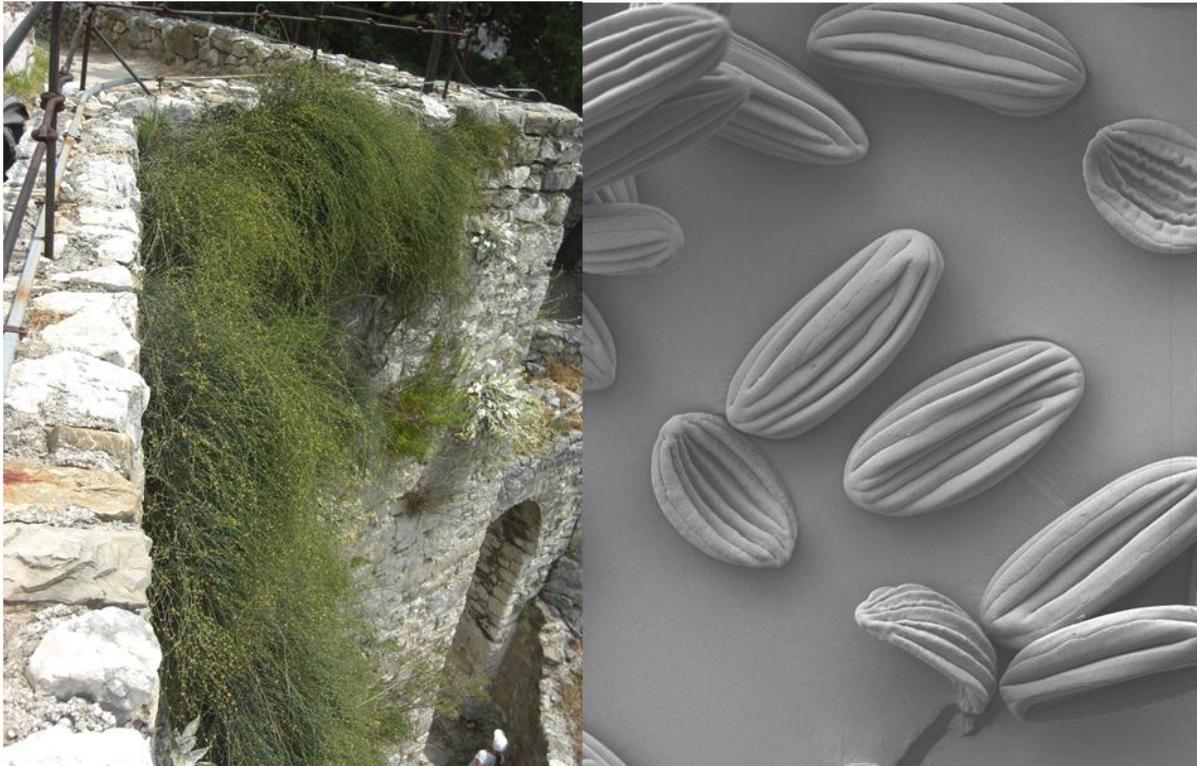


# Pollen morphology in *Ephedra* (Gnetales) and implications for understanding fossil ephedroid pollen from the Tibetan Plateau, using a phylogenetic approach

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## Abstract

Polylicate pollen, commonly referred to as “ephedroids”, is known since the Permian, but the morphology of these dispersed grains and their affinity to the Gnetales have not been rigorously investigated in a phylogenetic context. Here, I investigate pollen morphology in 11 extant species of *Ephedra* (Gnetales) using SEM. The results show that there are at least two types of pollen in *Ephedra*: the *foeminea* type with straight valleys and the *distachya* type with branched valleys. Other features, such as the number of exine ridges and the size of grains, vary considerably within species and overlap between species. Among investigated species, the *foeminea* type of pollen is present in *E. foeminea*, *E. alata*, *E. fragilis*, *E. altissima*, “the Mediterranean” *E. major*, *E. aphylla*, *E. milleri*, *E. foliata*, and *E. ciliata*. The *distachya* type is found in *E. distachya*, *E. major* and *E. viridis*. Ancestral state reconstruction show that the *foeminea* type of pollen is ancestral in *Ephedra*, whereas the *distachya* type evolved later, within the core living clade. This initial study of extant *Ephedra* formed the basis for a subsequent study of fossil pollen from the Tibetan Plateau. In these samples, ephedroid pollen similar to the *foeminea* type or the *Welwitschia* type is found from the Late Jurassic about 160 Ma. From mid-Cretaceous a type of pollen with a checkered morphology is found, however, although these grains are referred to as “ephedroids” and assumed to have been produced by gnetalean plants, the pollen type is not present among living species and I cannot confirm their affinity to the Gnetales. Ephedroid pollen similar to the extant *distachya* type with branched valleys appears much later in the fossil record and is first found in samples with an age of 75-50 Ma. To my knowledge, this is the earliest record of this type of pollen, which otherwise is commonly known from the mid-Eocene and onwards. Pollen morphology in *Ephedra* is promising as a key character for fossil calibration of analyses of divergence times of clades within *Ephedra*. The findings of the present study indicate that the core *Ephedra* clade evolved earlier than previously thought.

**Keywords:** *Ephedra*, ephedroids, fossil pollen, Gnetales, pollen morphology

### Cover:

Left: *Ephedra foeminea* in Omiš, Croatia, July 2013.

Right: SEM image of *Ephedra foeminea* pollen. Photo: Lena Norbäck Ivarsson.

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## Introduction

The relationship among seed plants is to this day an unresolved question (Rydin et al. 2002, Mathews 2009, Mathews et al 2010). One of the problems in resolving this issue is probably that the living diversity constitutes small remnants of once much more diverse groups. Most of the historical diversity is missing and therefore it becomes difficult to reconstruct the evolution, regardless of whether molecular or morphological data is utilized. Fossils can help us to get an insight into what has gone lost in the history, and can make taxon sampling more complete.

### Gnetales and the genus *Ephedra*

The Gnetales are a group of seed plants with a long evolutionary history (e.g. Arber and Parkin 1908, Crane 1996, Rydin et al. 2004). They show great morphological, molecular and ecological diversity, which probably is a result of them being remnants of a former much more diverse group (Crane 1996). Almost all possible proposals regarding their relationship to other seed plants have been made, e.g. Gnetales as sister to angiosperms (Crane 1985, Chase et al. 1993, Stefanovic et al. 1998), Gnetales as sister to Cupressophyta “the Gne-cup hypothesis” (Raubeson et al. 2006), Gnetales as sister to all other seed plants (Källersjö et al. 1998), Gnetales as sister to all other gymnosperms (Schmidt and Schneider-Poetsch 2002), Gnetales as sister to Pinaceae (Chaw et al. 2000) and Gnetales as sister to all Conifers, “the Gnetifer hypothesis” (Rydin et al. 2002, Rydin and Korall 2009).

The monophyly of Gnetales was recognized early (Hooker 1863, Arber and Parkin 1908) and this was later confirmed using cladistics analysis (e.g. Crane 1985, Chase et al 1993). *Ephedra* is sister to *Gnetum* and *Welwitschia* (Rydin and Korall 2009). *Gnetum* comprises about 30 species in tropical areas of America, Africa and Asia. They are dioecious trees, shrubs or vines (Kubitzki 1990). *Welwitschia* is a monotypic genus with its only species native to the Namib dessert. It is a dioecious plant with a somewhat bizarre morphology, with only two leaves with continuous basal growth (Kubitzki 1990).

Extant *Ephedra* comprises about 50-55 species, which are distributed in arid regions in Europe, Asia and North and South America (Kubitzki 1990). They are used as fodder plants for goat, sheep and camel and some species are used for medical purposes due to their high content of alkaloids (ephedrine) (Freitag and Maier-Stolte 1994). The genus shows little morphological variation and the species delimitations are often unclear (Freitag and Maier-Stolte 1994). Without reproductive organs (preferably female cones) species identification is very difficult, and even when using these characters, mistakes are easily made (Freitag and Maier-Stolte 1994). The plants are dioecious (rarely monoecious) shrubs, climbers or small trees. Phyllotaxis is decussate or in whorls of three, and the leaves are highly reduced in most species. Photosynthesis occurs mostly in the green stems of annual shoots (Kubitzki 1990). The female cones consist of 2-8 pairs or whorls of bracts, of which only the distal-most are fertile. The cone bracts of female cones may be fleshy and brightly coloured at seed maturity, but may also become dry, sometimes with a pronounced wing (Kubitzki 1990). Male cones may have more cone bracts than female cones, and most of them are fertile. Each male reproductive unit consists of two opposite scales and a microsporangiophore with 2-8 synangia, which each consists of 2 (or rarely more) fused microsporangia that open by horizontal slits.

