



From near extinction to diversification by means of a shift in pollination mechanism in the gymnosperm relict *Ephedra* (Ephedraceae, Gnetales)

KRISTINA BOLINDER^{1*}, AELYS M. HUMPHREYS^{1,2}, JOHAN EHRLÉN¹,
RONNY ALEXANDERSSON³, STEFANIE M. ICKERT-BOND^{4,5} and CATARINA RYDIN¹

¹*Department of Ecology, Environment and Plant Sciences, Stockholm University, SE-106 91 Stockholm, Sweden*

²*Comparative Plant & Fungal Biology, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK*

³*Biology Education Center, Evolutionary Biology Center, Uppsala University, Norbyvägen 14, SE-752 36 Uppsala, Sweden*

⁴*Department of Biology and Wildlife and Institute of Arctic Biology, University of Alaska Fairbanks, 907 Yukon Drive, PO Box 756960, Fairbanks, AK 99775-6960, USA*

⁵*School of Life Sciences, Arizona State University, PO Box 874501, Tempe, AZ 85287-4501, USA*

Received 18 August 2015; revised 14 December 2015; accepted for publication 20 December 2015

Pollination in gymnosperms is usually accomplished by means of wind, but some groups are insect-pollinated. We show that wind and insect pollination occur in the morphologically uniform genus *Ephedra* (Gnetales). Based on field experiments over several years, we demonstrate distinct differences between two *Ephedra* species that grow in sympatry in Greece in pollen dispersal and clump formation, insect visitations and embryo formation when insects are denied access to cones. *Ephedra distachya*, nested in the core clade of *Ephedra*, is anemophilous, which is probably the prevailing state in *Ephedra*. *Ephedra foeminea*, sister to the remaining species of the genus, is entomophilous and pollinated by a range of diurnal and nocturnal insects. The generalist entomophilous system of *E. foeminea*, with distinct but infrequent insect visitations, is in many respects similar to that reported for *Gnetum* and *Welwitschia* and appears ancestral in Gnetales. The *Ephedra* lineage is well documented already from the Early Cretaceous, but the diversity declined dramatically during the Late Cretaceous, possibly to near extinction around the Cretaceous–Palaeogene boundary. The clade imbalance between insect- and wind-pollinated lineages is larger than expected by chance and the shift in pollination mode may explain why *Ephedra* escaped extinction and began to diversify again. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016, **180**, 461–477.

ADDITIONAL KEYWORDS: anemophily – ants – Diptera – entomophily evolution – Lepidoptera.

INTRODUCTION

Gymnosperms are typically reported as wind-pollinated, probably because this is the prevailing state in the largest extant group, the conifers (Owens, Takaso & Runions, 1998). However, insect pollination has been documented for several living as well as fossil gymnosperm taxa (Tang, 1987; Labandeira, Kvacek & Mostovski, 2007). We study the

gymnosperm relict *Ephedra* L. of the monogeneric family Ephedraceae (Gnetales), for which the pollination biology has been a matter of debate but is surprisingly poorly known (Endress, 1996; Gorelick, 2001). Based on early observations of *E. distachya* L. subsp. *helvetica* (C.A.Mey) Asch. & Graebn., Jaccard (1894) suggested that *Ephedra* is wind-pollinated. More recently, comprehensive aerodynamic experiments have shown that cone and pollen morphology in *E. nevadensis* S.Watson and *E. trifurca* Torr. ex S.Watson are consistent with wind pollination

*Corresponding author. E-mail: Kristina.Bolinder@su.se

(Niklas, Buchmann & Kerchner, 1986; Niklas & Kerchner, 1986; Niklas & Buchmann, 1987; Buchmann, O'Rourke & Niklas, 1989; Niklas, 2015). However, insect pollination has also been proposed, for example for *E. foeminea* Forssk. (Porsch, 1910), *E. aphylla* Forssk. (Bino, Dafni & Meeuse, 1984a; Bino, Devente & Meeuse, 1984b; Meeuse *et al.*, 1990) and *E. distachya* (Moussel, 1980), and several of these studies discuss parallels between entomophily in *Ephedra* and angiosperms. Bino *et al.* (1984a, b) argued for an evolutionary trend from anemophily to entomophily in *Ephedra*, which they suggested may represent a precursor to the evolution of entomophily in angiosperms (see also discussions in Porsch, 1910).

Note that most of these studies were conducted at a time when methodological practice and views of seed-plant relationships were different from today. Several studies are based exclusively on observations and interpretations of circumstantial evidence or focus on a limited aspect of the pollination biology (Table 1). Interpretation of published results is further complicated by nomenclatural confusion and problematic species identifications and delimitations, well known in *Ephedra* (e.g. Freitag & Maier-Stolte, 1994). Thus, the inconsistent and partly contradictory reports call for further investigations as they indicate misconceptions and/or variation in pollination mechanism in this small family. Pollination biology in *Ephedra* has never been rigorously explored using field experimentation or been placed in a modern evolutionary context.

The *Ephedra* lineage has a long evolutionary history, extending at least to the late Mesozoic. *Ephedra*-like pollen increases in abundance in the fossil record during the Early Cretaceous (Crane & Lidgard, 1989) and a relatively large diversity of *Ephedra*-like plants has been documented in the fossil record, mainly from the Aptian (Krassilov, 1986; Yang *et al.*, 2005; Rydin, Wu & Friis, 2006b; Wang & Zheng, 2010), including taxa that share uniquely derived features with the extant clade (Rydin, Pedersen & Friis, 2004; Rydin *et al.*, 2006a). However, the Cretaceous diversity declined dramatically during the latter part of the period (Crane & Lidgard, 1989) and *Ephedra*-like megafossils are currently unknown from after the Early Cretaceous (Rydin *et al.*, 2006b). It has therefore been suggested that the Cretaceous diversity went almost entirely extinct towards the end of the period, with the resulting bottleneck effect reflected in extant *Ephedra* by an unusually low amount of genetic and morphological divergence among species (Rydin, Khodabandeh & Endress, 2010).

Today the family comprises 55–65 species in a single genus, distributed in arid regions of the world (Kubitzki, 1990). Extant species have been suggested

to be the result of relatively recent diversification of the crown group, currently dated to the earliest Oligocene (Ickert-Bond, Rydin & Renner, 2009). *Ephedra* diversity has thus fluctuated considerably over geological time. Although temporal fluctuations in diversity are a widely recognized phenomenon across the Tree of Life, less is known about what causes some lineages to prosper and others to vanish.

In the present study, we investigate the pollination biology in *Ephedra* in an evolutionary context and evaluate its potential implications for the temporal diversity fluctuations of the lineage. The results are based on several years of field experimentation and observations of two carefully selected *Ephedra* species. We test the mode of pollen transfer in these species to establish whether their pollination mechanisms differ and we explore the hypothesis that a shift in pollination syndrome promoted diversification in the core group of the extant clade of *Ephedra*.

MATERIAL AND METHODS

STUDY SYSTEM

To study the mode of pollen transfer, a series of experiments was conducted on two species: *Ephedra foeminea* (synonym *E. campylopoda* C.A.Mey.) and *E. distachya*. *Ephedra foeminea* was selected because it is sister to all other species of the genus (Rydin & Korall, 2009; Thureborn & Rydin, 2015) (Fig. 1A). Furthermore, it has been historically interpreted as insect-pollinated (Porsch, 1910; Bino *et al.*, 1984a) and its male cones are morphologically bisexual (functionally unisexual with male and sterile female reproductive units), whereas those of the remaining *Ephedra* spp. are unisexual. We wanted to be able to contrast the results for *E. foeminea* against those for a second species that grows under the same environmental conditions. To meet these requirements, we chose to conduct fieldwork in the province of Macedonia, north-eastern Greece, where *E. foeminea* and *E. distachya* grow in sympatry. Phylogenetically, *E. distachya* is not part of the Mediterranean grade of early-diverging lineages, but is nested in core *Ephedra*, as a member of the Asian clade (Rydin & Korall, 2009).

Both *E. foeminea* and *E. distachya* are functionally dioecious, despite the morphological differences of the male cones. *Ephedra foeminea* (Fig. 1B) occurs only in the eastern Mediterranean area (Freitag & Maier-Stolte, 1989) and is relatively common in north-eastern Greece. It is a large climber, often hanging from cliffs or partially supported by olive trees or small oaks. Female cones are reddish to salmon-coloured at the pollination stage of development, whereas male cones are greenish, but with

Table 1. Studies in the literature containing statements on pollination mode in *Ephedra* spp.

