E. distachya*. For pollen grains to successfully fertilize the plant they do not only need to reach the plant, they need to enter the pollination drop as well. How likely is it that the very few pollen that reach the female plant also enter the pollination drop? To investigate the capture efficiency of the *E. foeminea* female cone we explored the aerodynamic features of the pollen grains and the female cone (see Paper II and further below for details).

To investigate how important insect pollination is for *E. foeminea* we enclosed fertile branches in insect net of different kind and found that significantly more embryos are produced by female cones not enclosed in bags of any kind than by cones that are. Cones enclosed in closed bags blocking access of both wind- and insect-borne pollen were never fertilized. This suggests that insect pollination is very important for the reproductive success of *E. foeminea*. Insect observations around the clock during several days and field seasons revealed that both diurnal and nocturnal insects belonging to Lepidoptera, Diptera and Hymenoptera visit both male and female cones of

this species. All insects collected on the female cones had pollen on their bodies. *Ephedra foeminea* does thus have a generalist pollination system involving several insect species from distantly related groups, active both during day and night. This stand in sharp contrast with *E. distachya,* for which no insects were observed visiting both male and female cones. For *E. distachya*, the bagging experiments were difficult to interpret due to an overall poor and low seed set. We did not consider the results trustworthy and had to disregard them. During all years of investigation, except 2014, the field site suffered from severe drought during the spring (see Papers I and IV for more details), and this appears to affect the seed set of *E. distachya* (which has an earlier anthesis period than *E. foeminea*). During 2014, the precipitation was higher in the spring, and so was the seed set. In addition to a possible climate effect, seed set was negatively influenced by the infestment of parasitic larvae (Chalcidoidea, Hymenoptera) in as many as 22% of the cones of *E. distachya*. Our conclusion was that overall seed set, and the sexual reproduction, is poor in *E. distachya* in Greece. The conclusion goes in concert with the fact that *E. distachya* is rare in Greece (in comparison with *E. foeminea*), and probably at the border of its distribution area (Rydin and Korall, 2009). Whereas *E. foeminea* is native and endemic to the Mediterranean area, *E. distachya* has a broader distribution extending well into Central Asia.

Parasitic wasps –Wasps of the Chalcidoidea were observed visiting the female cones of *E. distachya* at all stages of the reproductive cycle, both prior to the pollination drop production (even before the outbreak of the micropylar tube), at anthesis (without any interest in the drop it self) and when pollination drop production had ceased (Fig. 5). Male cones were never visited. Therefore, I conclude that the Chalcidoidea visits the cones only to use it for its own reproduction, not for foraging, and they are probably not important pollinators. The larvae found in seeds of *E. distachya* were reared during two seasons using standard procedures (H. Vårdhal pers. com.) with a cold period (3°C) of three months between the seasons. The adult insects were identified by Hege Vårdahl (Swedish Natural History Museum) and Richard Askew (prof. emer.). During the first season insects belonging to *Aprostocetus* and *Baryscapus* hatched and during the second season *Idiomacromerus pallistigmus* Askew, *Blascoa ephedrae* Askew and *Bruchophagus ephedrae* hatched (Bolinder et al. work in progress). *Blascoa ephedrae* is phytophagous and probably serve as the host of the two other species reared during the second season, although *Bruchophagus ephedrae* can be partly phytophagous as well (R. Askew pers. com). There are small morphological differences in the *I. pallistigmus* and *B. ephedrae* from Greece and the paratypes collected in Spain (Askew & Blasco-Zumeta 1997; Askew & Blasco-Zumeta 1998), but this is most likely due to intraspecific variation common among widely separated populations of Chalcidoideae (R. Askew pers comm.). Lit-

tle is known about the ecology and distribution of these insects but clearly *E. distachya* has a central and complicated role in the life cycle of many Mediterranean parasitic insects, at the expense of its own sexual reproduction.

For *E. foeminea* Chalcidoideae were observed mainly during the smaller second peak in pollination drop production in August (see Paper IV). Some Chalcidoidea were observed visiting both the male and female cones at anthesis of *E. foeminea,* with some interest in the pollination drops (Fig. 5). There is thus a possibility that these insects function as an effective pollinator of *E. foeminea* during the second pollination peak. However, and perhaps more importantly, the primary interest of the Chalcidoidea was the older female cones that had produced drops during the anthesis period of July and now were about to set seeds. It is likely that the so far undetermined species of Chalcidoidea use fertilized *E. foeminea* cones as a position for egg laying, which would explain why they were not observed during the anthesis period of July. We collected cones in August that had produced their pollination drops in July and reared several individuals of Chalcidoidea, so far of undetermined species identity (Bolinder et al., work in progress).

Figure 5: Parasitic wasps of the Chalcidoidea visiting *Ephedra* cones. A: a visit at an *E. distachya* cone after pollination drop production has ceased. B: a visit prior to the outbreak of the micropylar tube. C: visit at a fertilized female cone of *E. foeminea.* D: visit at a functionally male (but morphologically bisexual) cone of *E. foeminea.*

Pollination drops - The pollination drops of *Ephedra* are high in sugar, especially sucrose (Ziegler 1959) and as high as 80% has been reported (Bino et al. 1984). In many conifers the pollination drops contain defense proteins that protects the ovule from foreign pollen, pathogenes and fungal hyphae (Gelbart & von Aderkas 2002). The pollination drops of *Ephedra* contain very small amounts of proteins and instead the high osmotic pressure formed by the high sugar content is thought to create an environment unsuitable for the germination of foreign pollen and the growth of pathogens and fungal hyphae (Hou & Rydin 2014; von Aderkas et al. 2015). In Paper I we investigated how the presence of ants affects the germination of *Ephedra* pollen. Ants are thought to be rare as pollinators since the formicid acid many ants produce suppresses the germination of many pollen and spores (Beattie et al. 1984; Beattie 2006). This is however not the case here. *Ephedra foeminea* pollen do actually germinate better when they have been in contact with local ants, than when they have not. We also observed an increased growth of fungal hyphae in the absence of ants. Therefore, we hypothesized that in this case fungal hyphae are suppressed by the presence of ants, which benefits the germination of *E. foeminea* pollen. *Ephedra* pollen seems more sensitive to the presence of fungal hyphae than to the presence of ants, therefore ants cannot be ruled out as pollinator of this species. Instead we considered them to provide an indirect enhancer of pollen germination by suppressing the growth of fungal hyphae.