The phylogeny within the genus has been difficult to resolve because of low sequence divergence and a distant relationship to outgroups. Several attempts have been made e.g.

Ickert-Bond and Wojciechowski (2004), Rydin et al. (2004), and Rydin and Korall (2009) who analyzed the phylogeny of *Ephedra* using seven plastid and nuclear loci and a big dataset with 104 specimens of *Ephedra* and 100 outgroup taxa. Their result indicates that *E. foeminea* is sister to all other species of *Ephedra*. The remaining clade comprises three lineages in a trichotomy: *E. alata*, a clade comprising *E. fragilis*, *E. altissima*, *E. aphylla*, and “the Mediterranean” *E. major*, and the remaining species in a clade. *E. milleri* is then sister to the remaining species (“the core *Ephedra*”). In core *Ephedra*, *E. foliata* (including *E. ciliata*) is sister to the two remaining sister clades, which comprises American species and species with a mainly Asian distribution, respectively.

*Ephedra foeminea*, the sister to all other *Ephedra* species, has some special features. The male cones have sterile female organs that produce pollination drops (pers. comm. Catarina Rydin). Further, this species has been proven to be insect pollinated (Bolinder 2011), and it is likely that these two features are interlinked (pers comm. Kristina Bolinder).

## Pollen

Pollen is the highly reduced microgametophyte of seed plants. They are haploid dispersal units that consist of three to five cells (Hesse 2009), five in *Ephedra* (Maheshwari 1935). The study of pollen and spores is called palynology (Punt et al. 2007), and comprise morphological studies of shape and size, type and position of apertures, as well as the structure of the pollen wall (Hesse 2009). The wall of a pollen grain consists of different layers, of which the outer “exine” is impregnated with sporopollenin. This makes the pollen grains resistant and due to this they have a high preservation potential. In pollen analysis the assemblages of dispersed pollen, for example in lake sediments or peats, is studied (Punt et al. 2007) and often used e.g. for reconstruction of historical vegetation and paleoclimate (Moore et al. 1991). As with many other biological questions, these questions need to be addressed in a phylogenetic framework.

Pollen of *Ephedra* are polyaplicate and inaperturate. Grain size ranges from 20 to 80  $\mu\text{m}$  in length (Steeves and Barghoorn 1959). They are prolate in shape and have longitudinal ridges and valleys sculptured by the outermost layer of the exine, the ectexine (Steeves and Barghoorn 1959, Kubitzki 1990). Pollen of *Welwitschia* are similar to that of *Ephedra* pollen but a sulcus is present; the grains are monosulcate (Rydin and Friis 2005). Pollen of *Gnetum* are small (about 9-22  $\mu\text{m}$ ) and spherical. The exine is thin and forms spines (Yao et al. 2004). Pollen germination experiments in *Ephedra* have shown that the grains shed their exines when germinating (Mehra 1938), leaving the gametophytes naked and the exines curled up in a characteristic way (El-Ghazaly et al 1998). This is not the case in *Welwitschia*, where the exine remains as a cap on the gametophyte (Rydin and Friis 2005). Attempts have been made to divide the pollen of *Ephedra* into subgroups. Steeves and Barghoorn (1959) recognizes four types (A-D) based on the characters size, shape, number of furrows and ridges, and exine structure, while for example Freitag and Maier-Stolte (1994) only divides the pollen into two groups: the “distachya type” and the “fragilis type”, without giving clear morphological definitions of the types. It remains unclear to what extent these subgroups of pollen reflect clade-specific variation.

## The evolutionary history of *Ephedra* and the aim of the study

Interpreting the evolutionary history of the Gnetales using dispersed pollen involves assuming that *Ephedra*-like (“ephedroid”) pollen is a synapomorphy for the Gnetales (Crane 1996, Friis et al. 2011). Ephedroid pollen are found from the Perm (Wilson 1962, Wang 2004) and onwards, and is incompletely understood. Conceivably, not all palynomorphs that

traditionally have been considered “ephedroid” belong to the Gnetales, however, at least some pollen described from the Permian could have been produced by gnetalean plants, or conceivable extinct sister lineages such as *Paleognetaleana* described by Wang (2004).

As currently understood and interpreted, the fossil record of dispersed pollen indicates that the diversity of Gnetales and *Ephedra* has fluctuated through history, with a peak during mid-Cretaceous. The diversity declines dramatically towards Late Cretaceous (Crane 1996). Macrofossils that share uniquely derived characters with extant *Ephedra* are found in the Early Cretaceous (Rydin et al. 2006). Shared characters are a combination of the features seeds with papillae on the inner surface of the seed envelope, in situ ephedroid pollen and associated shed exines (Rydin et al. 2006). These findings indicate that the *Ephedra* lineage is of significant age. However, the *Ephedra* fossils from the Cretaceous belong to extinct sister lineages to the living clade (Rydin et al. 2010), and extant *Ephedra* is estimated to have evolved during Oligocene, i.e. about 30 Ma, and onwards (Ickert-Bond et al. 2009). These results, in combination with the dramatic decline in gnetalean diversity during the Late Cretaceous as estimated from microfossil data (Crane and Lidgaard 1989), and from the absence of known macrofossils after the Early Cretaceous, have led to the hypothesis of at least two major radiations in *Ephedra*, one in the Early Cretaceous, and one beginning in the Oligocene. Between these radiations a significant part of the diversity is estimated to have gone extinct and this bottleneck effect might explain the poor molecular and morphological diversity in the genus today (Rydin et al. 2010).

The aim of this study is to detect and document inter- and intraspecific variation in pollen morphology in the genus *Ephedra*, focusing on *Ephedra foeminea* and the other early diverging lineages, all of which comprise species with a distribution around the Mediterranean area. Those species are: *E. alata*, *E. fragilis*, *E. altissima*, “the Mediterranean” *E. major*, *E. aphylla*, *E. ciliata*, *E. foliata* and *E. milleri*. For comparison studies of *E. distachya*, *E. major* and *E. viridis* will be made. Additionally, a pollen germination study of *Ephedra foeminea* will be carried out to explore if the germination process is the same as in the rest of *Ephedra*.

These initial studies of pollen morphology in extant *Ephedra* will then form the basis for studies of Mesozoic and Cenozoic “ephedroid” microfossils from the Tibetan Plateau. Here a first assessment of selected samples will be conducted. The hope is that together, all of these pieces of the puzzle will contribute to an increased understanding of the diversity and evolution of the genus *Ephedra*.

## Material and methods

### Field trip to Croatia

Between the dates 1st and 10th of July 2013 a field trip to Croatia was conducted. The aim was to collect both vegetative and reproductive parts of *Ephedra foeminea*, mainly male cones with pollen, and to reconnoiter the area for potential future pollination studies. Vegetative parts of *Ephedra major* were also collected. The field trip was concentrated to the mainland of Dalmatia, around the area of Split and Omiš. For detailed information about the collections made, see appendix 1.

The initial intention was to conduct two field trips; one in Croatia and one in Spain, in order to be able to collect as many Mediterranean species of *Ephedra* as possible. We had extensive

discussions in the research group about regulations regarding collection of plant material in other countries. With the UN Convention on Biological Diversity many new regulations came and we wanted to make sure we would not break any of these. Since no one was sure what actually applies, we decided to contact the authorities in Sweden with this question. In March 2013 an initial inquire regarding the regulations for collecting *Ephedra* in Croatia and Spain was sent to the Ministry of the Environment in Sweden. Their answer was that we should use the international website of the Convention on Biological Diversity ([www.cbd.int](http://www.cbd.int)) to find the appropriate person in the two countries, respectively. This website gathers information about the convention and, for example, contact information to the relevant authorities in each country, and it proved to be a very useful source of information to us.

In the beginning of April we had received an answer from the contact person in Croatia and an official request was sent to the Ministry of Environmental and Nature Protection, Republic of Croatia. On the 30<sup>th</sup> of April we received the permit for collecting protected wild taxa on territory of the Republic of Croatia for scientific/non-commercial research and for export of the same biological material from Croatia. The entire process took about two and a half months.

A request was also sent to the contact person in Spain, who would forward our official request to the proper authorities in Andalucia. Unfortunately, we never received an answer from these authorities, and hence the field trip to Spain was cancelled.

### **Pollen germination**

Immediately upon return to Stockholm, freshly collected pollen of *Ephedra foeminea* from Dalmatia was germinated using the culture medium of Brewbaker and Kwack (1963).