Species	Geographical origin	Pollination mode	Conclusion based on:	Reference(s)
<i>Ephedra aphylla</i> Forssk.	Mediterranean	Possibly insect	Insect observations	Bino <i>et al.</i> (1984a, b); Meeuse <i>et al.</i> (1990)
<i>Ephedra foeminea</i> Forssk.	Mediterranean	Insect	Insect observations, field experimentations and aerodynamics	Porsch (1910); Bolinder <i>et al.</i> (2015a); present study
<i>Ephedra fragilis</i> Desf.	Mediterranean	Wind	Dispersal distance of pollen	Navarro <i>et al.</i> (2001)
<i>Ephedra nevadensis</i> S.Watson	North America	Wind	Aerodynamics	Niklas <i>et al.</i> (1986); Niklas & Kerchner (1986); Niklas & Buchmann (1987); Buchmann <i>et al.</i> (1989)
<i>Ephedra trifurca</i> Torr.	North America	Wind	Aerodynamics	Niklas <i>et al.</i> (1986); Niklas & Kerchner (1986); Niklas & Buchmann (1987); Buchmann <i>et al.</i> (1989)
<i>Ephedra viridis</i> Coville	North America	Wind	Field observations and pollen ultrastructure	Freeman, Klikoff & Harper (1976); Bolinder <i>et al.</i> (2015a)
<i>Ephedra breana</i> Phil.	South America	Wind	Field observations	Arroyo <i>et al.</i> (1982)
<i>Ephedra distachya</i> Forssk.	Asia	Wind	Field studies and interpretation of pollen ultrastructure	Jaccard (1894); Bolinder <i>et al.</i> (2015a, b); present study
<i>Ephedra likiangensis</i> Florin	Asia	Wind	Interpretation of pollen ultrastructure	Bolinder <i>et al.</i> (2015a)
<i>Ephedra major</i> Puller	Asia	Wind	Dispersal distance of pollen	Navarro <i>et al.</i> (2001)

conspicuously yellow microsporangiophores at anthesis. *Ephedra distachya* (Fig. 1C) is widely distributed from central Asia to western Europe (Markgraf, 1964; Fu, Yu & Riedl, 1999). In Greece, it is restricted to coastal areas of the north-east (Strid, 1996, and the Flora Hellenica Database). It tends to grow as a prostrate dwarf shrub in disturbed habitats, but it can also be upright. Branch tips are often curled in a characteristic way. Male and female cones are inconspicuously green at the pollination stage of development, but male cones display brightly yellow microsporangiophores at anthesis.

We studied *E. foeminea* and *E. distachya* near the village of Asprovalta (Macedonia, Greece) during three field seasons. In 2011, studies were conducted regularly from early March to late September. In 2012, studies were conducted on 21–26 May and 29 June – 15 July, and in 2014 on 17–20 May, 1–7 July, 11–15 July and 8–11 August. We also received photos of the plants from local field assistants to keep track of the phenology. Two populations were selected for each species based on information in the Flora Hellenica Database (*E. foeminea*: 40°43'N, 24°05'E and

40°52'N, 24°19'E; *E. distachya*: 40°44'N, 23°43'E and 40°70'N, 23°70'E). The two species may grow within tens of metres of each other in this area, but the populations were selected to ensure that they contained only individuals from one of the species (at least 500 m from the study population to the nearest plant of the other species).

POLLINATION BIOLOGY

Pollen dispersal and receipt

To investigate the presence of *Ephedra* pollen in the air around male plants, pollen traps consisting of a microscopic slide covered with Vaseline were placed on a pole 1 m above ground, in each cardinal direction from a male plant at 0, 1, 2, 4, 6, 10 and 16 m. Pollen traps were set up from 07:00 to 19:00 h for 5 days in the periods 13–17 May 2011 and 22–25 May 2012 for *E. distachya* and 1–11 July 2012 for *E. foeminea*. During the selected days the weather was dry, sunny and windy and the majority of individuals in each respective population were at anthesis. Pollen traps were replaced every evening and,



Figure 1. *Ephedra* (Gnetales). A, simplified phylogeny of Gnetales. A shift to wind pollination is thought to have occurred during the early evolution of crown-group *Ephedra*. B, *Ephedra foeminea*; male cone with sterile but pollination-drop-producing ovules (left), and two young female cones with poorly developed micropylar tubes and weak colouring (right) which, nevertheless, produce pollination drops (see further information in Rydin & Bolinder, 2015). C, *Ephedra distachya*; male cones without sterile ovules (left), and female cone displaying pollination drops (right). D, *Ephedra aphylla*; cluster of male cones that lack sterile ovules and pollination drops but display drops of unknown source (nectar?) (left and right).

after transport to Stockholm University, screened carefully under a 20 \times objective using a Leitz Orthoplan microscope. Pollen grains were documented via scanning electron microscopy for morphological characterization and comparison.

To test if pollen dispersal differs between the two species, we used a general linear model with species identity as a fixed factor and distance and the interaction between species and distance as continuous predictors. A significant effect of species would indicate a difference between the two species concerning the amount of pollen dispersed. A significant interaction between species and distance would indicate differences in pollen dispersal ability between species, where shallower slopes correspond to longer dispersal distances. As the response variable we used the mean number of pollen grains at each distance averaged over the four different directions in each trial. To ensure normality, data were log-transformed ($\log(x + 1)$).

To test the extent to which pollen grains are transported by air to a female cone, five pollen traps with Vaseline on each side were placed 1 m apart in a pure stand of female plants (at least 30 m from the nearest male plant); details and dates were as above. The number of pollen grains counted per slide was compared between the two species using a one-way ANOVA.

Pollination drop production and insects as pollen vectors

To document the effect of excluding insects from female cones on embryo production, 30 branches of female plants in each population were randomly chosen and enclosed in bags of different fabrics. The bags were carefully sealed towards the branches using duct tape. To exclude entomophilous pollen, but allow anemophilous pollen access to the cones, two plus two branches in each plant were enclosed in 5 \times 20-cm bags of insect netting types that allow wind-borne pollen to pass through: type A (mesh 1 \times 1 mm) and type B (mesh 0.3 \times 0.3 mm). To investigate if seeds are set by apomixis, an identical set-up was made using bags of a close cotton fabric, netting type C, which completely excludes pollen from the cones. As a control, branches were marked and left alone with no bagging or any other treatment. Towards the end of the season all cones on selected branches were harvested. To assess fertilization success, seeds were checked for the presence of embryos with a dissecting microscope. The presence/absence of embryos under the different treatments was compared with Fisher's exact test.

To document pollination drop production and potential pollinators, observations of female and male cones at anthesis were conducted at randomly

chosen plants of each population. Male and female cones were observed during 10-min intervals at all hours of the day and night. At night-time, observations were made using a torch, weak red light, an ultraviolet camera and the moonlight alone (if any). Potential pollinators were photographed on site and captured, placed in a container with paper soaked in diethyl ether and transferred individually to Petri dishes for further study and identification.

Ants are considered rare as pollinators, among the reasons being the fact that ant secretions are thought to significantly reduce pollen viability and germination (Beattie, 1982, 2006; Beattie *et al.*, 1984). We therefore decided to make a first assessment of pollen viability in *E. foeminea* subsequent to contact with ants from the field locality. Anthetic male cones of *E. foeminea* were collected and placed in small containers together with ants from the field locality (about ten cones and three or four ants of one of the four detected species per container). In addition to the four containers with ants, one container with cones was left without ants and used as a control. The ants were kept in the containers for 24 h and then put in 70% ethanol for subsequent identification. Next, pollen from each container, including the control, was placed in Petri dishes containing germination medium prepared as described by Brewbaker & Kwack (1963). The Petri dishes were kept at room temperature. The germination process was monitored regularly using a dissection microscope, every hour for the first 8 h, and then every second hour until the experiment was terminated at 50 h when development had completely ceased.

RESULTS

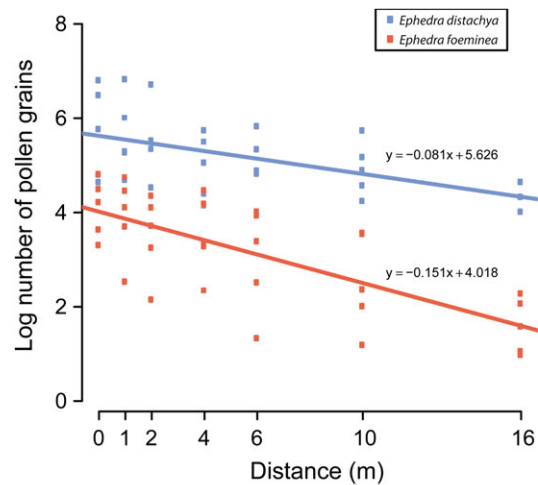
POLLINATION BIOLOGY

Pollen dispersal and receipt

Pollen trap experiments showed that pollen grains of *E. distachya* were more abundant at all distances and dispersed farther from the male cones than pollen grains of *E. foeminea* (Species: $F_{1,66} = 132.0$, $P < 0.0001$; Distance: $F_{1,66} = 49.7$, $P < 0.0001$; Species \times Distance: $F_{1,66} = 4.6$, $P = 0.04$, Fig. 2A). Experiments near female plants showed that more wind-dispersed conspecific pollen reaches individuals of *E. distachya* than individuals of *E. foeminea* ($F_{1,46} = 28.6$, $P < 0.001$, Fig. 2B). Pollen grains of *E. distachya* were usually found as singletons in the pollen traps, whereas grains of *E. foeminea* often were found in clumps (Fig. 3).