Shift in pollination mechanism – The two other members of the Gnetales, *Welwitschia* and *Gnetum,* are repeatedly described as insect-pollinated (Hooker 1863; Baines 1864; Pearson 1907; van der Pijl 1953; Kato & Inoue 1994; Kato et al. 1995; Wetschnig & Depish 1999; Gong et al. 2015) (see further discussions below), and insect pollination is probably ancestral within the *Ephedra*-lineage. The clade imbalance (Slowinski & Guyer 1993) between the insect-pollinated and the wind-pollinated linages of *Ephedra* is larger than expected by chance and we therefore concluded that a shift in pollination biology, perhaps during a time of climatic instability in the early Paleogene, might have played a crucial role as a precursor of the Cenozoic re-radiation of the clade.

Paper II – Aerodynamics and pollen ultrastructure

The settling velocity of a particle highly affects the time spent in the air and therefore the dispersal distance of the particle (Vogel 1981; Niklas 1985c). It is therefore not surprising that wind-pollinated species tend to have pollen with a lower settling velocity and therefore spend longer time in the air and thus disperse longer distances than do insect-pollinated species (Hall & Walter 2011, Paper I). And this is also what we found in *Ephedra* (Paper I

and II). The density of the protoplasm does not vary between closely related species (Niklas 1985c). We therefore hypothesized that variation in the structure of the pollen wall may account for the aerodynamic differences between *E. foeminea* and *E. distachya* pollen observed in Paper I and the relatively high settling velocity of *E. foeminea* pollen. We therefore decided to investigate pollen wall ultrastructure in some key-species of *Ephedra*. The results showed that pollen of *E. foeminea* has a dense ultrastructure resulting in a high density of the grain and a higher settling velocity compared to other investigated species. The pollen is therefore incapable of travelling long distances. In contrast, the wind-pollinated *E. distachya* and several other species of the genus have a spacious ultrastructure of the pollen wall and their pollen is thus capable of traveling longer distances by wind (see also Paper I).

Welwitschia has long been assumed to be insect-pollinated (Hooker 1863; Baines 1864; Pearson 1906) and field experimentation performed by Wetschnig and Depish (1999) shows that only a very limited amount of pollen of *Welwitschia* can travel more than a few meters away from the male plant. Therefore it is not surprising that the pollen wall ultrastructure of *Welwitschia* (Paper II) resembles that of the entomophilous *E. foeminea*. While working with Paper II we tried to investigate the aerodynamic features of *Welwitschia* pollen, in addition to *E. foeminea* pollen. However, the pollen was so sticky that it was impossible to measure the settling velocity with currently available methods. Based on the density of the pollen wall and the poor flight capability of the pollen we concluded the settling velocity of *Welwitschia* pollen to be high, had it been possible to measure. Pollen of the Gnetales is considered to lack pollenkitt (Hesse 1980; Hesse 1984) and the origin of the stickiness needs further investigation (see further discussion below). Nevertheless, the stickiness and the dense ultrastructure of *Welwitschia* pollen provide further support for insect-pollination in this species.

Studied species of *Gnetum* are also interpreted as insect-pollinated (van der Pijl 1953; Kato & Inoue 1994; Kato et al. 1995) or at least partly so (Gong et al. 2015) but the aerodynamic features of pollen of *Gnetum* are not yet studied. Gong et al. (2015) investigated the pollination biology of *Gnetum parvifolium* and investigated among other things the number of pollen grains in the air at certain distances from a male plant. The number of pollen grains in the traps decreases exponentially with distance from the male plant, and it can therefore be concluded that pollen of this species can travel only very short distances by wind although there are no reports further than 5 meters away from the male plant (Gong et al. 2015). The ultrastructure of *G. parvifolium* is highly dense (Yao et al. 2004) probably correlated with a high settling velocity resulting in the bad flight capability observed by Gong et al. (2015). Also the insect-pollinated *G. gnemon* (van der Pijl 1953; Kato &

Inoue 1994; Kato et al. 1995) shows a high density of granules in the infratectum (Gullvåg 1966; Hesse 1980; Kurmann 1992) as does *G. cleistostachyum* (Yao et al. 2004), *G. indicum* (Bernard & Meyer 1972), *G. pendulum* (Yao et al. 2004) and *G. ula* (Gullvåg 1966). Among studied species of *Gnetum*, only *G. africanum* has a different ultrastructure with only a few large granules (Tekleva & Krassilov 2009), resulting in a more spacious infratectum. Other studied species have numerous small granules in the dense infratectum (Gullvåg 1966; Bernard & Meyer 1972; Hesse 1980; Kurmann 1992; Yao et al. 2004; Tekleva & Krassilov 2009). The aerodynamics of *G. africanum* is not investigated and there are to date no pollination studies performed on African *Gnetum* species. However, the comparatively spacious ultrastructure of *G. africanum* pollen lends support for a lower settling velocity of this species, thus indicating wind-pollination for this species (see also discussion in Jörgensen and Rydin 2015, Rydin and Hoorn 2016).

Capture efficiency - Pollen capture can be aerodynamically specialized in very elegant ways. In the wind-pollinated *E. trifurca* and *E. nevadensis* the pollen gets streamlined towards the pollination drop in such a precise manner that the species can sort out conspecific pollen (Niklas & Buchmann 1987). In the entomophilous *E. foeminea* on the other hand the capture efficiency is nearly none-existent and even if pollen grains manage to travel to the female plant the chance of them fertilizing an ovule is negligible. Investigating the capture efficiency of the female cones of *G. africanum* using aerodynamics would therefore be an efficient way to predicting its pollination biology.

Pollen clumping and pollenkitt – Clump formation and ability to stick to a vector are essential for insect pollination. Capability of forming clumps is actually even considered a good indicator of pollination syndrome (Hall & Walter 2011). In angiosperms the stickiness of pollen is explained by the presence of pollenkitt at the surface of the exine. Pollenkitt is produced by plastids of the anther tapetum during and after the tetrad stage in microsporogenesis and consists of a lipid component and osmiophiolous carotenoid droplets (Pacini & Hesse 2005). In the angiosperms, pollenkitt is present in all sub-groups, also among anemophilous angiosperm groups like the Poales (Hesse 1981; Furness & Rudall 1998). In gymnosperms, on the other hand, pollenkitt is absent in all group, entomophilous as well as anemophilous (Hesse 1984). Nevertheless is pollen of the insect-pollinated gymnosperms investigated in this thesis indeed sticky. Pollen of *Welwitschia* is even sticky to the level that the experimental approach in Paper II was impossible to complete. What makes the pollen of *E. foeminea* and *Welwitschia* so sticky? According to Hesse (1984) some of the stickiness in this species could be accounted for by tapetal cytoplasm debis, perhaps in combination with the polyplicate morphology. The morphology with a high number of

ridges shown by *E. foeminea* and *Welwitschia* would facilitate the attachment to the thorax of a hairy insect. However, since not all *Ephedra* species with the ancestral pollen type (see Paper III and below) have sticky pollen, and *Welwitschia* pollen is a lot stickier than pollen of *E. foeminea* despite the similar morphology, this does not explain the whole situation and further research of the stickiness of Gnetales pollen is needed.