In 2 dl deionized water the following was added:

- 20 g Sucrose
- 20 mg Boric acid,  $H_3BO_3$
- 60 mg Calcium nitrate,  $Ca(NO_3)_2$
- 40 mg Magnesium sulfate,  $MgSO_4$
- 20 mg Potassium nitrate,  $KNO_3$

Cones were sampled in Croatia on the 10<sup>th</sup> of July (voucher: Lena Norbäck Ivarsson and Olle Thureborn 103) where they were put in a petri dish on top of moist paper to keep them fresh. Approximately 24 hours after sampling anthers were put in a clean petri dish, where the synangia were punctured to release the pollen and the culture medium was added. The petri dishes were left in room temperature. The pollen was examined after 1.5 and 24 hours. The results from the pollen germination were documented using a Nikon ECLIPSE 80i light microscope, a Nikon DS-Fi1 camera and the software NIS-Elements F 2.30.

### **Test of SEM preparation methods**

To assess the reliability in using herbarium material a test was carried out using pollen from an *Ephedra viridis* plant cultivated in the greenhouse of the Department of Ecology, Environment and Plant Sciences, Stockholm University. Six different preparation methods were tested:

- “Fresh” material: sampled the same morning and placed directly on stubs, examined in scanning electron microscopy (SEM) approximately one hour later.
- Dried material: cones were sampled one week in advance and dried in an envelope.

- 70 % alcohol (ethanol): samples were collected one week in advance in 70% alcohol and stamens were then directly placed on stubs.
- 70 % alcohol followed by dehydration: samples were collected one week in advance and put in 70% alcohol. Before mounting on stubs the samples were dehydrated using alcohol series of 80%, 90%, 95% and 100% concentration. The samples were put in beakers with respective alcohol concentration for 10 minutes (2\*20 minutes in the 100% alcohol).
- Dried material was washed with Phosphate buffered saline (PBS) followed by dehydration using 20%, 50%, 70%, 80%, 90%, 95% and 100% alcohol. The samples were put in beakers with respective alcohol concentration for 10 minutes (2\*20 minutes in the 100% alcohol).
- Material was kept in 70% alcohol and then washed with PBS followed by dehydration using 20%, 50%, 70%, 80%, 90%, 95% and 100% alcohol. The samples were put in beakers with respective alcohol concentration for 10 minutes (2\*20 minutes in the 100% alcohol).

Length and width of 30 pollen grains of each preparation method were measured. T-tests assuming unequal variances were carried out using Microsoft Excel.

### SEM analysis of extant pollen

Three stamens were carefully picked from cones on herbarium sheets and placed on stubs with carbon tape. The microsporangia were then punctuated using a needle so that the pollen grains spread out on the stubs. These preparations were carried out under a dissecting microscope. The tools were cleaned in 70% alcohol between samples to avoid contamination. The samples were coated with a thin layer (30 seconds) of gold using a gold sputter. The SEM studies were carried out at the Department of Material and Environmental Chemistry, Stockholm University, using a JEOL JSM-7401F at 2,0 kV, and a working distance of 8 mm.

For each sample, a minimum of 10 pollen grains were measured (length; polar axis and width; equatorial axis) and the ridges were counted. The total number of ridges was calculated by counting the number of ridges visible in equatorial view of pollen grains. This number was then multiplied by two. Voucher information of examined specimens is given together with the results (table 1).

An ACCTRAN ancestral state reconstruction as described in Farris (1970) was conducted based on phylogenetic results in Rydin and Korall (2009). *Welwitschia* was selected as outgroup.

### The sampling sites of the Tibetan Plateau and the fossil pollen studies

The sampling sites are located at the northeastern corner of the Tibetan Plateau, in the vicinity of the cities Xining and Lanzhou. The area consists of Mesozoic-Cenozoic basins and subbasins, and the nomenclatures of these are complicated (Horton et al. 2004). The stratigraphy in this region comprises Lower Jurassic through Miocene strata. The material has been deposited in “lacustrine and distal fluvial environments with subordinate proximal fluvial and alluvial fan environments” (Horton et al. 2004).

The microscope slides were prepared by Horton et al. (2004) where the method of pollen preparation is described as follows: “*For each sample, 5-10 grams of siltstone was dissolved in HCl and HF acids. Resistant pollen grains were sieved with 10 µm mesh, floated in a solution of ZnBr<sub>2</sub> (specific gravity of 2.0), mounted on standard slides, and examined using a*

*binocular microscope.*” The sampling in Tibet was carried out by Brian Horton and the samples were kindly lent to us by Carina Hoorn.

Samples from Late Jurassic to Eocene were thoroughly investigated using a Nikon ECLIPSE 80i light microscope in the search for *Ephedra* or *Ephedra* like pollen. In selected samples where the *Ephedra* or *Ephedra* like pollen were not just a few grains but thought to constitute a substantial part of the total amount of pollen grains, the relative abundance was calculated. A minimum of 300 pollen grains were then counted in these samples. Results from the light microscopy were documented using a Nikon DS-Fi1 camera and the software NIS-Elements F 2.30.

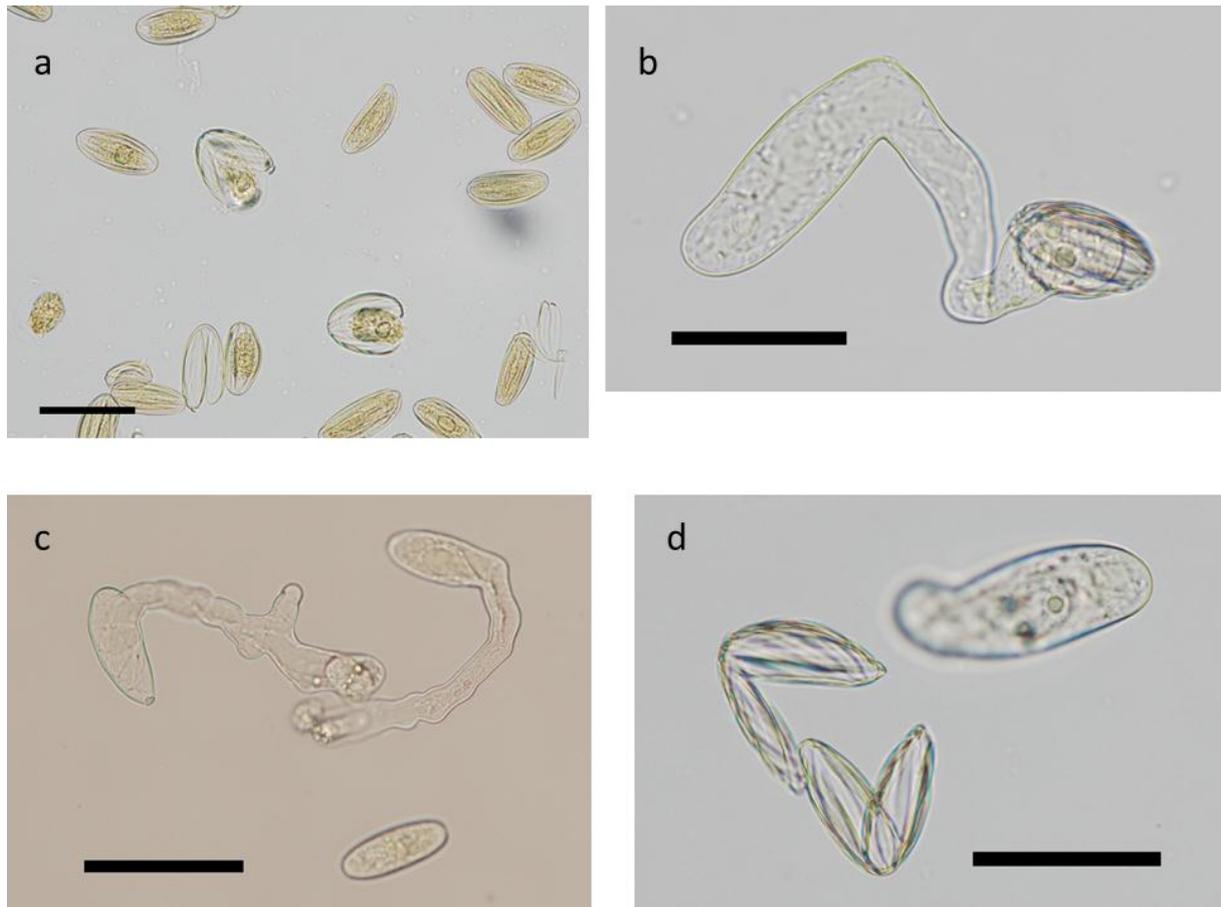
## Results

### Pollen germination in *Ephedra foeminea*

After 1.5 hours in the culture medium the pollen had started to germinate. The exines split longitudinally and were in some cases discarded from the rest of the gametophytes, and in other cases still attached to the gametophytes. The gametophytes had swollen up to a more sphere-like shape (figure 1a).