Female cones of *E. foeminea* were fertilized to significantly different degrees dependent on the bagging treatment (Fisher's exact test: $P < 0.001$). Female

A Decrease in number of pollen grains with distance



B Pollen grains that reach the ovulate plants by wind

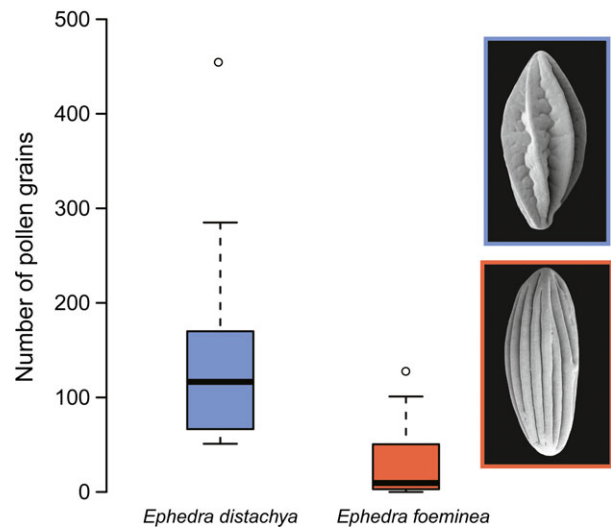


Figure 2. Pollen grains of *Ephedra distachya* are air-borne to a greater extent than pollen grains of *Ephedra foeminea*. A, number of pollen grains in the traps decreases with distance from the male plant. The decrease is significantly more pronounced for *E. foeminea* (red) than for *E. distachya* (blue). B, significantly more pollen grains of *E. distachya* (blue) reach the female plant than do pollen grains of *E. foeminea* (red). Box-and-whisker plots show number of pollen grains with upper and lower box bounds representing 75th and 25th percentiles, respectively. The median is represented by the black line within the box and outliers are shown as circles.

cones enclosed in the two types of bags excluding insects were fertilized in 5.0% (type A, $N = 80$) and 5.6% (type B, $N = 89$) of the cases. Cones enclosed in bags blocking access of both wind- and insect-borne pollen (type C) were never fertilized ($N = 87$) Fig. 4.



Figure 3. Tendency of pollen grains to form clumps. A, *Ephedra foeminea* pollen grains were sticky, adhered to each other and were often found in clumps; B, by contrast, pollen grains of *Ephedra distachya* were usually found as singletons in the pollen traps. The mechanism behind the stickiness and tendency to form clumps in *E. foeminea* is not understood. Scale bars: 100 μ m.

Of the cones without bags, 95.2% ($N = 84$) were fertilized. There was no statistical difference between cones enclosed in bag types A and B. These treatments were therefore combined and contrasted against the control to investigate the effect of insect pollination. For *E. distachya*, no obvious difference in embryo production among different bagging treatments was observed. Seeds of *E. distachya* were, however, often poorly developed (possibly because of severe drought), and 22% of the seeds were infested with larvae of several different species of parasitic wasps (Chalcidoidea, Hymenoptera). The embryo counts for *E. distachya* are therefore disregarded although the results do not contradict other results of the present study.

Pollination drop production and insects as pollen vectors

Pollination drops were produced around the clock. If collected by an insect, they were sometimes regenerated within 20–30 min, but not always. Our interpretation is that they are not regenerated if pollination

has occurred, but we have not explicitly tested this. Pollen was also released around the clock during anthesis. In *E. foeminea*, pollination drop production started abruptly in association with the full moon of July and continued for about 10 days. A second pollination phase occurred in August, but with fewer cones involved. By contrast, *E. distachya* was always at anthesis during the same dates in late May in every year of the experiment. Different populations of *E. distachya* entered the pollination phase during slightly different dates, and these differences were constant among years and appeared to be correlated with micro-climatic variations (e.g. wind exposure).

Ephedra foeminea: Daytime observations revealed visits of several potential pollinators and additional insects not interpreted as pollinators (Table 2). The female cones are salmon-red and the male cones show bright yellow microsporangia (Fig. 5A, C–E). No scent was detected. A member of Syrphidae (Diptera; *Paragus quadrifasciatus*, Fig. 5A, B), was observed on both male and female cones, actively seeking and consuming the pollination drops. During its visits to male cones, it was clear that it focused on the pollination drops, not the pollen. It even appeared as if contact between its mouthparts and the anthers was avoided. Individuals collected on female cones had pollen on their abdomens (Fig. 5B). Two additional members of Diptera (belonging to Ceratopogonidae, Fig. 5C, and Brachycera, respectively) were observed visiting both male and female cones. We were not able to catch or photograph any individuals of Brachycera. In addition, we observed four species of Formicidae (Hymenoptera): *Aphaenogaster* sp. (subfamily Myrmicinae), *Camponotus* sp. (subfamily Formicinae), *Cataglyphis* sp. (subfamily Formicinae) and one unidentified genus. The ants walked systematically from cone to cone to collect pollination drops on both male and female plants (Fig. 6A), but appeared in addition to be interested in pollen. Visits by other insects, e.g. pollen-collecting bees, occurred rarely (Table 2). However, in the second pollination peak in August, visits by parasitic wasps (Chalcidoidea, Hymenoptera) were frequently observed (Fig. 5D). As they mainly visit cones past the pollination phase they are not necessarily pollinators, even though their (relatively infrequent) visits also to pollen-receptive female cones and male cones may indicate that they contribute to pollination to some extent.

Night-time observations of *E. foeminea* also revealed visits by several potential pollinators (Table 3). As during the daytime, no scent was detected. Instead, we were amazed by the spectacular display produced by the glittering of the many pollination drops in the full moonlight, and we

Table 2. Number of insect visitations during the diurnal observation sessions ($N = 57 \times 10$ min) of male and female cones of *Ephedra foeminea* and *E. distachya*

Insect order: subgroup	<i>E. foeminea</i> female	<i>E. foeminea</i> male	<i>E. distachya</i> female	<i>E. distachya</i> male
Hymenoptera: Chalcidoidea	68	16	19	0
Hymenoptera: Formicidae*	460	437	31	8
Diptera: Brachycera*	6	10	0	0
Coleoptera: Mordellidae	1	10	0	0
Other Coleoptera	1	10	0	0
Coleoptera: Dermestidae	0	0	22	7
Diptera: Syrphidae (<i>Paragus quadrifasciatus</i>)*	2	9	0	0
Diptera: Muscidae	1	1	0	0
Diptera: Ceratopogonidae*	Numerous	Numerous	0	0
Hymenoptera: Vespoidae	0	1	0	0

*Hypothesized pollinator of *E. foeminea* (see the text for details).

believe this phenomenon constitutes the nocturnal means of attraction. A moth (*Cnephasia* sp., Lepidoptera, Fig. 5E, F) was frequently observed on male cones. It flew from cone to cone, landed on the distal end of the cone and consumed the pollination drop with its proboscis, and individuals caught had pollen on their bodies. These individuals were, however, caught on male plants; only on one occasion was this moth observed on a female cone and we did not manage to catch the individual. On a few occasions, we also observed visits by larger moths of the Geometridae (Lepidoptera) and small flies (Sciaridae, Diptera, Fig. 5G, H) on both female and male cones. The moths visited cones high up in the shrubs and details of their behaviour were difficult to observe in the darkness, but they clearly flew from cone to cone. The flies were observed in large numbers. They were foraging on the pollination drops but did not show any interest in the pollen. Ants (Formicidae, Hymenoptera) were frequently observed also during the night. All putative pollinators caught on female cones (*Paragus quadrifasciatus*, Formicidae, Sciaridae and *Cnephasia* sp.) had *E. foeminea* pollen on their bodies (Fig. 5).

Ephedra distachya: No putative pollinators were observed. No insects were observed visiting both female and male cones. Ants and parasitic wasps were observed but only on female plants. Visitations by other insects were recorded, but only rarely and only during the daytime (Table 2). At night, no insect visitations were recorded for *E. distachya* (Table 3).

Effect of ants on pollen germination

In all Petri dishes, including the control dish, pollen had started to germinate after 31 h in the

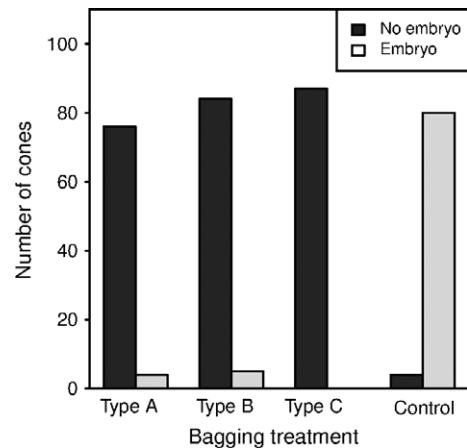


Figure 4. Embryo formation in cones of *Ephedra foeminea* under different treatments. There are significantly more embryos produced in *E. foeminea* cones when both wind- and insect-borne pollen has access to the ovulate cones (control) than when insects are excluded (types A, B and C). When cones were enclosed in bags that exclude insects, but wind-borne pollen is capable of reaching the plant, only 5.1% ($N = 80$; type A; mesh 1×1 mm) and 5.9% ($N = 89$; type B; mesh 0.3×0.3 mm) of the seeds were fertilized. When cones were enclosed in bags of fabric that blocks access of both wind- and insect-borne pollen (type C), 0% ($N = 87$) of the ovules contained embryos. For the control group, where both wind- and insect-borne pollen have access to the plant, 95.9% ($N = 84$) of the cones were fertilized.