Paper III – Pollen morphology

In Paper III we recognized three different pollen types in *Ephedra*; 1) without side-branches on the pseudosulci, 2) with first order side-branches on the pseudosulci, and 3) with first and second order side-branches on the pseudosulci (Fig. 3). The different pollen types of *Ephedra* are clade specific and pollen without side-branches is the ancestral state and it is known in the fossil record since the Permian (Wilson 1962). Pollen with first order branches is derived and this pollen type is only documented in the fossil record a handful of times prior to the K-Pg boundary (Steeves & Barghoorn 1959; Chlonova 1981; Bercovici et al. 2012; Norbäck Ivarsson 2014). Pollen with first and second order branches is to my knowledge not described from strata older than the K-Pg boundary. In the early Cenozoic, pollen with branches on the pseudosulci becomes more and more abundant and is in most samples after the Eocene the only occurring type (Bolinder and Rydin in progress). This new knowledge about pollen evolution in *Ephedra* provides for the first time an opportunity for a calibration point within *Ephedra* for the purpose of molecular dating analyzes (Thureborn et al. in progress), the results of which indicates that the crown group *Ephedra* is much older than the median age of ~30 Ma previously suggested by Ickert-Bond et al. (2009).

Furthermore, the different pollen types indicate adaptations to different pollination syndromes. Pollen with branches on the pseudosulci is associated with a more spacious structure of the pollen wall and consequently lower settling velocities that facilitates long distance dispersal and, thus, wind pollination (see Paper II). The different pollen types are also suggested to be related to the degree of dehydration at the time of dispersal of the grain and germination of the male gametophyte. Pollen grains with branches on the pseudosulci are more dehydrated when dispersed than is pollen without branches on the pseudosulci. Pollen of the derived type (with branches on the sulci) is, therefore, lighter at dispersal than those of the ancestral type, and can disperse longer distances (see Paper II). As a trade-off, however, germination occurs slower in the derived type as they first have to re-hydrate in the pollination drop upon arrival at the female cone.

Paper IV – Moonlight pollination

Night-pollinated angiosperms do generally have white flowers that produce a strong scent to attract pollinators to the flower, and we were puzzled by the apparent complete absence of scent produced by the non-showy cones of *E. foeminea*. How do the pollinators find their way to these inconspicuous cones in the pitch black night? Also day-pollinated entomophilous angiosperms have some means of attraction, and the same question could be asked regarding the daytime pollinators of *Ephedra*. Why are they attracted to the cones? Although it is of course possible that the cones present some kind of scent or other attractant undetectable to us, we found no such indications. We were also puzzled by the difficulties to predict the phenology of *E. foeminea* compared to *E. distachya*. Most research regarding phenology is performed with angiosperms as a study object and most phenological patterns can be explain either by day-length or patterns of light intensity, or by climate variables, in most cases temperature. For *E. foeminea* no known factor for phenology could explain the observed variable pattern in "flowering time" from year to year. *Ephedra distachya,* on the other hand, produced its pollination drops at the same time every year, always around the 20th of May during our years of observation in Greece, possibly linked to the arrival of the summer in the Balkans. Extended studies of phenology and pollinators of the entomophilous *E. foeminea* revealed a strong correlation between the date of the full moon in July and the peak in pollination drop production. *Ephedra foeminea* is pollinated partly by nocturnal insects, which often use the full moon for navigation (Danthanarayana 1986). Furthermore, after observing the reflections of the full moon in the pollination drops we no longer considered the cones of *E. foeminea* non-spectacular. It was all of a sudden completely clear how the insects find their way to the cones. Also at daytime is the attraction probably entirely visual, and provided by the beautifully salmon-red female cones and the brightly yellow microsporangia of male cones (Figs 1A-B).

Our observations are, however, peculiar in many respects. The day before the full moon clearly mature cones did not produce any drops at all. In contrast, at the day of the full moon all cones were flooding out their pollination drops, also cones that were evidently immature. In a low-frequent pollination system like that of *E. foeminea*, with relatively few insect visitations, it is conceivably important to maximize the length of the effective pollination period. Only at full moon is the moon up during the entire night, and thus, only at full moon do the plants have access to well-navigating pollinators around the clock. We therefore suggest that the correlation to the lunar cycle is essential for the reproduction of this species. For wind-pollinated species,

a lunar correlation would have no advantage, and it is therefore not surprising that the anemophilous *E. distachya* lacks this system. How the plants detect the lunar cycle is not clear. It can either be due to gravity or light intensity and the question needs further exploration, preferably by plant physiologists. It is however clear that the moon is important in many respects for many organisms, including navigation and reproduction. Lunar-correlated reproduction has previously been described for both animals such as reef corals (Harrison et al. 1984) and for brown algae (Andersson et al. 1994).

Future perspectives

To further explore the evolution of pollination biology in *Ephedra*, as well as the evolution of insect pollination as phenomenon, we need to further explore the pollination biology of the two other members of the Gnetales. The pollination biology of *Gnetum* have only been studied with field experimentations and observations for a few species; i.e. *G. gnemon, G. cupsidatum* and *G. parvifolium* (van der Pijl 1953; Kato & Inoue 1994; Kato et al. 1995; Gong et al. 2015) and more studies regarding the pollination biology of this taxa is needed, especially for African species considered the indication of wind-pollination mentioned above. For *Welwitschia* the pollination biology has been sufficiently studied by means of field experimentation by Wetschnig and Depish (1999). However, they did only briefly make nocturnal observation of the plants. Considering the pollination biology and the correlations with the full moon discovered in this thesis there are reasons to extend the nocturnal studies of *Welwitschia* in the future.