After 24 hours the pollen tubes were fully developed and the exines were in some cases shed (figure 1c and d), in other cases not (figure 1b). The shed exines were slightly contracted.

After the last examination the pollen was left in the lab over the weekend. When examined on Monday, the culture was already overgrown with mold. The germination experiment was repeated again about three months later using dry cones. A fresh batch of culture medium was mixed and the experiment went on for five days, both in room temperature and using a heating lamp, but the pollen did not germinate.



**Figure 1. Pollen germination in *Ephedra foeminea*. a: after 1,5 hours, b, c and d: after 24 hours. In b, the exine is still attached to the gametophyte, which has developed a pollen tube. In c, the gametophytes are “naked”, i. e. the exine has been shed. Photo d shows a naked gametophyte next to two shed exines. Scale bars = 50  $\mu\text{m}$ .**

### **Test of SEM preparation methods**

T-tests comparing fresh material with dried material revealed no significant difference between them when it comes to length ( $p=0.06$ ), width ( $p=0.95$ ) and length/width ratio ( $p=0.09$ ).

T-tests comparing fresh material with material put in alcohol followed by dehydration series showed significant differences in length ( $p=6.0 \cdot 10^{-20}$ ) and length/width ratio ( $p=1.6 \cdot 10^{-13}$ ). No difference in width was observed ( $p=0.6$ ).

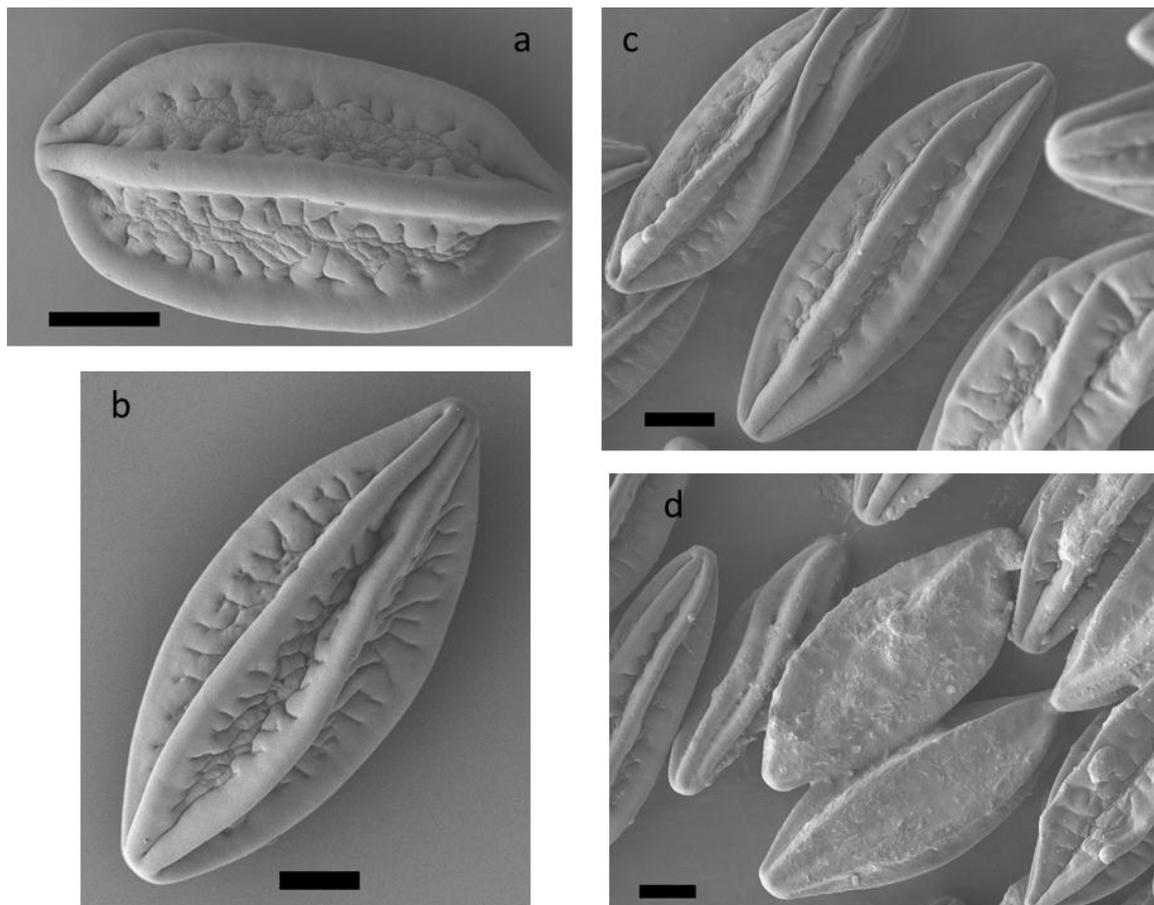
T-tests comparing dried material with material put in alcohol followed by dehydration series showed significant differences in length ( $p=2.6 \cdot 10^{-13}$ ) and length/width ratio ( $p=2.97 \cdot 10^{-11}$ ). No difference in width was observed ( $p=0.67$ ).

The average length, width and length/width ratio is given in table 2.

**Table 2. Results from the methodology test.**

Preparation method	Average length ( $\mu\text{m}$ )	Average width ( $\mu\text{m}$ )	L/W ratio
"Fresh" material	64.3	24.2	2.7
Dried for a week	60.9	24.1	2.6
Stored in alcohol – no dehydration series before observations	48.2	22.5	2.2
Stored in alcohol – dehydration series before observations	45.4	23.8	1.9

The test of different preparation methods showed that the dry, untreated pollen grains were most similar in shape and size to fresh pollen grains (figure 2b and c). Pollen grains that have been placed in alcohol have a different shape and length/width ratio, and this is true also after dehydrating series (figure 2a). Among the fresh material, a coating of uncertain origin was observed on some pollen grains (figure 2d). This coating was not observed in any other samples of *Ephedra viridis* or from the following studies of herbarium material. This study did not show that washing with PBS buffer made the pollen grains cleaner of e.g. orbicules and other tapetum rests.



**Figure 2. Pollen grains of *Ephedra viridis*. a: Pollen grain stored in alcohol for a week, b: Pollen grain dried for a week, c and d: Fresh material. Note the coating of the pollen grains in d. Scale bars = 10  $\mu\text{m}$ .**

### **SEM analysis of extant pollen and ancestral state reconstruction**

The length, width, valley pattern and number of ridges for each examined specimen are given in table 1. Only pollen grains interpreted as viable are included in the results. Thus, in addition to the reported results, a great number of collapsed and/or aborted pollen grains were often found (figure 3m and n). Orbicules were observed in all studied species.

The ancestral state reconstruction shows straight valleys to be the ancestral state in *Ephedra*. The result is illustrated in figure 4.