germination medium, albeit more slowly and at a lower frequency in the control dish compared with the others. After 45 h, pollen grains in all Petri dishes had shed their exines and the gametophytes had started to develop (Fig. 6B). After 50 h

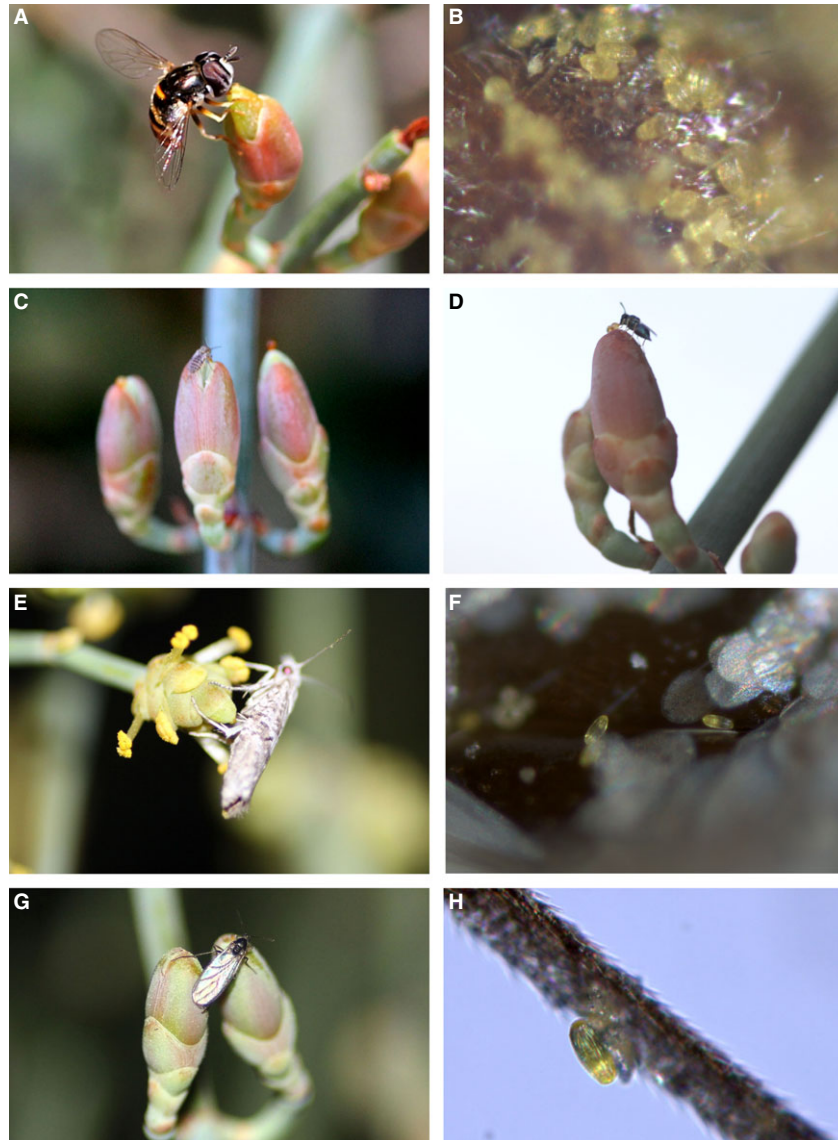


Figure 5. Pollinators and other visitors of *Ephedra foeminea*. A–D, diurnal pollinators and other visitors; E–H, nocturnal pollinators. A, *Paragus quadrifasciatus* (Syrphidae, Diptera), pollinator visiting a female cone. B, pollen present on the ventral side of the abdomen of *Paragus quadrifasciatus* collected on a female cone. C, insect of Ceratopogonidae (Diptera), pollinator visiting a female cone. D, insect of Chalcidoidea (Hymenoptera) visiting a female cone. Chalcidoidea are parasitic wasps and are not interpreted as pollinators. E, *Cnephasia* sp. (Lepidoptera) visiting a male cone. F, pollen present on the abdomen of *Cnephasia* sp. (Lepidoptera) collected on a male cone. G, insect of Sciaridae (Diptera), pollinator visiting a female cone. H, pollen present on the leg of an insect of Sciaridae collected on a female cone.

the germination process had ceased in all Petri dishes, probably due to the presence of fungal hyphae. The amount of fungal hyphae was higher in the control dish than in the dishes with pollen that had been in contact with ants. The pollen in the control dish had a lower germination success (i.e. fewer pollen germinated) than the pollen that had been exposed to ants, where almost all pollen germinated.

DISCUSSION

POLLINATION BIOLOGY

Extant *Ephedra* is a small, uniform clade, in which morphological and genetic divergence is low (Ickert-Bond, 2003; Ickert-Bond & Wojciechowski, 2004; Rydin & Korall, 2009; Rydin *et al.*, 2010; Ickert-Bond & Rydin, 2011). Remarkably, therefore, our study provides strong mechanistic evidence for the exis-

tence of two pollination mechanisms among living species: entomophily in *E. foeminea* and anemophily in *E. distachya*. We demonstrate several distinct differences between these two species in features related to their pollination biology: number of pollen grains in the air around male and female plants and dispersal distances of pollen (Fig. 2); the tendency of pollen grains to form clumps (Fig. 3); and the nature and frequency of insect visitation (Tables 2, 3). Diurnal and nocturnal pollinators belonging to Lepidoptera, Diptera and probably also Hymenoptera, showing the distinctive behaviour of a foraging pollinator (Levin, 1979), were observed visiting both male and female cones of *E. foeminea*, suggesting that several insect species from distantly related groups are pollinators of this species. For *E. distachya*, no insects were observed visiting both male and female cones.

Based on these results, and the low degree of fertilization in *E. foeminea* when insects are excluded from the cones (Fig. 4), we conclude that insects are the main pollen vectors in *E. foeminea*, whereas wind is the main mode of pollen transfer in *E. distachya*. These conclusions are in agreement with Jaccard's (1894) opinion regarding *E. distachya* subsp. *helvetica* and Porsch's (1910) observations of *E. foeminea*, but differ from Mousset's (1980) conclusions regarding *E. distachya*. The pollination system of *E. foeminea* can be characterized as a generalist entomophilous system. Insect visits are, however, not frequent and a correlation between the initiation of the pollination phase and the full moon has been demonstrated and suggested to have evolved to maximize the efficiency of pollination in this species (Rydin & Bolinder, 2015). Many nocturnal insects use the moon to navigate efficiently (Danthanarayana, 1986; Dacke *et al.*, 2003) and only during full moon is the moon visible all night.

Five per cent of the ovules of *E. foeminea* were also fertilized when insects were prevented from accessing the cones. It is possible that some insects managed to deliver pollen despite the covering bag, but it is also possible that wind is responsible for a

small fraction of the pollen transfer in *E. foeminea*. A small amount of pollen in *E. foeminea* appears to actually travel far enough by wind to reach a female plant (Fig. 2B). However, although the morphology of female cones of studied wind-pollinated *Ephedra* spp. (*E. trifurca* and *E. nevadensis*) is aerodynamically adapted to efficiently capture wind-borne pollen and direct it to the micropylar opening (Niklas & Kerchner, 1986; Niklas *et al.*, 1986; Niklas & Buchmann, 1987; Buchmann *et al.*, 1989; Niklas, 2015), the opposite has recently been demonstrated for *E. foeminea* (Bolinder, Niklas & Rydin, 2015a). To fertilize the ovule successfully the pollen needs not only to reach the female plant by air, it also needs to enter the pollination drop. In *E. foeminea*, pollen does not enter the pollination drop unless the pollen grain accidentally settles just by the drop (Bolinder *et al.*, 2015a). It is therefore clear that wind pollination is highly inefficient in *E. foeminea* (Bolinder *et al.*, 2015a) and can only play a peripheral role in the pollination biology of this species.

INSECT VISITATION AND REPRODUCTIVE SUCCESS

Previous studies of the pollination biology in *Ephedra* mention involvement of diurnal dipterans and hymenopterans (Porsch, 1910; Bino *et al.*, 1984a; Meeuse *et al.*, 1990). Our observations revealed a range of diurnal and nocturnal pollinators in *E. foeminea* (Fig. 5) (but none in *E. distachya*; Tables 2, 3). Pollinators were typically observed on both male and female cones and all pollinators caught on female cones had *E. foeminea* pollen on their bodies (see examples in Fig. 4). Pollinator visits were, however, infrequent during the day and night and several pollinators, for example Sciaridae, were observed only during a single night. We agree with Porsch's (1910) conclusion that an extended observation period would no doubt lead to detection of additional kinds of pollinators.

All insects interpreted as pollinators systematically moved from cone to cone and were clearly consuming the pollination drops. In addition, we observed several kinds of hymenopterans and coleopterans during

Table 3. Number of insect visitations during the nocturnal observation sessions ($N = 54 \times 10$ min) of male and female cones of *Ephedra foeminea* and *E. distachya*

Insect group	<i>E. foeminea</i> female	<i>E. foeminea</i> male	<i>E. distachya</i> female	<i>E. distachya</i> male
Hymenoptera: Formicidae*	290	395	0	0
Lepidoptera: Geometridae*	13	8 (+ 2 larvae)	0	0
Diptera: Sciaridae*	8	8	0	0
Lepidoptera, <i>Cnephasia</i> sp.*	1	8	0	0

*Hypothesized pollinator of *E. foeminea* (see the text for details).