Interpretation of pollination mode in extinct ephedroid taxa would if possible provide extremely important data for the dating of the shift in pollination mechanism and the re-radiation of the clade. In Paper II we found a correlation between the density of the ultrastructure and the pollination mode in *Ephedra*; anemophilous taxa have a lower density of the granules in the infratectum and as a consequence a lower settling velocity and better flight capability. The ultrastructure of fossil taxa can be investigated and based on the information; the settling velocity can be reconstructed. The ultrastructure has been investigated only for a few fossil *Ephedra*-like pollen (Trevisan 1980; Osborn et al. 1993; Tekleva & Krassilov 2009; Tekleva 2015), all of the ancestral pollen type and older than the K-Pg boundary. Next step is to investigate the ultrastructure of pollen of the derived type found in younger strata. Does also the oldest pollen of the derived type have a spacious ultrastructure? However, the settling velocity of the pollen grains does not provide the whole picture. Also the capture efficiency needs to be addressed and, thus, the aerodynamic impaction of the corresponding female cone. Only rarely is it possible to asses which pollen and female cone that belongs together, but there are examples of pollen found *in situ* in the micropylar tube of fossil ovules/seeds (Rydin et al. 2006b). It would nevertheless be difficult to make correct assessments of the capturing efficiency of the female structure because the ovules/seeds are dispersed and may have original-

ly been situated in a cone with different aerodynamic properties than the naked ovule. Another factor that provides an obstacle when it comes to reconstruction of the aerodynamics of fossil taxa is the angle between the stem and the cone that often has been modified in the fossilization process. The angle between stem and cone is one of the factors that affects the capture efficiency of the female cone the most. Investigated anemophilous species show a larger tilt of the cone in relation to the stem (Niklas et al. 1986) compared to *E. foeminea* (Paper II).

All indisputably insect-pollinated members of the Gnetales have sterile female organs in the male cone; i.e. *Welwitschia*, *Gnetum* and *E. foeminea* (Endress 1996; Jörgensen & Rydin 2015) whereas all wind-pollinated *Ephedra* species do not (Rydin & Endress in progress). The morphology of the male cones can thus provide some indirect evidence for the pollination biology. Unfortunately, the male cones are short-lived and fragile and are, thus, only rarely preserved as fossils. To date there are no ephedroid fossils that can be safely assigned to the lineage and at the same time provide sufficient information about the male cone for indirect inference of the pollination biology (C. Rydin pers. com.). However, in early-diverging lineages of the extant *Ephedra* clade, and in certain Cretaceous fossils, the female cones show a characteristic thickening, perhaps lignification, of the inner epidermis of the micropylar tube (Rydin & Friis 2010; Rydin et al. 2010). It is possible that this character provides support for the tube and prevents it from breaking or bending during an insect visitation and thus provides an adaptation to insect-pollination. The feature has been lost in wind-pollinated members of the living *Ephedra* clade (see also Rydin et al. 2010). Better knowledge of at least some of the above mentioned features, i.e. ultrastructure of the pollen, bi- or unisexual morphology of the male cone, and estimated aerodynamic properties of the corresponding female cone, would provide very interesting indirect evidence for inference of the pollination biology of extinct members of the *Ephedra* lineage.

Svensk sammanfattning

Den här avhandlingen handlar om *Ephedra,* en liten växtgrupp på omkring 50 arter tillhörande den gåtfulla ordningen Gnetales. Det som gör det så intressant att studera Gnetales i allmänhet och *Ephedra* i synnerhet, är dels dess långa evolutionära historia samt det oklara släktskapet mellan Gnetales och de andra fröväxterna; det vill säga blomväxter, kottepalmer, barrträd och tempelträd. Det tidigaste fossila spåren av *Ephedra* är pollen som är omkring 270 millioner år gamla men det var först under tidig Krita (145-100 Ma), samtidigt som dinosaurierna härskade på jorden, som mångfalden av *Ephedra*-liknande växter nådde sin höjdpunkt. Från tidig Krita finns det en rad megafossil beskrivna som påvisar stor morfologisk diversitet men ändå unika likheter med dagens *Ephedra*. Därför anses det helt klarlagt att åtminstone vissa av dessa fossil representerar utdöda systergrupper till dagens *Ephedra*. Mot slutet av Krita-perioden sjönk diversiteten drastiskt och i och med meteoritnedslaget som orsakade dinosauriernas utdöende för 65 millioner år sedan, var det nära att de *Ephedra*-liknande växterna också dog ut. Men så, för ungefär 30 miljoner år sedan enligt rådande datering hände något. Mångfalden inom gruppen ökade igen vilket resulterade i de ungefär 50-60 arter vi ser idag. Frågan är vad var det som hände? Varför dog de inte ut som så många andra arter gjorde efter meteoritnedslaget? Det verkar ha varit nära.

Ett övergripande mål med den här avhandlingen har varit att utvärdera huruvida ett skifte i pollinationsmekanism kan ha medverkat till återväxten av *Ephedra* för 30 millioner år sedan. Första steget var därför att sätta upp ett pollinationsexperiment och undersöka om det finns flera olika pollinationsmekanismer inom gruppen idag. *Ephedra* är så kallade tvåbyggare, vilket innebär att de har skilda han- och honindivider. Från honkotten sekreteras en söt pollinationsdroppe där pollen tas emot för att sedan transporteras till fröämnets inre där befruktningen sker. Hos *E. foeminea*, som är endemisk till medelhavsområdet och systerart till alla andra *Ephedra*-arter, sekreteras en likadan söt droppe från sterila fröämnen också i hankotten, något som inte sker hos någon annan *Ephedra*-art. Denna morfologiska skillnad samt systerskapet till resten av gruppen motiverade *E. foeminea* som självklart studieobjekt för pollinationsstudien. Vi ville dock studera ytterligare en *Ephedra*-art, och helst en som växer i samma område men som ändå inte är nära släkt med just *E. foeminea*. Det gjorde att valet föll på nordöstra Grekland där *E. foeminea* växer i samma områden som *E. distachya*, en art som släktskaps-