**Table 1. Voucher information and results from the SEM studies of extant *Ephedra* pollen.**

Taxon	Voucher	Herbarium	Sampling location	Sampling date	Polar axis ( $\mu\text{m}$ )	Equatorial axis ( $\mu\text{m}$ )	Number of ridges	Valleys
<i>E. alata</i>	Ibrahim and Mahdi	(S)	Egypt	1971	39-48 (42)	16-21 (18)	12-14	s
<i>E. alata</i>	A. A. Anderberg 480	(S)	Algeria	1980	39-50 (46)	18-24 (20)	6-10	s
<i>E. alata</i>	K. H. Rechinger 102	(S)	Iraq	1957	50-68 (60)	15-21 (19)	8-12	s
<i>E. altissima</i>	A. Faure s.n.	(S)	Algeria	1915	44-51 (48)	15-25 (19)	12-20	s
<i>E. altissima</i>	Staudinger 6714	(W)	Morocco	1999	35-40 (38)	16-20 (18)	10-16	s
<i>E. aphylla</i>	G. Samuelsson 2696	(S)	Jordan	1933	36-62 (50)	18-30 (23)	8-16	s
<i>E. aphylla</i>	Ibrahim & Mahdi s.n.	(S)	Egypt	1971	38-48 (45)	17-27 (22)	10-14	s
<i>E. ciliata</i>	H. T. Яковлева	(W)	Iran	1956	33-44 (38)	16-22 (18)	10-20	s
<i>E. ciliata</i>	E. K. Balls B2487	(S)	Morocco	1936	34-46 (40)	15-23 (17)	12-18	s
<i>E. distachya</i>	Kristina Bolinder		Greece	24/5-2012	46-52 (49)	19-22 (20)	6-8	b
<i>E. distachya</i>	Racz I., Nemeth J. and Kun A. Coll#35308	(S)	Hungaria	1993	45-60 (50)	15-23 (21)	4-8	b
<i>E. foeminea</i>	Kristina Bolinder		Asprovalta, Greece	27/9-2011	37-41 (39)	14-17 (15)	8-12	s
<i>E. foeminea</i>	Kristina Bolinder		Asprovalta, Greece	30/6-2012	35-46 (42)	15-22 (18)	6-14	s
<i>E. foeminea</i>	Fred S. Meyers and J. E. Dinsmore 8124	Leiden	Palestine, Jericho, Jerusalem	1912	45-49 (47)	20-22 (21)	16-22	s
<i>E. foeminea</i>	R. Pampanino and R. Pichi-Sermolli 139	Leiden	Libia, Cyrenaica	1934	35-54 (42)	15-24 (18)	8-16	s
<i>E. foeminea</i>	Lena Norbäck Ivarsson and Olle Thureborn 103		Croatia, Dalmatia, Pisak	3/7-2013	39-49 (44)	15-18 (17)	8-12	s
<i>E. foliata</i>	Wendelbo and Assadi 1604	(W)	Iran	1975	38-51 (43)	15-18 (17)	12-20	s
<i>E. foliata</i>	Brahmadatta Tiagi	(S)	India	1948	34-39 (37)	15-19 (17)	10-20	s
<i>E. fragilis</i>	E. Reverchon 205	(S)	Spain	1889	44-49 (46)	18-22 (21)	8-12	s
<i>E. fragilis</i>	Botany department, Univ. of Stockholm	(S)	Tunisia	1972	41-47 (45)	18-22 (21)	10-12	s
<i>E. major</i>	Elisée Reverchon 969	(S)	Spain	1895	50-65 (55)	20-28 (25)	4-8	b
<i>E. major</i>	Erik Wall	(S)	Morocco	1934	45-53 (50)	18-25 (22)	4-8	b
<i>E. major</i>	Akhani 10462	(W)	Iran	1995	49-63 (55)	20-26 (22)	4-6	b
<i>E. major</i>	Ronniger	(W)	Croatia, Split	1930	49-66 (56)	19-27 (24)	6-12	b
<i>E. major ssp. major</i>	Erik Julin	(UPS)	Spain	1979	35-54 (46)	15-21 (19)	8-16	s
<i>E. milleri</i>	P. Hein, H. Kütschner and M. Reisch YP 1110	(E)	Yemen	2001	40-57 (51)	19-24 (21)	10-20	s
<i>E. viridis</i>	Lena Norbäck Ivarsson, 25/3-2013		The green house, SU	25/3-2013	46-74 (61)	17-32 (24)	4-10	b

s = straight valleys, b = branched valleys.

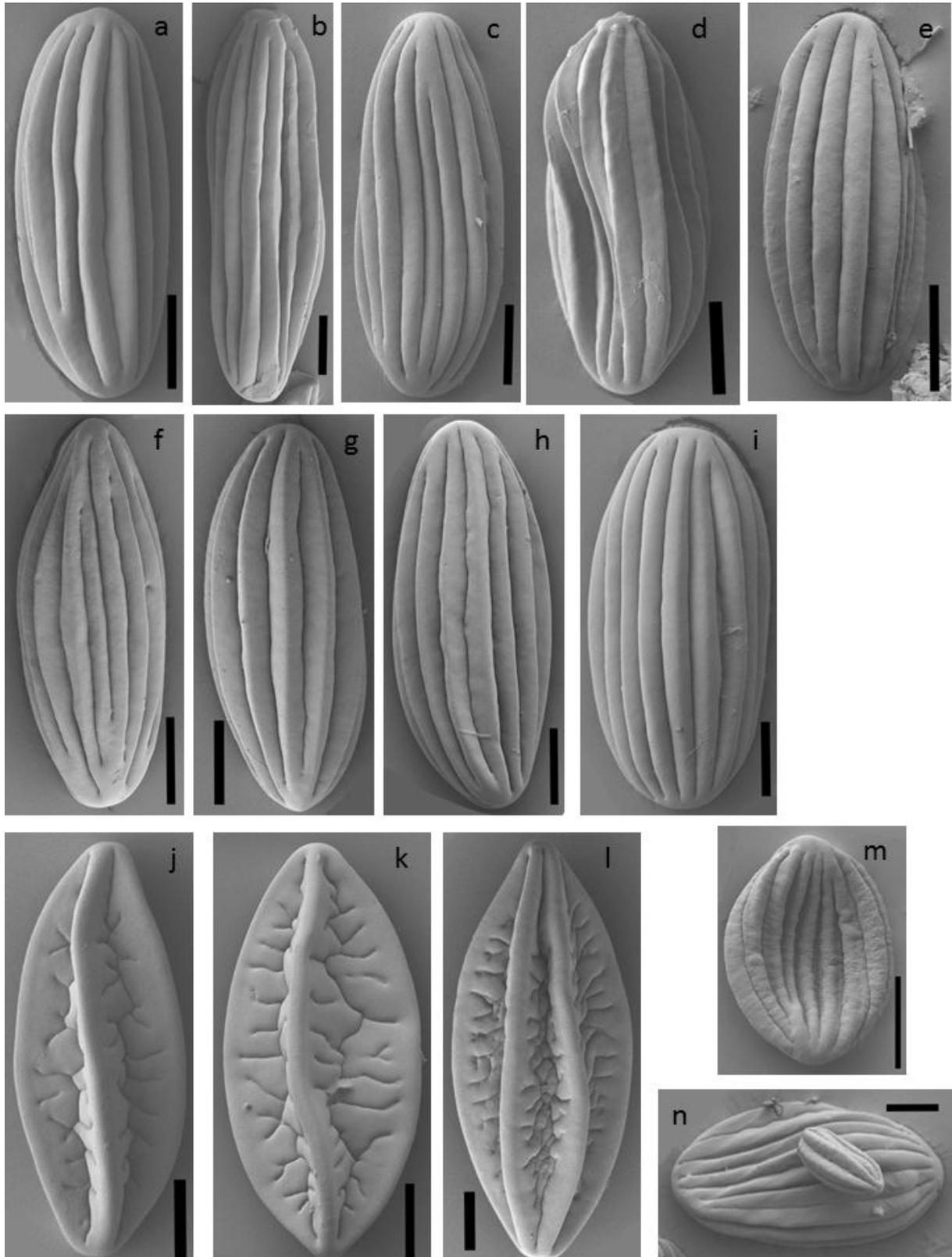
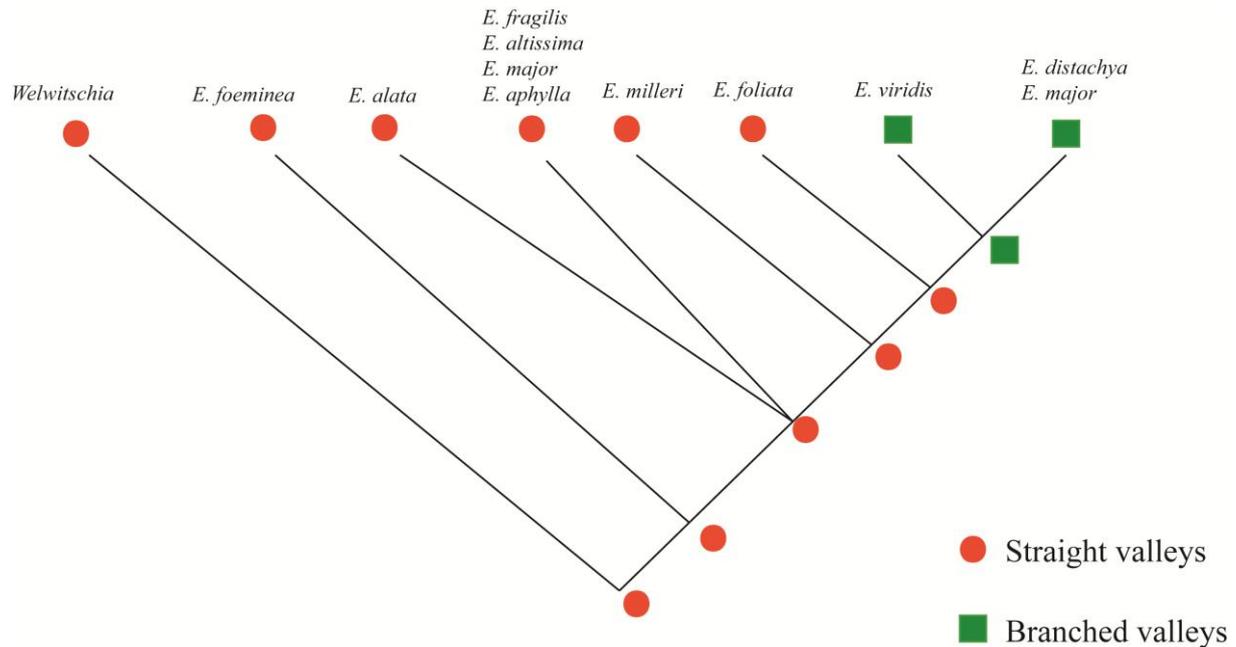


Figure 3. SEM photos of *Ephedra* pollen grains. a: *E. foeminea*, b: *E. alata*, c: *E. altissima*, d: *E. aphylla*, e: *E. ciliata*, f: *E. foliata*, g: *E. fragilis*, h: *E. major*, i: *E. milleri*, j: *E. distachya*, k: *E. major*, l: *E. viridis*, m: *E. foeminea*, collapsed pollen grain, n: *E. aphylla*, note the size variation. Scale bars = 10µm.