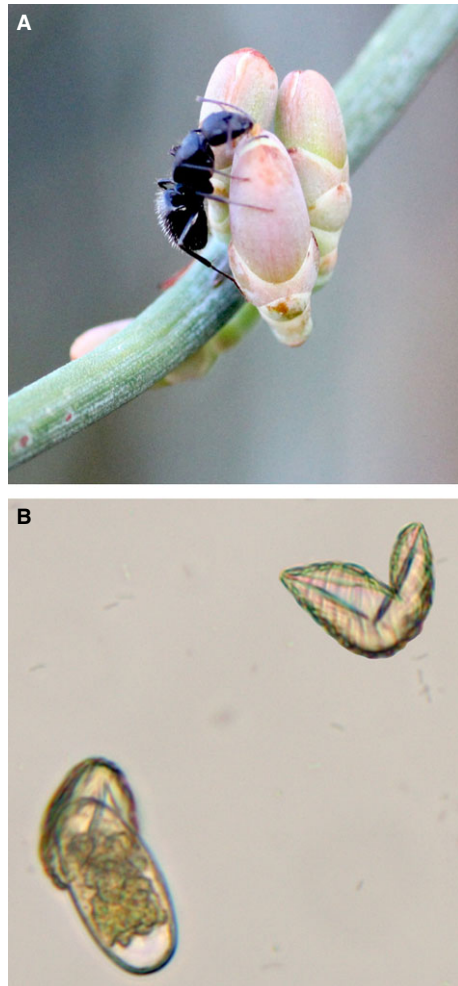


Figure 6. Ants are interpreted as pollinators of *Ephedra foeminea* as they are by far the most frequent visitors of both male and female cones (Table 2) and because our pilot study shows that local ants facilitate the germination rate of pollen grains. A, *Camponotus* sp. (Formicidae, Hymenoptera) visiting a female cone, feeding on the pollination drop. B, germinating pollen grains. A naked gametophyte (lower left), and a shed exine (upper right), derived from pollen grains that had been exposed to ants of the genus *Camponotus* (see text for details). Pollen grains germinated equally efficiently also after having been exposed to ants of *Aphaenogaster* and *Cataglyphis* and one unidentified genus (Formicidae, Hymenoptera).

our diurnal observation sessions (Table 2), but their visits were typically rare, brief and not targeting the pollination drops. With the exception of ants, we do not consider any of the hymenopterans and coleopterans to be pollinators. Among the visiting hymenopterans were parasitic wasps of Chalcidoidea that lay eggs in cones of both *Ephedra* spp. studied here (Fig. 7). For *E. foeminea* they were mostly seen



Figure 7. Parasitic wasps of Chalcidoidea (Hymenoptera) lay eggs in female cones of (A) *Ephedra distachya* during its pollination phase in May and (B) *E. foeminea* during the second peak of pollination drop production in August (see text for details).

in August and although some of them infested premature cones not yet receptive to pollen, others infested already fertilized cones. We conclude that their contribution as pollinators would be highly inefficient.

Previous observations (Moussel, 1980; Askew & Blasco-Zumeta, 1997) indicating that parasitism by chalcidoideans may be common in European populations of *E. distachya* are supported by our observations; 22.0% of the seeds of *E. distachya* were infested with parasitic larvae (Fig. 8). It is thus possible that they have a substantially negative effect on reproduction in *E. distachya*. Drought appears to be another severe constraint on sexual reproduction in *E. distachya* in Greece. When mature, the cones of *E. distachya* are red and fleshy

and probably bird-dispersed. During 2011, when our bagging experiments were set up, many seeds did not develop fully and the cones never became fleshy (Fig. 9A). Although pollination and early seed development were successful as assessed by our field observations, later developmental stages that take place in July and August appeared to be suppressed by drought and/or parasitic wasps. The same was true in 2012 (based on field observations of cone development) and we believe this suppression of sexual reproduction may occur in most years in Greece. In 2014, however, April and June were unusually wet (Rydin & Bolinder, 2015) and cone development in *E. distachya* was strikingly different, with mature and brightly red cones being common in July at all our field sites (Fig. 9B). We believe that sexual reproduction of *E. distachya* in Greece varies between years, with sexual reproduction being more commonly successful in years with high summer precipitation. These observations are in sharp contrast to those found for *E. foeminea*, which appears to successfully reproduce sexually each year. Our conclusions go in concert with the fact that *E. distachya* is rare in Greece. In contrast to *E. foeminea*, which is common and apparently phylogenetically native to the Mediterranean area, *E. distachya* (a member of the Asian clade) has its westernmost outposts in southern Europe and may be less well adapted to the Mediterranean climate.

ARE ANTS POLLINATORS OF *EPHEDRA FOEMINEA*?

Ants (Formicidae, Hymenoptera) were frequently observed visiting both male and female cones of *E. foeminea* and female cones of *E. distachya* (visits to male cones of *E. distachya* were only rarely observed; Table 2). In *E. foeminea*, ants were seen on the plants during both the day and the night, feeding on the pollination drops of male and female cones and on pollen of male cones. The ants systematically walked from cone to cone to collect pollination drops and one of the individuals caught on female plants had a few pollen grains on its ventral abdomen. It is thus probable that some pollen transfer occurs with ants as vectors.

Ants are, however, considered rare as pollinators. This is partly because secretions of the ants are thought to significantly reduce pollen viability and germination (Beattie, 1982, 2006; Beattie *et al.*, 1984), but the matter is clearly understudied, in particular for ants with non-specialized biology (Beattie, 2006; Yek & Mueller, 2011). Metapleural glands are unique to ants, but the feature has been lost repeatedly and is missing in c. 20% of the species (Yek & Mueller, 2011). The chemistry of the secretions from the metapleural glands differs significantly among

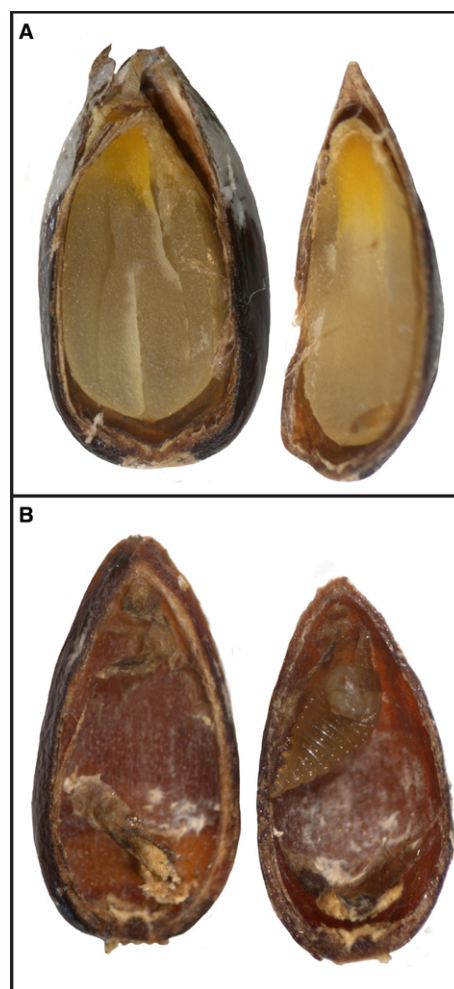


Figure 8. Seeds of *Ephedra distachya* (A) with normal embryo development and (B) infested with a parasitic larva.

subgroups, although they are all acidic (Yek & Mueller, 2011). Like many other social insects ants also have other kinds of exocrine glands, and ants of the subfamily Formicinae have poison glands that produce formic acid. Formic acid is known to affect biological processes and antibiotic properties have been documented against yeast, bacteria and fungi (Vieira *et al.*, 2012; Fernández-Marín *et al.*, 2015). It typically targets early stages of infection and is even shown to negatively affect non-germinated spores and pollen grains by preventing them from remaining viable (Beattie *et al.*, 1986; Veal, Trimble & Beattie, 1992; Bot *et al.*, 2002; Yek & Mueller, 2011). This destruction of pollen has been one of the main arguments against ants as effective pollinators (Beattie *et al.*, 1984; Beattie, 2006). However, ant pollination does occur, even by ants that possess metapleural and formic acid secretions (Beattie, 2006; Yek & Mueller, 2011). It is thus unclear to

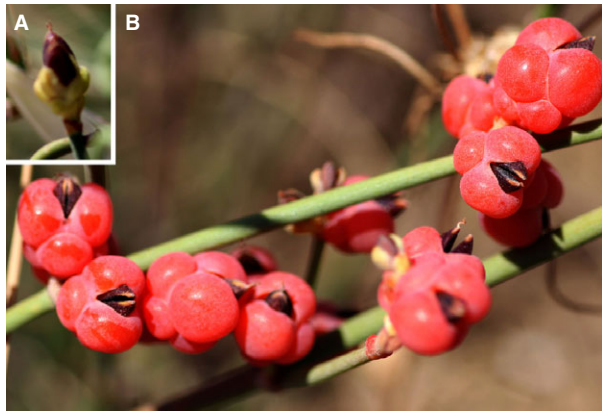


Figure 9. Mature cones of *Ephedra distachya*. A, cone at maturity in July during a normal, dry year (2011). B, during an unusually wet year (2014) the cone bracts become fleshy and red when mature; see text for details.

what extent metapleural secretions and formic acid can be claimed to generally reduce pollen viability. Yek & Mueller (2011) concluded that, although the subject needs more research, ants with functional metapleural glands can also be effective pollinators.