mässigt hör hemma i den asiatiska *Ephedra*-gruppen. Genom att jämföra hur långt pollen kan flyga, hur många kottar som befruktas om insekter utesluts med insektsnät, samt observationer av besökande insekter under både dag och natt kunde vi dra slutsatsen att *E. foeminea* är insektspollinerad medan *E. distachya* är vindpollinerad. Genom en slump upptäckte vi under arbetet med den här avhandlingen att de sockriga dropparna hos *E. foeminea* sekreteras i samband med fullmånen i juli, vilket var lika förvånande som intressant. *Ephedra foeminea* pollineras bland annat av nattaktiva malar men också av andra insekter, både dag- och nattaktiva. Många nattaktiva insekter använder sig av fullmånen för sin navigering och troligtvis är det de glittrande reflektionerna av månljuset i dropparna som gör de annars ganska oansenliga kottarna attraktiva för insekterna och ser till att insekterna hittar fram till kottarna. Det är troligen på grund av det, och för att fullmånenätterna är de enda nätterna när *E. foeminea* har tillgång till väl-navigerande pollinatörer hela natten som den så specifika kopplingen till fullmånen har uppstått och blivit kvar. Hur växten känner av fullmånen är ännu inte känt och kräver vidare studier. Hos den vindpollinerade *E. distachya* avges de sockriga dropparna inte alls i korrelation med mån-cykeln utan kring mitten av maj varje år, vilket snarare verkar spegla vädret och sommarens ankomst till nordöstra Grekland. Ytterligare en art, *E. aphylla,* som liksom *E. foeminea* släktskapsmässigt placerar sig utanför den stora huvudgruppen av *Ephedra*-arter, har också beskrivits som åtminstone delvis insektspollinerad. Idag finns det alltså en (*E. foeminea*)*,* eller möjligtvis två (*E. foeminea* och *E. aphylla*) arter som är insektspollinerade. Att jämföra med ungefär 50 vindpollinerade arter. Denna obalans i antal arter är större än vad som är förväntat på grund av slump vilket tyder på att artbildningshastigheten är högre inom den vindpollinerade linjen än inom den insektspollinerade linjen. Detta ger stöd åt tanken att ett historiskt och evolutionärt skifte i pollinationsmekanism, kanske på grund av förändrat klimat och minskad tillgång till pollinatörer, underlättat artbildning och arters fortlevnad inom *Ephedra*.

Vilken pollinationsmekanism en växt har återspeglas ofta i hur pollenkornen ser ut. Vindpollinerade växter har generellt sett mindre pollenkorn med en slät yta medan insektspollinerade växter har större pollen med ornamenterad yta. Hos *Ephedra* speglas pollinationsmekanismen i pollenväggens struktur. Alla arter har inte undersökts, men baserat på de data som finns har den insektspollinerade *E. foeminea* en tätare pollenvägg än vindpollinerade arter. En tätare struktur på väggen ger pollenkornet en högre densitet och därför en högre fallhastighet vilket gör att de inte kan flyga lika långt som pollenkorn med luftigare struktur i väggen. Hur pollenkornen tas emot hos honkotten är också av största betydelse för lyckad befruktning hos vindpollinerade arter. Tidigare studier har visat att honkottarna hos vindpollinerade *Ephedra*-arter är strömlinjeformade och placerade i en sådan vinkel att pollen av samma art leds mot droppen på ett mycket effektivt sätt. Hos *E. foeminea* är honkottarna inte alls anpassade för att aerodynamiskt leda pollen mot droppen.

Ephedra-pollen är båtformade med 4-25 ribbor (plicae) som löper längs med den långa ekvatorialaxeln på pollenkornet. Mellan varje plicae-par löper en fåra, en pseudosulcus, som antingen är grenad eller ogrenad. Pseudosulci ska ses som svaghetszoner och strukturen på pollenväggen är annorlunda där eftersom vissa lager saknas. När pollenkornet hamnat i pollinationsdroppen börjar hangametofyten gro och pollenkornet spricker i en av svaghetszonerna och skalet ramlar av. Man kan dela upp *Ephedra*-pollen i två typer, sådana med ogrenade pseudosulci, vilket är det ursprungliga inom gruppen, och sådana med grenade pseudosulci. De allra äldsta pollenkornen som tros tillhöra *Ephedra*-liknande växter är av den ursprungliga typen och har ogrenade pseudosulci. Först för 90 millioner år sedan dyker de första pollenkornen med grenade pseudosulci upp vid ett par lokaler på norra halvklotet. Från att ha varit nere på mycket låga nivåer precis efter meteoritnedslaget för 65 miljoner år sedan ökar också den relativa förekomsten av *Ephedra*-pollen under Paleogen-perioden, speciellt på det norra halvklotet. Pollentypen med grenade pseudosulci blir också allt mer vanlig för att tillslut vara den enda förekommande på många lokaler på det norra halvklotet. Pollen med grenade pseudosulci har aldrig hittats på det södra halvklotet, varken i nutid eller som fossil. Vi tror att skiftet i vilken pollentyp som är den vanligast förekommande speglar skiftet i pollinationsmekanism som verkar ha skett under Paleogen. För att ytterligare undersöka när skiftet skedde hoppas jag nu på att kunna snitta pollen med grenade pseudosulci från Krita och undersöka deras pollenvägg för att kunna rekonstruera och modellera deras fallhastighet. Detta skulle ge oss en ledtråd till pollinationsmekanismen hos dessa utdöda växter.

Tack till

Först och främst vill jag tacka min alltid lika engagerade och stöttande handledare Catarina Rydin. TACK för att du trodde på mig när jag kom till dig som masterstudent med galna idéer och sen aldrig har slutat tro på mig och min förmåga, inte ens när jag själv tvivlat. TACK för att ingen fråga, oavsett hur liten, någonsin varit oviktig. TACK för att du lärt mig att tänka fritt och förutsättningslöst och TACK för att du så frikostigt delat med dig av all din kunskap och för all den glädje och entusiasm du sprider för vetenskap i allmänhet och för Gnetales i synnerhet. Jag kunde verkligen inte önskat mig en bättre handledare och förebild. TACK!

Johan Ehrlén; TACK för perfekt statistikhandledning och för alla peppande ord.

Aelys Humphreys; min akademiska storasyster, alltid med ett uppmuntrande ord, en ny infallsvinkel på ett problem och ett svar på varför i ******* mina R-skript inte fungerar, TACK.

Stefanie Ickert-Bond and Carina Hoorn; Steffi; thank you for all your enthusiasm and for sharing your deep knowledge of *Ephedra*. Carina; thank you for training me in palynology and for sharing fossil material, knowledge and ideas.

Karl Niklas; thank you for having me as a guest in your lab at Cornell, for showing me the beauty of aerodynamics and for great supervision and support during the work with Paper II.

Jürg Schönenberger; thank you for being the opponent of my lic thesis. Your questions really opened my eyes for new ways of thinking and gave me many new ideas. Thank you!

James Doyle; thank you for being the carful reviewer of almost all my papers; your comments have really improved the manuscripts tremendously, thank you!