**Figure 4. Ancestral state reconstruction of the pollen morphology character “straight or branched valleys” in *Ephedra*, using *Welwitschia* as outgroup. The phylogeny is redrawn from Rydin and Korall (2009).**

### Fossil pollen from the Tibetan Plateau

Here follows a list of selected “key fossils”. As for living species, the number of ridges was counted in equatorial view and then multiplied by 2. The coordinates have been attained using an England Finder.

Late Jurassic, Xining sect 006251 7D, coordinates: H14;3, figure 5a.

In the samples from Late Jurassic *Ephedra*-like pollen are unusual. This pollen grain is about 40\*23 µm in size and do not have branched valleys. Number of ridges is 12.

Late Jurassic, Xining sect 006251 7A, coordinates: F13;3, figure 5b.

This pollen grain is about 42\*19 µm in size and do not have branched valleys. Number of ridges is 8 (10?). The grain appears to have a sulcus.

In the samples from the Early Cretaceous, no *Ephedra* like pollen was observed.

Mid-Cretaceous (Aptian-Cenomanian), Xining 02x351-P 21, coordinates: J23;1, figure 5c.

The pollen grain is about 52\*26 µm in size and appears to have twisted ridges, or ridges in two opposite directions, which gives the grain a checkered morphology. All pollen with a checkered morphology together represents 29 % of the pollen in this sample.

Mid-Cretaceous (Aptian-Cenomanian), Xining 02x351-P 21, coordinates: N20;1, figure 5d.

The pollen grain is about 56\*24 µm in size and appears to have twisted ridges, or ridges in two opposite directions, which gives the grain a checkered morphology.

Late Cretaceous – early Eocene (75-50 Ma), East Xining section 02x535-P 24C, coordinates: S13;3, figure 5e.

The pollen grain is about 59\*23 µm in size and has 4 ridges. The pollen grain has branched patterns in the valleys between the ridges. In this sample, ephedroid pollen with branched patterns in the valley makes up for 30% of the pollen grains.

Paleocene-middle Eocene, Pingan 02PC1-P 36A, coordinates: Q13;2, figure 5f.

The pollen grain is about 45\*15 µm in size and has 8 (10?) ridges. No branches in the valleys are visible. A sulcus may be present, but this observation is uncertain.

Eocene, Pingan C sect. 006202 5A, coordinates: J13;3, figure 5g.

The pollen grain is about 46\*19 µm in size and has 4 ridges, with branched patterns in the valleys.

Eocene, Zhangxian 02ZX15-P 6A, coordinates: P28, figure 5h.

The pollen grain is about 67\*32 µm in size and has 6 ridges, with branched patterns in the valleys.

Eocene, Pingan C sect. 006202 5A, coordinates: M22;4, figure 5i.

The pollen grain is about 34\*22 µm in size and has 6 ridges. Indistinct branching pattern in the valleys seems to be present.

Eocene, Pingan C sect. 006202 5A, coordinates: F18;3, figure 5j.

The pollen grain is about 59\*20 µm in size and has 8 ridges. The grain does not have a branched pattern in the valleys. A sulcus was not observed but may be present.

Eocene, Pingan C sect. 006201 4A, coordinates: L28;2, figure 5k.

The pollen grain is about 78\*17 µm in size and has 8 ridges. The grain does not have branched pattern in the valleys.

Eocene, Zhangxian 02ZX15-P 6A, coordinates: V39, Figure 5l.

The pollen grain is about 68\*27 µm in size and has 8 ridges. There may be a tendency of branched patterns in the valleys, but this observation is uncertain.

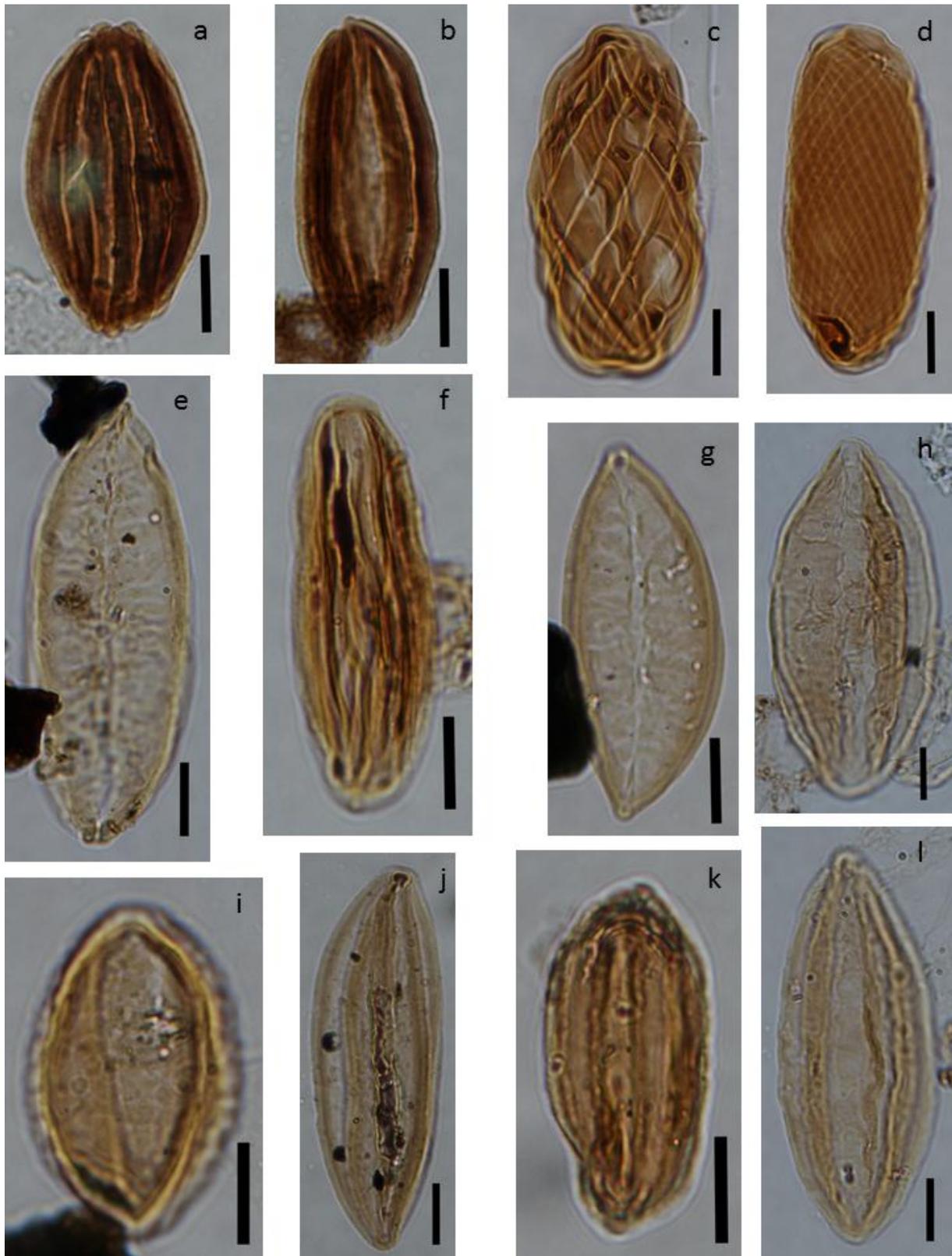


Figure 5. Fossil pollen grains from the Tibetan plateau. a and b: Late Jurassic, c and d: Aptian-Cenomanian, e: Cenomanian-Maastrichtian, f: Paleocene-Middle Eocene, g-l: Eocene.