The ants we collected on *Ephedra* cones at our field sites are members of *Camponotus* (carpenter ants) and *Cataglyphis* (desert ants), both of the formic-acid-producing subfamily Formicinae, and *Aphaenogaster* (funnel ants) of Myrmicinae, which do not produce formic acid. Whereas all species of *Camponotus* (Yek & Mueller, 2011) lack metapleural glands, *Cataglyphis* has them (Keegans *et al.*, 1992). In *Aphaenogaster*, some species have metapleural glands, while others do not (Yek & Mueller, 2011). Further details about the identity, morphology and physiology of the ants we caught, and their association with *E. foeminea*, have to await substantial zoological and ecological investigations; however, our pilot study shows that even though the local ants we collected belong to different subfamilies that have different secretory systems, they all had the same positive effect on pollen germination in *E. foeminea*. Species of both *Aphaenogaster* and *Camponotus* have previously been shown to reduce pollen viability in certain angiosperms (Beattie *et al.*, 1984, 1985), but our experiments show that their presence enhances pollen viability in *E. foeminea* rather than suppressing it. Pollen that had been in contact with ants had a higher germination rate compared with the control and the amount of fungal hyphae was lower when pollen had been in contact with ants.

It is thus possible that the lower germination success of pollen in the control is explained by absence of natural fungicides provided by the ants. This is potentially relevant because the pollination drops of *Ephedra* (in contrast to other gymnosperms) contain

negligible amounts of defence proteins against pathogens and fungal hyphae (von Aderkas *et al.*, 2015). A high sugar concentration has been suggested to compensate for the absence of defence proteins by creating an osmotic pressure that prevents fungal and bacterial growth and germination of foreign pollen (von Aderkas *et al.*, 2015). The standard germination medium used in our experiments contains 20% sucrose (Brewbaker & Kwack, 1963), which is less than the reported sugar content of pollination drops in *Ephedra* (Ziegler, 1959; Bino *et al.*, 1984b). Future studies that experiment with germination media with different sugar concentrations should be conducted. A higher percentage of sugar in the medium, more comparable to that of natural conditions, would perhaps prevent fungal growth with an increased pollen germination rate as a consequence. However, it is also possible that the presence of ants (i.e. their antibiotic secretions) improves pollen germination rates in *Ephedra*. Based on current knowledge, it cannot be ruled out that ants play an important role for pollination in *E. foeminea*, perhaps as both anti-fungal agents and pollen vectors.

POLLINATOR ATTRACTION AND REWARD

Pollinator attraction in *E. foeminea* appears entirely visual; we have not detected any scent or other means of non-visual attraction. Diurnal insects are probably attracted by the colourful cones and nocturnal insects by the reflection of moonlight in the many pollination drops. Pollination drops are secreted from the nucellus (Takaso, 1990; Owens *et al.*, 1998) and are therefore only present in female structures, specifically the ovules. In *Ephedra*, only *E. foeminea* has retained the putatively ancestral state in Gnetales with sterile, pollination-drop-producing ovules also in male cones (ongoing studies and Jørgensen & Rydin, 2015). *Ephedra foeminea* is thus the only *Ephedra* sp. that can use pollination drops as reward for pollinators in both female and male plants. The pollination drop is an ancestral feature in seed plants, presumably documented even for some of the earliest, now extinct taxa from the Late Devonian (Rothwell & Scheckler, 1988). Pollination drops are also present in most extant gymnosperms, in which they function primarily as a medium for pollen capture, transportation and germination (Owens *et al.*, 1998; Gelbart & von Aderkas, 2002), although an additional function of pollination drops as a pollinator reward may have been established early in the evolutionary history of seed plants (Doyle, 1945). Pollination drops differ markedly among gymnosperm groups in sugar concentration and composition (Nepi *et al.*, 2009). The sugar concentration in the pollination drops of *Pinus* L. is, for example, only 1–2%

(McWilliam, 1958), whereas *c.* 25% or much more has been reported for *Ephedra* (Ziegler, 1959; Bino *et al.*, 1984b). Similarly, pollination drops of investigated gymnosperms typically contain high amounts of fructose (Gelbart & von Aderkas, 2002; Nepi *et al.*, 2009), which is essential for pollen germination (Nepi *et al.*, 2009). In pollination drops of *Ephedra* the dominant sugar is instead sucrose (Ziegler, 1959), often associated with insect pollination (Baker & Baker, 1983; Nepi *et al.*, 2009).

Insect pollination has previously been suggested for one additional species of *Ephedra*, *E. aphylla* (Bino *et al.*, 1984a, b; Meeuse *et al.*, 1990), which like *E. foeminea* is among the early-diverging species (Fig. 1). The results and conclusions of these studies are somewhat ambiguous and abiotic pollination is also claimed to be important (Bino *et al.*, 1984a, b; Meeuse *et al.*, 1990). Furthermore, the mode by which this species could attract pollinators to male cones remains unclear. Bino *et al.* (1984b) suggested that extrafloral nectaries ('hydathode-like' pores, Bino *et al.*, 1984b) are present in the cone bracts of *E. aphylla*, serving as pollinator attractants, particularly in male plants that lack pollination drops. The structures taken as evidence for this (figures in Bino *et al.*, 1984b) represent, in fact, ordinary (sunken) stomata, but it is possible that nectar is produced from stomata-like pores, as has been shown for some angiosperms (Vogel, 1997). The evolutionary origin of nectar has been hypothesized to be leaking phloem sap (de la Barrera & Nobel, 2004), but little is known about nectar outside the angiosperms. *Ephedra aphylla* and *Gnetum cuspidatum* Blume (Gnetales) are among the few gymnosperms that have been suggested to have nectar (Bino *et al.*, 1984b; Kato, Inoue & Nagamitsu, 1995). Male plants of *E. aphylla* sometimes display droplets of unknown origin on sterile parts of the inflorescence (Fig. 1D) and these drops may represent nectar. The presence of extraovular nectar in *G. cuspidatum* has, however, recently been questioned (Jørgensen & Rydin, 2015) and the nature and origin of the liquid observed on male cones of *E. aphylla* need more research, as do other aspects of the pollination biology of this species.

EVOLUTIONARY IMPLICATIONS

All currently available knowledge of pollination biology in Gnetales indicates that insect pollination and bisexual cones, although of different organization, are ancestral characters in the order (ongoing studies and Jørgensen & Rydin, 2015). Cones of *Gnetum* L. have long been reported to be visited by various insects (van der Pijl, 1953), and observations by Kato *et al.* (1995) suggested that *G. gnemon* L. is pollinated by nocturnal moths and

G. cuspidatum by dipterans. *Welwitschia* Hook.f. has repeatedly been described as insect-pollinated (Hooker, 1863; Baines, 1864; Pearson, 1907; Wetschnig & Depish, 1999), with dipterans, hemipterans and hymenopterans suggested as pollinators (Pearson, 1907; Wetschnig & Depish, 1999). For *Ephedra*, however, pollination mechanisms have been poorly understood (Endress, 1996; Gorelick, 2001). In this study, we show that the sister of the remaining members of the genus (*E. foeminea*; Rydin & Korall, 2009; Thureborn & Rydin, 2015) is insect-pollinated, whereas all currently available data (see Table 1, and results of the present study) indicate that wind pollination is the prevailing state in core *Ephedra*. Placed in a temporal, phylogenetic context our findings suggest that an evolutionary shift from insect pollination to wind pollination occurred in *Ephedra* prior to diversification of the core clade (Fig. 1). As mentioned in the Introduction, Gnetales have sometimes been viewed as an intermediate stage, a 'stepping stone', between 'primitive anemophily' in gymnosperms and 'advanced entomophily' of angiosperms (e.g. Bino *et al.*, 1984a: p. 10). However, the evolutionary trend in *Ephedra* (if any) is the opposite: from entomophily to anemophily.

Cretaceous members of *Ephedra* may thus have been entomophilous. The fossil record of Gnetales is rich, but assessing the pollination biology of extinct species is generally difficult (Friis, Crane & Pedersen, 2011). As in *Ephedra*, all other indisputably insect-pollinated species of Gnetales have morphologically bisexual male cones and are able to attract pollinators also to male plants using pollination drops. In contrast, species known to be wind-pollinated have exclusively unisexual cones. The morphology of male cones (bisexual or unisexual) could therefore be used for indirect inference of the pollination biology. However, based on currently published interpretations (Bino *et al.*, 1984a, b; Meeuse *et al.*, 1990), *E. aphylla* (as an at least partly insect-pollinated species that lacks sterile ovules in the male cones) constitutes an exception. Furthermore, staminate structures are fragile and short-lived and rarely preserved in fossils. We are not aware of any ephedroid fossil that can safely be assigned to the ephedran lineage with male cones of sufficient preservation to be used for indirect inference of the pollination biology. There are, however, other features that may indicate entomophily in extinct members of *Ephedra* and Gnetales as a whole. Thickening and/or ovular support of the micropylar tube (Rydin *et al.*, 2010) appears particularly pronounced in certain fossil taxa and some early-diverging extant lineages of *Ephedra* (Rydin *et al.*, 2006a, 2010; Rydin & Friis, 2010). This feature may represent an early adaptation

to insect pollination by preventing the micropylar tube from breaking or bending during insect visitation, an adaptation that has subsequently been lost in wind-pollinated species (see also Rydin *et al.*, 2010).