Kjell Jansson; alltid med en ny idé till att få till den perfekta SEM-bilden, tack för att du delat med dig av all din kunskap och för allt ditt engagemang.

References

Andersson S, Kautsky L, Kalvas A. 1994. Circadian and lunar gamet release in *Fucus vesculosus* in the atidal Baltic sea. Marine Ecology Progress Series 110: 195-201.

Askew R, Blasco-Zumeta J. 1998. Insects associated with galls of a new species of Eurytomidae (Hymenoptera: Chalcidoidea) on *Ephedra nebrodensis* in Spain. Journal of Natural History 32: 805-821.

Askew RR, Blasco-Zumeta J. 1997. Parasitic Hymenoptera inhabiting seeds of *Ephedra nebrodensi*s in Spain, with descriptions of a phytophagous pteromalid and four other new species of Chalcidoidea. Journal of Natural History 31: 965-982.

Baines T 1864. Explorations in South-West Africa: being an account of a journey in the years 1861 and 1862 from Walvisch Bay, on the western coast, to Lake Ngami and the Victoria Falls. London: Longman Green Longman Roberts & Green.

Beattie AJ. 2006. The evolution of ant pollination systems. Botanische Jahrbücher für Systematik 127: 43-55.

Beattie AJ, Turnbull C, Knox EM, Williams EG. 1984. Ant inhibition of pollen function: a possible reason why ant pollination is rare. American Journal of Botany 71: 421-426.

Beattie AJ. 1982. Ants and gene dispersal in flowering plants. In: Armstrong JA, JM Powell & AJ Richards, eds., Pollination and Evolution, 1-8. Kew: The Royal Botanic Gardens.

Bercovici A, Vajda V, Sweet A. 2012. Palynostratigraphy of the cretaceous-paleogene mass-extinction interval of the Northern Hemisphere. Journal of Stratigraphy 36: 165-178.

Bernard V, Meyer N. 1972. Pollen grains of *Ephedra, Welwitschia* and *Gnetum*. Moskva University Series VI Biology Pochvoved 27: 86-88.

Bernhardt P, Thien LB. 1987. Self-isolation and insect pollination in the primitive angiosperms: new evaluations of older hypotheses. Plant Systematics and Evolution 156: 159-176.

Bino RJ, Devente N, Meeuse ADJ. 1984. Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forssk. (=*E. alte* C.A. Mey.), with some notes on *E. campylopoda* C.A. Mey. II. Pollination droplets, nectaries, and nectarial secretion in *Ephedra*. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen. Series C: Biological and medical sciences 87: 15-24.

Bolinder K. 2014. Pollination in *Ephedra* (Gnetales). Licentiate thesis, Stockholm University, Stockholm, Sweden.

Bond WJ. 1989. The tortoise and the hare: ecology of angiosperm dominance and gymnosperm persistence. Biological Journal of the Linnean Society 36: 227-249.

Brewbaker JL, Kwack BH. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. American Journal of Botany 50: 859-865.

Buchmann SL, O'Rourke MK, Niklas KJ. 1989. Aerodynamics of *Ephedra trifurca*. III. Selective pollen capture by pollination droplets. Botanical Gazette 150: 122-131.

Burger WC. 1981. Why are there so many kinds of flowering plants. BioScience 31: 572-581.

Chase MW, Soltis DE, Olmstead RM, Morgan D, Les DH, Mishler BD, Duvall MR, Price RA, Hills HG, Qiu YL. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. Annals of the Missouri Botanical Garden 80: 528-580.

Chaw S, Parkinson C, Cheng Y, Vincent T, Palmer J. 2000. Seed plant phylogeny inferred from all three plant genomes: monophyly of extant gymnosperms and origin of Gnetales from conifers. Proceedings of the National Academy of Sciences of the United States of America 97: 4086-4091.

Chlonova AF. 1981. Senonian (Late Cretaceous) Palynofloral provinces in circumpolar areas of the Northern Hemisphere. Review of Palaeobotany and Palynology 35: 315-324.

Crane PR, Lidgard S. 1989. Angiosperm diversification and paleolatitudinal gradients in Cretaceous floristic diversity. Science 246: 675-678.

Crane PR. 1985. Phylogenetic analysis of seed plants and the origin of angiosperms. Annals of the Missouri Botanical Garden 72: 716-793.

Crepet WL. 1979. Insect pollination: a paleontological perspective. BioScience 29: 102-108.

Crepet WL. 1984. Advanced (constant) insect pollination mechanisms: pattern of evolution and implications vis-à-vis angiosperm diversity. Annals of the Missouri Botanical Garden 71: 607-630.

Crepet WL, Friis EM. 1987. The evolution of insect pollination in angiosperms. In: Friis EM, WG Chaloner & PR Crane, eds., The origins of angiosperms and their biological consequences, 181-201. Cambridge: Cambridge University Press.

Danthanarayana W. 1986. Lunar periodicity of insect flight and migration. In: Danthanarayana W, ed., Insect Flight, 88-119. Berlin Heidelberg: Springer Verlag.

Dodd ME, Silvertown J, Chase MW. 1999. Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families. Evolution 53: 732-744.

Doyle JA, Donoghue MJ. 1986. Seed plant phylogeny and the origin of angiosperms: an experimental cladistic approach. Botanical Review 52: 321-431.

Doyle JA, Donoghue MJ. 1993. Phylogenies and angiosperm diversification. Paleobiology 19: 141-167.

Doyle JA, Donoghue MJ. 1992. Fossil and seed plant phylogeny reanalyzed. Brittonia 44: 89-106.

Doyle JA. 2008. Integrating molecular phylogenetic and paleobotanical evidence on origin of the flower. International Journal of Plant Sciences 169: 816-843.

El-Ghazaly G, Rowley JR, Hesse M. 1998. Polarity, aperture condition and germination in pollen grains of *Ephedra* (Gnetales). Plant Systematics and Evolution 213: 217-231.

Endress PK. 1996. Structure and function of female and bisexual organ complexes in Gnetales. International Journal of Plant Sciences 157: 113-125.

Furness CA, Rudall PJ. 1998. The tapetum and systematics in monocotyledons. The Botanical Review 64: 201-239.

Gelbart G, von Aderkas P. 2002. Ovular secretions as part of pollination mechanisms in conifers. Annals of Forest Science 59: 345-357.

Gong YB, Yang M, Vamosi JC, Yang HM, Mu WX, Li JK, Wan T. 2015. Wind or insect pollination? Ambophily in a subtropical gymnosperm *Gnetum parvifolium* (Gnetales). Plant Species Biology 31: 272-279.