## Discussion

### Pollen morphology of extant *Ephedra* in a phylogenetic context

From the results of the present study it is evident that there are at least two types of *Ephedra* pollen, defined by the morphology of the valleys between the exine ridges; a) the “*foeminea* type” with straight valleys and b) the “*distachya* type” with branched valleys. All other examined characters (shape and size of grains, and number of exine ridges) overlap within and between species, and are not useful for species delimitation, nor are there indications of evolutionary trends in these features. Even an obvious and traditionally used feature such as number of exine ridges appears problematical to use for clade or species delimitations because of substantial intra-specific variation. Even though pollen grains of the *distachya* type typically have fewer ridges than pollen grains of the *foeminea* type, it is not consistently so (see table 1). Among investigated species, the *foeminea* type of pollen morphology is present in *E. foeminea*, *E. alata*, *E. fragilis*, *E. altissima*, “the Mediterranean” *E. major*, *E. aphylla*, *E. milleri*, *E. foliata* and *E. ciliata* (figure 3a-i). The *distachya* type is in this study found in *E. distachya*, *E. major* and *E. viridis* (figure 3j-l), which are all members of the core *Ephedra* clade; *E. distachya* and *E. major* are nested within the clade with a mainly Asian distribution, and *E. viridis* belongs in the American clade in Rydin and Korall (2009). From the ancestral state reconstruction (figure 4) it is evident that the *foeminea* type of pollen represents the ancestral state in *Ephedra*.

Substantial variation in pollen morphology has previously been reported for *Ephedra*, but the information in the literature is partly conflicting and sometimes difficult to interpret. Steeves and Barghoorn (1959) recognized four groups of *Ephedra* pollen based on the features size, shape, number of furrows and ridges, and exine structure. The descriptions of the pollen types are long and circumstantial and I have trouble understanding them. For example, *E. foeminea*, *E. alata* and *E. fragilis* are reported to have different pollen types (B, D and C, respectively). I have tried to understand this using the illustrations and descriptions from their study and the results from my study (figure 3a, b and c), but failed to do so. Nothing in my results supports the recognition of these four groups. My conclusion is that at least a part of the variation they report is artificial and can be explained by the preparation methods employed, or by the fact that scanning microscopy was not available in the 1950ies.

Later, El-Ghazaly et al. (1997) argued that all four pollen types of Steeves and Barghoorn (1959) may be found within one single microsporangium of *E. foliata*. However, although I too find intra-specific variation, I for example never find the *foeminea* type and the *distachya* type of pollen in the same species. Unfortunately there are no photos in the article by El-Ghazaly et al. (1997) to support their argument. Freitag and Maier-Stolte (1994) mention two types of pollen, the “Fragilis-type” and the “Distachya-type” but provide no definitions or descriptions of the types. Although it therefore is difficult to make comparisons, the conclusions of Freitag and Maier-Stolte (1994) appear more in line with my results. Ickert-Bond et al. (2003) report a unique dimorphism in three American species; *E. torreyana*, *E. trifurca* and *E. funerea* ↔ *E. torreyana*. They report two distinct pollen types (straight or folded, zig-zag ridges) to be present in pollen grains from the same specimen. In addition, both straight and branched valleys were observed in pollen of the same specimen. Morphological variation as considerable as reported in Ickert-Bond et al. (2003) was not observed in the present study. It is possible that there is more variation in pollen morphology in American taxa than in the species examined in the present study, but it is also possible that

the extraordinary dimorphism found by Ickert-Bond et al. (2003) can be explained by hybrid origin of examined specimens.

Bolinder (2011) carried out pollination studies of *E. foeminea* and *E. distachya* in North-Eastern Greece. The results show that the two species differ in pollination syndrome, *E. foeminea* is insect-pollinated (entomophilous) and *E. distachya* is wind-pollinated (anemophilous). Since both *Gnetum* and *Welwitschia* are insect-pollinated and *E. foeminea* is the sister to all other species, the conclusion is that while insect pollination is the ancestral state in *Ephedra*, most extant species are wind-pollinated (Bolinder et al. in progress), although with a various efficiency (Niklas and Buchmann 1987). And interestingly, pollen grains of *E. foeminea* differ from other investigated species in that they appear not to consistently shed their exines during germination as described in El-Ghazaly et al. (1998), nor were the exines observed to curl up in the characteristic way described in the same study (figure 7e in El-Ghazaly et al. 1998). They do however, split longitudinally during germination, as other *Ephedra* pollen does, and the germination process in *E. foeminea* is not comparable to that of *Welwitschia* where the exine remains attached to the gametophyte as a cap (Rydin and Friis 2005). Otherwise, *E. foeminea* pollen is very similar to pollen of other Mediterranean species. The pollination syndrome shift that has occurred in *Ephedra* (from entomophily to anemophily) may reflect the two pollen types, but the pollen morphology shift did apparently not occur simultaneously with the shift in pollination syndrome.

#### *Specific details and methodological notes*

In Rydin and Korall (2009) *E. major* is not monophyletic, but constitutes two different clades, one among the Mediterranean species and one nested within the Asian clade. They therefore suggest a taxonomic revision of this species. The results from the present study support this conclusion. The same specimen of *E. major* ssp. *major* as constitutes a part of the Mediterranean grade in Rydin and Korall (2009) was examined in the present study and its pollen has straight valleys in contrast to the other specimens of *E. major*, which had branched valleys. The pollen morphology seems to be a useful character when distinguishing the two taxa. In addition to a revision of *E. major*, it might be necessary with a revision of the whole *E. aphylla*, *E. altissima*, *E. fragilis* and *E. major* species complex in order to assess species delimitations and taxonomic problems (see also Rydin and Korall 2009, Rydin et al. 2010, Thureborn, unpublished results).

The test of SEM preparation methods showed that dried pollen grains are most similar to fresh grains. The ridges of *Ephedra* pollen might be an adaptation to moisture stress (Osborn et al. 1993). It is possible that both the dry and the “swollen up” morphology of the pollen exist in natural environments, depending on the degree of moisture. The swollen up morphology was not observed among fresh pollen, and my suggestion based on results in this study is to continue working with untreated herbarium material. The most important thing to keep in mind is probably to not compare e.g. herbarium material with material kept in alcohol, and to be aware of the fact that artificial morphological differences may arise if different preparation methods are utilized in a study. It would have been interesting to know more about preparations techniques used in the study by Steeves and Barghoorn (1959), but the information in the paper regarding this is unfortunately very sparse.

Finally, a great number of collapsed, aborted and/or small pollen grains were observed in all specimens. From this study I cannot conclude whether this is correlated with the preservation, the timing of sampling, or some other variables.

## Ephedroid pollen in the fossil record

In the literature, the first ephedroid pollen is reported from Perm (Wilson 1962, Wang 2004). Wang (2004) describes a cone (*Palaeognetaleana auspicia*) with in situ ephedroid pollen. These grains have straight valleys, similar to the extant *foeminea* type of pollen. The cone is suggested to be of gnetalean affinity, but the similarity to conifers is also pointed out as a fossil evidence for the Gnetales-Conifer relationship suggested by some authors (e.g. Chaw et al. 2000, Rydin et al. 2002, Rydin and Korall 2009). It is likely that a wide range of extinct plants produced polyplicate pollen during the late Paleozoic and the Mesozoic, perhaps including the suggested common ancestor of Gnetales and Conifers.

In my samples from the Late Jurassic, pollen with an affinity to the Gnetales is rare. For the grains in figure 5a and 5b, a gnetalean affinity cannot be ruled out. In figure 5b, a sulcus appears to be present and this grain is thus probably of *Welwitschia* type, but may also belong to an extinct group within the Gnetales. A sulcus could in theory also be present in the pollen grain in 5a, but on the invisible side of the grain, facing downwards. Otherwise this grain is very similar to the *foeminea* type of pollen.

In *Ephedra portugallica* from Early Cretaceous, ephedroid pollen is found in situ on *Ephedra* seeds (Rydin et al. 2006). This pollen appears to be of the *foeminea* type. The fossils share synapomorphies with extant *Ephedra*, such as seeds with papillae on the inner surface of the seed envelope, in situ ephedroid pollen and associated shed and curled up exines. Even so, the conclusion is that *E. portugallica* belongs somewhere on the stem lineage of *Ephedra* (Rydin et al. 2010).