Furthermore, pollen morphology and ultrastructure indicate that at least some of the Cretaceous ephedroid pollen may have been produced by entomophilous plants (Bolinder *et al.*, 2015a, b). *Ephedra* pollen occurs in two major types. The ancestral type lacks branched pseudosulci and has a more dense ultrastructure (Bolinder *et al.*, 2015a, b). The derived type has branched pseudosulci and a more spacious ultrastructure (Bolinder *et al.*, 2015a, b). Among living plants, the ancestral pollen type is mostly found in early diverging lineages and is represented among both entomophilous and anemophilous taxa. The derived pollen type probably evolved as an adaptation to wind pollination and is today only found in the core clade of anemophilous *Ephedra* spp. In the fossil record, pollen of the type that is ancestral in *Ephedra* is first documented in the Permian (Wilson, 1959; Wang, 2004), became common in the Early Cretaceous and declined dramatically during the Late Cretaceous (Crane & Lidgard, 1989). The derived pollen type appears much later and is first documented from the Late Cretaceous (Steeves & Barghoorn, 1959; Norbäck Ivarsson, 2014). Based on this new information, Bolinder *et al.* (2015b) questioned current dating of the core clade and suggested a much older age than the previously estimated median age of *c.* 30 Ma (Ickert-Bond *et al.*, 2009).

In the Cenozoic, the ancestral pollen type becomes almost completely replaced by a strongly increasing amount of the derived pollen type in palaeopalynological assemblages (Bolinder *et al.*, 2015a; Han *et al.*, 2015). We therefore hypothesize that a shift to wind pollination promoted the diversification of the core clade of *Ephedra*. Sister clade comparisons (Slowinski & Guyer, 1993) show that the probability that the observed imbalance between the indisputably insect-pollinated *E. foeminea* and remaining *Ephedra* has been generated by stochasticity alone is low ($\chi^2 = 8.22$, d.f. = 2, $P = 0.02$). Taking into account the possibility that *E. aphylla* is (at least partly) insect-pollinated as well (Bino *et al.*, 1984a, b; Meeuse *et al.*, 1990) results in the same conclusion ($\chi^2 = 6.83$, d.f. = 2, $P = 0.03$). The phylogenetic structure and distribution of pollination syndrome therefore support our hypothesis.

CONCLUDING REMARKS

In angiosperms, pollinator-driven speciation is thought to be one of the primary mechanisms

promoting diversification (Grant, 1949; Crepet, 1979; van der Niet & Johnson, 2012), but transitions to wind pollination have occurred repeatedly also in species-rich clades such as Poales and Fagales (Linder, 1998; Gorelick, 2001). Here we provide an analogous example from gymnosperms. Plant–insect interactions were most probably important for evolution in Gnetales in earlier geological times, but few insect-pollinated *Ephedra* spp. have persisted to the present. Furthermore, ongoing studies based on molecular phylogenetic analyses (A. M. Humphreys & C. Rydin, unpubl. data) indicate a negative net diversification rate in the early-diverging (insect-pollinated) lineages and a positive net diversification in the core (wind-pollinated) clade of *Ephedra*. Although *Ephedra* has not fully recovered from the Mesozoic–Palaeogene extinction(s), wind pollination may have facilitated reproductive assurance in *Ephedra* during the climatically unstable (Zachos *et al.*, 1993, 2001) times of the Palaeogene and afforded the group a means by which to diversify again.

ACKNOWLEDGEMENTS

We thank James A. Doyle, Jakub Těšitel and one anonymous reviewer for constructive comments on the text. We also thank Arne Strid (prof. emer.) for locality maps and specimen lists, Richard Askew (prof. emer.), Peter Halvarsson (Uppsala University), Julia Stigenberg, Bert Gustavsson and Hege Vårdahl (Swedish Museum of Natural History) for insect identifications; Linda Andersson, Julia Ferm (Uppsala University), Lena Norbäck Ivarsson (Södertörn University), Olle Thureborn (Stockholm University), and Markus Karlsson for field and laboratory assistance; Kathleen Pigg (Arizona State University), Birgitta Bremer (the Bergius Botanic Garden, Stockholm) and Jürg Schönenberger (University of Vienna) for comments on earlier drafts of the text, Moshira Hassan (Free University of Berlin) for photographs of *E. aphylla* and the Doulikeridis family for allowing us to conduct research on their property. Funding was provided by the Swedish Research Council, the Faculty of Science, Stockholm University, Magnus Bergvalls Stiftelse (CR), Göransson-Sandviken stipendiefond, Tullbergs stiftelse för biologisk forskning and Stiftelsen Extensus (KB).

REFERENCES

- 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.

- Arroyo M, Primack, R, Armesto, J. 1982.** Community studies in pollination ecology in the high temperate Andes of central Chile. I. Pollination mechanisms and altitudinal variation. *American Journal of Botany* **69**: 82–97.
- Askew RR, Blasco-Zumeta J. 1997.** Parasitic Hymenoptera inhabiting seeds of *Ephedra nebrodensis* 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.
- Baker HG, Baker I. 1983.** Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ, eds. *Handbook of experimental pollination biology.* New York: Van Nostrand Reinhold Company, 117–141.
- de la Barrera E, Nobel PS. 2004.** Nectar: properties, floral aspects, and speculations on origin. *Trends in Plant Science* **9**: 65–69.
- Beattie AJ. 1982.** Ants and gene dispersal in flowering plants. In: Armstrong JA, Powell JM, Richards AJ, eds. *Pollination and evolution.* Royal Botanic Gardens: Kew, 1–8.
- 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, Turnbull C, Hough T, Jobson S, Knox RB. 1985.** The vulnerability of pollen and fungal spores to ant secretions: evidence and some evolutionary implications. *American Journal of Botany* **72**: 606–614.
- Beattie AJ, Turnbull CL, Hough T, Knox RB. 1986.** Antibiotic production: a possible function for the metapleural glands of ants (Hymenoptera: Formicidae). *Annals of the Entomological Society of America* **79**: 448–450.
- Bino RJ, Dafni A, Meeuse ADJ. 1984a.** Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forssk. (= *E. alte* C.A. Mey.), with some notes on *E. campylopoda* C.A. Mey. I. Aspects of the entomophilous syndrome. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen. Series C: Biological and Medical Sciences* **87**:1–13.
- Bino RJ, Devente N, Meeuse ADJ. 1984b.** 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, Niklas KJ, Rydin C. 2015a.** Aerodynamics and pollen ultrastructure in *Ephedra* (Gnetales). *American Journal of Botany* **102**: 457–470.
- Bolinder K, Norbäck Ivarsson L, Humphreys AM, Ickert-Bond SM, Han F, Hoorn C, Rydin C. 2015b.** Pollen morphology of *Ephedra* (Gnetales) and its evolutionary implications. *Grana* (in press).
- Bot ANM, Ortius-Lechner D, Finster K, Maile R, Boomsma JJ. 2002.** Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Sociaux* **49**: 363–370.
- 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.
- Crane PR, Lidgard S. 1989.** Angiosperm diversification and paleolatitudinal gradients in Cretaceous floristic diversity. *Science* **246**: 675–678.
- Crepet WL. 1979.** Insect pollination: a paleontological perspective. *BioScience* **29**: 102–108.
- Dacke M, Nilsson D-E, Scholtz CH, Byrne M, Warrant EJ. 2003.** Animal behaviour: insect orientation to polarized moonlight. *Nature* **424**: 33.
- Danthanarayana W. 1986.** Lunar periodicity of insect flight and migration. In: Danthanarayana W, ed. *Insect flight.* Berlin: Springer Verlag, 88–119.
- Doyle J. 1945.** Developmental lines in pollination mechanisms in the Coniferales. *Scientific Proceedings of the Royal Dublin Society* **24**: 43–62.
- Endress PK. 1996.** Structure and function of female and bisexual organ complexes in Gnetales. *International Journal of Plant Sciences* **157**: 113–125.
- Fernández-Marin H, Nash DR, Higginbotham S, Estrada C, van Zweden JS, d'Ettorre P, Weislo WT, Boomsma JJ. 2015.** Functional role of phenylacetic acid from metapleural gland secretions in controlling fungal pathogens in evolutionarily derived leaf-cutting ants. *Proceedings of the Royal Society of London B: Biological Sciences* **282**: 1–9.
- Freeman D, Klikoff, L, Harper, K. 1976.** Differential resource utilization by the sexes of dioecious plants. *Science* **193**: 597–599.
- Freitag H, Maier-Stolte M. 1989.** The *Ephedra*-species of P. Forsskål: identity and typification. *Taxon* **38**: 545–556.
- Freitag H, Maier-Stolte M. 1994.** Characterization of areas, Ephedraceae. In: Browicz K, ed. *Chorology of trees and shrubs in South-West Asia and adjacent regions.* Kornik: Polish Academy of Sciences, Institute of Dendrology, 5–16.
- Friis EM, Crane PR, Pedersen KR. 2011.** *Early flowers and angiosperm evolution.* New York: Cambridge University Press.
- Fu L, Yu Y, Riedl H. 1999.** Ephedraceae. In: Wu CY, Raven P, eds. *Flora of China.* Beijing: Science Press, 97–101.
- Gelbart G, von Aderkas P. 2002.** Ovular secretions as part of pollination mechanisms in conifers. *Annals of Forest Science* **59**: 345–357.
- Gorelick R. 2001.** Did insect pollination cause increased seed plant diversity? *Biological Journal of the Linnean Society* **74**: 407–427.
- Grant V. 1949.** Pollination systems as isolating mechanisms in angiosperms. *Evolution* **3**: 82–97.
- Han F, Rydin C, Bolinder K, Dupont-Nivet G, Abels HA, Koutsodendris A, Zhang K, Hoorn C. 2015.** Radical