Gottsberger G. 1988. The reproductive biology of primitive angiosperms. Taxon 37: 630-643.

Gullvåg BM. 1966. The fine structure of some gymnosperm pollen walls. Grana 6: 435-475.

Hall JA, Walter GH. 2011. Does pollen aerodynamics correlate with pollination vector? Pollen settling velocity as a test for wind versus insect pollination among cycads (Gymnospermae: Cycadaceae: Zamiaceae). Biological Journal of the Linnean Society 104: 75-92.

Harrison PL, Babcock RC, Bull GD, Oliver JK, Wallace CC, Willis BL. 1984. Mass spawning in tropical reef corals. Science 223: 1186-1189.

Hesse M. 1981. The fine structure of the exine in relation to the stickiness of angiosperm pollen. Review of Palaeobotany and Palynology 35: 81-92.

Hesse M. 1980. Pollenkitt is lacking in *Gnetum gnemon* (Gnetaceae). Plant Systematics and Evolution 136: 41-46.

Hesse M. 1984. Pollenkitt is lacking in Gnetatae: *Ephedra* and *Welwitschia*; further proof for its restriction to the angiosperms. Plant Systematics and Evolution 144: 9-16.

Hickey LJ, Doyle JA. 1977. Early Cretaceous fossil evidence for angiosperm evolution. Botanical Review 43: 3-104.

Hooker JD. 1863. On *Welwitschia*, a new genus of Gnetaceae. Transactions of the Linnean Society of London 24: 1-48.

Hou C, Rydin C. 2014. Proteome of pollination drops in *Ephedra* (Gnetales). In: Hou C, ed., Proteomics and phylogentics of the Gnetales, 23-39. Licentiate thesis: Stockholm University, Stockholm, Sweden.

Hu S, Dilcher DL, Jarzen DM, Taylor DW. 2008. Early steps of angiosperm pollinator coevolution. Proceedings of the National Academy of Sciences of the United States of America 105: 240-245.

Ickert-Bond SM, Rydin C, Renner SS. 2009. A fossil-calibrated relaxed clock for *Ephedra* indicates an Oligocene age for the divergence of Asian and New World clades and Miocene dispersal into South America. Journal of Systematics and Evolution 47: 444-456.

Jörgensen A, Rydin C. 2015. Reproductive morphology in the *Gnetum cuspidatum* group (Gnetales) and its implications for pollination biology in the Gnetales. Plant Ecology and Evolution 148: 387-396.

Kato M, Inoue T. 1994. Origin of insect pollination. Nature 368: 195.

Kato M, Inoue T, Nagamitsu T. 1995. Pollination biology of *Gnetum* (Gnetaceae) in a lowland mixed dipterocarp forest in Sarawak. American Journal of Botany 82: 862-868.

Krassilov VA. 1986. New floral structures from the Lower Cretaceous of Lake Baikal area. Review of Palaeobotany and Palynology 47: 9-16.

Kubitzki K. 1990. Ephedraceae. In: Kubitzki K, ed., The families and genera of vascular plants. I. Pteridophytes and Gymnosperms, 379-382. Berlin: Springer.

Kurmann MH. 1992. Exine stratification in extant gymnosperms: a review of published transmission electron micrographs. Kew Bulletin 47: 25-39.

Källersjö M, Farris JS, Chase MW, Bremer B, Fay MF, Humphries CJ, Petersen G, Seberg O, Bremer K. 1998. Simultaneous parsimony jackknife analysis of 2538 *rbcL* DNA sequences reveals support for major clades of green plants, land plants, seed plants and flowering plants. Plant Systematics and Evolution 213: 259-287.

Labandeira CC, Kvacek J, Mostovski MB. 2007. Pollination drops, pollen, and insect pollination of Mesozoic gymnosperms. Taxon 56: 663-695.

Liu Z-J, Wang X. 2015. An enigmatic *Ephedra*-like fossil lacking micropylar tube from the Lower Cretaceous Yixian Formation of Liaoning, China. Palaeoworld in press:

Loconte H, Stevenson DW. 1990. Cladistics of the Spermatophyta. Brittonia 42: 197-211.

Mathews KG, Dunne N, York E, Struwe L. 2009. A phylogenetic analysis and taxonomic revision of *Bartonia* (Gentianaceae: Gentianeae), based on molecular and morphological Evidence. Systematic Botany 34: 162-172.

Mathews S, Clements MD, Beilstein MA. 2010. A duplicate gene rooting of seed plants and the phylogenetic position of flowering plants. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 365: 383-395.

Niklas KJ 1992. Plant biomechanics: an engineering approach to plant form and function. Chicago & London: University of Chicago Press.

Niklas KJ, Buchmann SL, Kerchner V. 1986. Aerodynamics of *Ephedra trifurca*. I. Pollen grain velocity fields around stems bearing ovules. American Journal of Botany 73: 966-999.

Niklas KJ. 1985a. Aerodynamics of wind pollination in *Simmondsia chinensis* (Link) Schneider. American Journal of Botany 72: 530-539.

Niklas KJ. 1985b. Wind Pollination—A study in controlled chaos: aerodynamic studies of wind-pollinated plants reveal a high degree of control in the apparently random process of pollen capture. American Scientist 462-470.

Niklas KJ. 2015. A biophysical perspective on the pollination biology of *Ephedra nevadensis* and *E. trifurca*. The Botanical Review 81: 28-41.

Niklas KJ. 1985c. The aerodynamics of wind pollination. Botanical Review 51: 328-386.

Niklas KJ, Kerchner V. 1986. Aerodynamics of *Ephedra trifurc*a II. Computer modelling of pollination efficiencies. Journal of Mathematical Biology 73: 966-979.

Niklas KJ, Buchmann SL. 1987. Aerodynamics of pollen capture in two sympatric *Ephedra* species. Evolution 41: 104-123.

Norstog KJ. 1987. Cycads and the origin of insect pollination. American Scientist 75: 270-279.

Norstog KJ, Stevenson DW, Niklas KJ. 1986. The role of beetles in the pollination of *Zamia furfuracea* L. fil. (Zamiaceae). Biotropica (USA) 18: 300-306.

Osborn JM, Taylor TN, de Lima MR. 1993. The ultrastructure of fossil ephedroid pollen with gnetalean affinities from the Lower Cretaceous of Brazil. Review of Palaeobotany and Palynology 77: 171-184.

Owens JN, Takaso T, Runions CJ. 1998. Pollination in conifers. Trends in Plant Science 3: 479-485.