- different steppe composition in Eocene Tibet. *Grana* (in press).
- Hooker JD. 1863.** On *Welwitschia*, a new genus of Gnetales. *Transactions of the Linnean Society of London* **24**: 1–48.
- Ickert-Bond SM. 2003.** *Systematics of New World Ephedra L. (Ephedraceae): integrating morphological and molecular data*. PhD Thesis, Arizona State University.
- Ickert-Bond SM, Rydin C. 2011.** Micromorphology of the seed envelope of *Ephedra* L. (Gnetales) and its relevance for the timing of evolutionary events. *International Journal of Plant Sciences* **172**: 36–48.
- Ickert-Bond SM, Wojciechowski MF. 2004.** Phylogenetic relationships in *Ephedra* (Gnetales): evidence from nuclear and chloroplast DNA sequence data. *Systematic Botany* **29**: 834–849.
- 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.
- Jaccard P. 1894.** Recherches embryologiques sur l'*Ephedra helvetica*. *Bulletin de la Société Vaudoise des Sciences Naturelles* **30**: 46–84.
- Jørgensen 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, Nagamitsu T. 1995.** Pollination biology of *Gnetum* (Gnetales) in a lowland mixed dipterocarp forest in Sarawak. *American Journal of Botany* **82**: 862–868.
- Keegans SJ, Morgan ED, Agosti D, Wehner R. 1992.** What do glands tell us about species? A chemical case study of *Cataglyphis* ants. *Biochemical Systematics and Ecology* **20**: 559–572.
- 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: K, K ed. *The families and genera of vascular plants. I. Pteridophytes and gymnosperms*. Berlin: Springer, 379–382.
- Labandeira CC, Kvacek J, Mostovski MB. 2007.** Pollination drops, pollen, and insect pollination of Mesozoic gymnosperms. *Taxon* **56**: 663–695.
- Levin DA. 1979.** Pollinator behavior and the breeding structure of plant populations. In: Richards AJ, ed. *The pollination of flowers by insects*. New York: Academic Press, 133–150.
- Linder HP. 1998.** Morphology and the evolution of wind pollination. In: Owens SJ, Rudall PJ, eds. *Reproductive biology in systematics, conservation and economic botany*. Kew: Royal Botanic Gardens, 123–153.
- Markgraf F. 1964.** *Ephedra* L. In: Tutin TG, Heywood VH, Burges NA, Valentine DH, Walters SM, eds. *Flora Europaea*. Cambridge: Cambridge University Press, 40.
- McWilliam JR. 1958.** The role of the micropyle in the pollination of *Pinus*. *Botanical Gazette* **120**: 109–117.
- Meuse ADJ, De Meijer AH, Mohr OWP, Wellinga SM. 1990.** Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forssk. (= *E. alte* C.A. Mey.), with some notes on *Ephedra campylopoda* C.A. Mey. III. Further anthecological studies and relative importance of entomophily. *Israel Journal of Botany* **39**: 113–123.
- Moussel B. 1980.** Gouttelette réceptrice du pollen et pollinisation chez l'*Ephedra distachya* L. Observations sur le vivant et en microscopies photonique et électronique. *Revue de Cytologie et de Biologie végétales, Le Botaniste* **3**: 65–89.
- Navarro C, Carrión JS, Munuera M, Prieto AR. 2001.** Cave surface pollen and the palynological potential of karstic cave sediments in palaeoecology *Review of Palaeobotany and Palynology* **117**: 245–265.
- Nepi M, von Aderkas P, Wagner R, Mugnaini S, Coulter A, Pacini E. 2009.** Nectar and pollination drops: how different are they? *Annals of Botany* **104**: 205–219.
- van der Niet T, Johnson SD. 2012.** Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* **27**: 353–361.
- Niklas KJ. 2015.** A biophysical perspective on the pollination biology of *Ephedra nevadensis* and *E. trifurca*. *The Botanical Review* **81**: 28–41.
- Niklas KJ, Buchmann SL. 1987.** Aerodynamics of pollen capture in two sympatric *Ephedra* species. *Evolution* **41**: 104–123.
- Niklas KJ, Kerchner V. 1986.** Aerodynamics of *Ephedra trifurca* II. Computer modelling of pollination efficiencies. *Journal of Mathematical Biology* **73**: 966–979.
- 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.
- Norbäck Ivarsson L. 2014.** *Pollen morphology in Ephedra (Gnetales) and implications for understanding fossil ephedroid pollen from the Tibetan Plateau, using a phylogenetic approach*. MSc Thesis, Stockholm University.
- Owens JN, Takaso T, Runions CJ. 1998.** Pollination in conifers. *Trends in Plant Science* **3**: 479–485.
- Pearson H. 1907.** The living *Welwitschia*. *Nature* **75**: 536–537.
- 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.
- Porsch O. 1910.** *Ephedra campylopoda* C.A. Mey., eine entomophile Gymnosperme. *Berichte der Deutschen Botanischen Gesellschaft* **28**: 404–412.
- Rothwell GW, Scheckler SE. 1988.** Biology of ancestral gymnosperms. In: Beck CB, ed. *Origin and evolution of gymnosperms*. New York: Columbia University Press, 85–134.
- Rydin C, Bolinder K. 2015.** Moonlight pollination in the gymnosperm *Ephedra* (Gnetales). *Biology Letters* **11**: 20140993.
- 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**: 183.

- 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 United States of America* **101**: 16571–16576.
- Rydin C, Pedersen KR, Crane PR, Friis EM. 2006a.** Former diversity of *Ephedra* (Gnetales): evidence from Early Cretaceous seeds from Portugal and North America. *Annals of Botany* **98**: 123–140.
- Rydin C, Wu SQ, Friis EM. 2006b.** *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, 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.
- 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.
- Steeves MW, Barghoorn ES. 1959.** The pollen of *Ephedra*. *Journal of the Arnold Arboretum* **40**: 221–255.
- Strid A. 1996.** *Flora Hellenica bibliography: a critical survey of floristic, taxonomic and phytogeographical literature relevant to the vascular plants of Greece, 1753–1994*. Krakow: W. Szafer Institute of Botany.
- Takaso T. 1990.** ‘Pollination drop’ time at the Arnold Arboretum. *Arnoldia* **50**: 2–7.
- Tang W. 1987.** Insect pollination in the cycad *Zamia pumila* (Zamiaceae). *American Journal of Botany* **74**: 90–99.
- Thureborn O, Rydin C. 2015.** *Phylogeny of Ephedra revisited*. Botany 2015. Edmonton, Alberta, Canada, Abstract ID: 1010.
- Veal D, Trimble JE, Beattie A. 1992.** Antimicrobial properties of secretions from the metapleural glands of *Myrmecia gulosa* (the Australian bull ant). *Journal of Applied Bacteriology* **72**: 188–194.
- Vieira AS, Morgan ED, Drijfhout FP, Camargo-Mathias MI. 2012.** Chemical composition of metapleural gland secretions of fungus-growing and non-fungus-growing ants. *Journal of Chemical Ecology* **38**: 1289–1297.
- Vogel S. 1997.** Remarkable nectaries: structure, ecology, organophyletic perspectives. I. Substitutive nectaries. *Flora: Morphologie, Geobotanik, Oekophysiologie* **192**: 305–333.
- Wang Z. 2004.** A new Permian gnetalean cone as fossil evidence for supporting current molecular phylogeny. *Annals of Botany* **94**: 281–288.
- 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. 1959.** Geological history of the Gnetales. *Oklahoma Geological Notes* **19**: 35–40.
- 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.
- Yek SH, Mueller UG. 2011.** The metapleural gland of ants. *Biological Reviews* **86**: 774–791.
- Zachos JC, Lohmann KC, Walker JCG, Wise SW. 1993.** Abrupt climate change and transient climates during the Paleogene: a marine perspective. *Journal of Geology* **101**: 191–213.
- Zachos JC, Pagani M, Sloan L, Thomas E, Billups K. 2001.** Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* **292**: 686–693.
- Ziegler H. 1959.** Über die Zusammensetzung des ‘Bestäubungstropfens’ und den Mechanismus seiner Sekretion. *Planta* **52**: 587–599.