Pacini E, Hesse M. 2005. Pollenkitt–its composition, forms and functions. Flora-Morphology, Distribution, Functional Ecology of Plants 200: 399-415.

Pearson HHW. 1907. The living *Welwitschia*. Nature 75: 536-537.

Pearson HHW. 1906. Some observations on *Welwitschia mirabilis,* Hooker, f. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 198: 265-304.

R Development Core Team. 2014. R- A language and environment for statistical computing, Version 3.1.1. Vienna, Austria, http://www.r-project.org/.

Regal PJ. 1977. Ecology and evolution of flowering plant dominance. Science 196: 622-629.

Rowley JR. 1995. Are the endexines of pteridophytes, gymnosperms and angiosperms structurally equivalent? Review of Palaeobotany and Palynology 85: 13-34.

Rydin C, Korall P. 2009. Evolutionary relationships in *Ephedra* (Gnetales), with implications for seed plant phylogeny. International Journal of Plant Sciences 170: 1031-1043.

Rydin C, Pedersen KR, Friis EM. 2004. On the evolutionary history of *Ephedra*: Cretaceous fossils and extant molecules. Proceedings of the National Academy of Sciences of the USA 101: 16571-16576.

Rydin C, Hoorn C. 2016. The Gnetales: Past and Present. Grana 55: 1-4.

Rydin C, Källersjö M. 2002. Taxon sampling and seed plant phylogeny. Cladistics 18: 485-513.

Rydin C, Wu SQ, Friis EM. 2006a. *Liaoxia* Cao et S.Q. Wu (Gnetales): ephedroids from the Early Cretaceous Yixian Formation in Liaoning, northeastern China. Plant Systematics and Evolution 262: 239-265.

Rydin C, Pedersen KR, Crane PR, Friis EM. 2006b. Former diversity of *Ephedra* (Gnetales): evidence from Early Cretaceous seeds from Portugal and North America. Annals of Botany 98: 123-140.

Rydin C, Friis EM. 2010. A new Early Cretaceous relative of Gnetales: *Siphonospermum simplex* gen. et sp. nov. from the Yixian Formation of Northeast China. BMC Evolutionary Biology 10: 1-6.

Rydin C, Khodabandeh A, Endress PK. 2010. The female reproductive unit of *Ephedra* (Gnetales): comparative morphology and evolutionary perspectives. Botanical Journal of the Linnean Society 163: 387-430.

Rydin C, Källersjo M, Friis EM. 2002. Seed plant relationships and the systematic position of Gnetales based on nuclear and chloroplast DNA: Conflicting data, rooting problems, and the monophyly of conifers. International Journal of Plant Sciences 163: 197-214.

Schmidt M, Schneider-Poetsch HAW. 2002. The evolution of gymnosperms redrawn by phytochrome genes: The Gnetatae appear at the base of the gymnosperms. Journal of Molecular Evolution 54: 715-724.

Slowinski JB, Guyer C. 1993. Testing whether certain traits have caused amplified diversification: an improved method based on a model of random speciation and extinction. American Naturalist 142: 1019-1024.

Sprengel CC 1793. Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen. Berlin: Vieweg.

Steeves MW, Barghoorn ES. 1959. The pollen of *Ephedra*. Journal of the Arnold Arboretum 40: 221-255.

Tekleva M. 2015. Pollen morphology and ultrastructure of several *Gnetum* species: an electron microscopic study. Plant Systematics and Evolution 1-13.

Tekleva MV, Krassilov VA. 2009. Comparative pollen morphology and ultrastructure of modern and fossil gnetophytes. Review of Palaeobotany and Palynology 156: 130-138.

Tiffney BH. 1984. Seed size, dispersal syndromes, and the rise of the angiosperms: Evidence and hypothesis. Annals of the Missouri Botanical Garden 71: 551-576.

Trevisan L. 1980. Ultrastructural notes and considerations on *Ephedrites, Eucommiidites* and *Monosulcites* pollen grains from Lower Cretaceous sediments of southern Tuscany (Italy). Pollen et Spores 22: 85-132.

Vamosi JC, Vamosi SM. 2010. Key innovations within a geographical context in flowering plants: towards resolving Darwin's abominable mystery. Ecology Letters 13: 1270-1279.

Van Campo M, Lugardon B. 1973. Structure grenue infratectale de l'ectexine des pollen quelques Gymnospermes et Angiospermes. Pollen et Spores 15: 171-187.

van der Pijl L. 1953. On the flower biology of some plants from Java with general remarks on fly-traps (species of *Annona, Artocarpus, Typhonium, Gnetum, Arisaema* and *Abroma*). Annales Bogorienses 1: 77-99.

Wang X, Zheng SL. 2010. Whole fossil plants of *Ephedra* and their implications on the morphology, ecology and evolution of Ephedraceae (Gnetales). Chinese Science Bulletin 55: 1511-1519.

Wetschnig W, Depish B. 1999. Pollination biology of *Welwitschia mirabilis* Hook. f. (Welwitschiaceae, Gnetopsida). Phyton 39: 167-183.

Wilson LR. 1962. Permian plant microfossils from the Flowerpot Formation, Oklahoma. Oklahoma Geological Survey Bulletin 49: 5-50.

Vogel S. 1981. Life in moving fluids: the physical biology of flow. Boston: Willard Grant Press.

von Aderkas P, Prior N, Gagnon S, Little S, Cross T, Hardie D, Borchers C, Thornburg R, Hou C, Lunny A. 2015. Degradome and secretome of pollination drops of *Ephedra*. The Botanical Review 81: 1-27.

Yang Y, Geng B-Y, Dilcher DL, Chen Z-D, Lott TA. 2005. Morphology and affinities of an Early Cretaceous *Ephedra* (Ephedraceae) from China. American Journal of Botany 92: 231-241.

Yang Y, Ferguson DK. 2015. Macrofossil evidence unveiling evolution and ecology of early Ephedraceae. Perspectives in Plant Ecology, Evolution and Systematics 17: 331-346.

Yao Y-F, Xi Y-Z, Geng B-Y, Li C-S. 2004. The exine ultrastructure of pollen grains in *Gnetum* (Gnetaceae) from China and its bearing on

the relationship with the ANITA Group. Botanical Journal of the Linnean Society 146: 415-425.

Zavada MS. 1984. Angiosperm origins and evolution based on dispersed fossil pollen ultrastructure. Annals of the Missouri Botanical Garden 71: 444-463.

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