In mid-Cretaceous samples, pollen grains with a checkered morphology (figure 5c and 5d) are abundant. The systematic affinity of these pollen grains is uncertain. Dispersed pollen with checkered morphology is found in Mesozoic strata, for example in Late Triassic sediments, and has been described under several names (palynomorphs), e.g. as *Equisetosporites* (Osborn 1993), *Ephedripites* (Balme in Krassilov 1986), and *Ephedra* (Scott 1960). Based on my experience from the work with the present study, there is nothing in these grains that would explicitly indicate a gnetalean affinity. However, Krassilov (1986) has reported pollen with checkered morphology in the pollen chamber of *Eoanta zherkhinii*, a lower Cretaceous plant with a probable affinity to the Gnetales. Osborn (1993) describes a diversity of dispersed pollen grains with gnetalean affinity from Lower Cretaceous sediments from Brazil, but the illustrations are difficult to interpret. It is possible that these grains are of gnetalean affinity and produced by subgroups that are now extinct, but it is also possible that this type of pollen was produced by other seed plants that are now extinct.

The pollen grain in figure 5e is interpreted as being of the *distachya* type and pollen of this type (with branched valleys) is abundant in the sample (constitutes 30%). According to the publication of Horton et al. (2004), the age of this sample is Late Cretaceous (Cenomanian-Maastrichtian), but new geological information indicates that the age of the sample is uncertain and ranges between 75-50 Ma (pers. comm. Guillaume Dupont-Nivet), which corresponds to Late Cretaceous – earliest Eocene. This type of pollen (the *distachya* type) is restricted to the subclade “core *Ephedra*”, and the discovery of the *distachya* type of pollen in samples from the earliest Eocene or older sharply contradicts results in analyses of divergence times of clades, which indicate that the extant *Ephedra* clade originated in the Oligocene. To

my knowledge, there are no previous reports of the *distachya* type of *Ephedra* pollen in samples this old, however, time has not permitted a complete review of the literature.

The samples from the Paleocene contain few pollen grains in general, also those with an ephedroid morphology. The pollen grain in figure 5f (Paleocene – middle Eocene) is similar to a *foeminea* type, but it may have a sulcus, which rather would indicate an affinity with *Welwitschia*.

In samples from the Eocene, the *distachya* type of pollen is present. Hoorn et al. (2012) studied palynomorphs in samples from the Eocene from the same location in Tibet, and divided the pollen into different types. The results from my study of the extant taxa of *Ephedra* suggest that at least some of this variation may be intraspecific variation. For example, the pollen in figures 5g, 5h and 5i, could be interpreted as different types based on size and number of ridges, but based on my studies of extant species, it cannot be ruled out that the variation is intraspecific and perhaps caused by environmental factors (e.g. moisture) during the release and sedimentation of the pollen.

According to my morphological results, the *distachya* type of pollen appears to have evolved only once; I find no differences between the *distachya* type of pollen from the Late Cretaceous – early Eocene, and from the latter Eocene, nor from that produced by extant *Ephedra*. Further, there are no macrofossils with the *distachya* type of pollen *in situ*, and there is to my knowledge no indications on that these pollen grains have been produced by any other plants than *Ephedra*. However, an extended study, preferable using SEM and/or TEM, is needed in order to confidently rule out the possibility that the similarities between *distachya* type of pollen from the Mesozoic, the Paleogene, and from recent material are only superficial.

Another noteworthy observation is that when the *foeminea* (or *Welwitschia*) type of pollen is present during the Late Jurassic and Paleocene-Eocene, there are only one or a few grains in the samples (e.g. fig 5a, b, f and k). In contrast, the *distachya* type of pollen is always abundant when present. In order for the pollen to be preserved as fossils they have to be transported to a suitable environment (e.g. lake sediments). Bolinder (2011) concludes that insect pollination is the ancestral state in *Ephedra*, and this may explain the sparse record of *foeminea* type of pollen in the stratigraphy. Conceivably, fossil pollen of the *foeminea* type was, unlike that of the *distachya* type, typically not transported by wind, and was consequently not deposited in environments suitable for sedimentation as often as the *distachya* type of pollen.

### **The ecology of fossil Gnetales and the use of ephedroid pollen as a dry climate indicator**

Data from dispersed ephedroid pollen indicate that the Gnetales were highly diverse at low paleolatitudes during the mid-Cretaceous (Crane 1996). Their increase in abundance and diversity coincides with the radiation of early angiosperms and it seems that the two groups had similar ecological tolerances (Crane 1996). The ephedroids then decreases during the Late Cretaceous, during which a global cooling of the climate took place (Friis et al. 2011). Macrofossils indicate that Cretaceous Gnetales were not restricted to arid environments. *Drewria potomacensis*, an Early Cretaceous member of the Gnetales, was probably an herbaceous plant associated with mesic environments (Crane and Upchurch 1987). There are also (unpublished) fossils, which indicate that Cretaceous Gnetales inhabited a wide range of

ecological niches, including wetland and aquatic environments, similar to those inhabited by angiosperms (for example some grasses, sedges and other monocots) today (pers. comm. Catarina Rydin). Both the scenario of a decrease in diversity of ephedroid plants, and them being outcompeted by angiosperms and confined to dryer habitats, would lead to the same result; fewer fossilized ephedroid plants.

Interesting to note is also that if a shift in pollination syndrome occurred in *Ephedra* during the Late Cretaceous or early Paleogene (from entomophily to anemophily, Bolinder 2011), this lend further support for the conclusion Crane (1996) made from dispersed pollen data: that the diversity of ephedroids decreased during this period. A shift to wind pollination in some species should result in an increase of the amount of dispersed pollen from these plants. The fact that such pollen instead decreases dramatically in Upper Cretaceous sediments (Crane 1996) indicates that the species diversity of ephedroid plants declined drastically during this time.

Also in extant *Ephedra*, there are differences in ecology among the species despite the small amount of diversity within the genus today. For example, *E. alata* is adapted to extremely dry desert climate (Freitag and Maier-Stolte 1994), whereas *E. foeminea* is found in Greece and Croatia where the precipitation is substantial during the winter months (Christopherson 2011). In the entire Gnetales, the ecological differences could not be greater, with *Gnetum* having a tropical distribution, *Ephedra* distributed in arid to sub-arid regions, and *Welwitschia* being native to the Namib Desert. When it comes to ecology of extinct Gnetales members, we still have a lot to learn. There is a risk for circular argumentation, i.e., that initial assumptions that the presence or absence of fossil *Ephedra* or ephedroid pollen in sediments indicate dry or moist climate respectively, and subsequent climate reconstructions based on this will then form the basis for further interpretations of the ecology of the fossils. In my opinion, conclusions about paleoclimate based on dispersed ephedroid pollen are unwarranted and do not have any scientific support. This conclusion is in line with e.g. Yang (2010) and Crane and Upchurch (1987).

### **One step closer towards a calibration point within *Ephedra*?**

As mentioned in the introduction, detailed studies of fossils and living species led to the hypothesis of (at least) two major radiations in *Ephedra*, and the intervening bottle neck period conceivably explains the lack of molecular and morphological diversity within the extant genus (Rydin et al. 2010). The age of the extant *Ephedra* clade has been estimate to about 30 Ma (Ickert-Bond et al. 2009), or possibly as young as 8 Ma (Huang and Price 2003).

However, the results from this study indicate that the *distachya* type of pollen, which I find in sediments as old as 75-50 Ma, has originated only once, and constitutes a synapomorphy for the America/Asia clade. Although this conclusion needs to be backed up by further pollen morphology studies of the whole genus, the present study clearly shows that pollen morphology is promising as a key character for fossil calibration of clades within *Ephedra*, and indicates that the extant *Ephedra* clade is much older than previously thought.

## Conclusions

Two types of *Ephedra* pollen were observed in this study; the *foeminea* type with straight valleys between the exine ridges, and the *distachya* type with branched valleys between the exine ridges. *E. foeminea*, *E. alata*, *E. fragilis*, *E. altissima*, “the Mediterranean” *E. major*, *E. aphylla*, *E. milleri*, *E. foliata* and *E. ciliata* (all of which are excluded from the so called core *Ephedra* clade) have the *foeminea* type of pollen morphology. The *distachya* type is in this study represented by *E. distachya*, *E. major* and *E. viridis*, all members of the core *Ephedra* clade. In my samples, ephedroid pollen, probably of the *foeminea* type, is found from the Late Jurassic. In the literature, similar pollen is described from even older strata (the Permian). *Ephedra* pollen of the *distachya* type appears much later in the fossil record and is in my material first found in a sample that has been difficult to date, but is of Late Cretaceous to early Eocene age. To my knowledge, this is the earliest report of the *distachya* type of pollen in the fossil record. Pollen morphology has potential of being a key character for dating clades within *Ephedra*.

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## References

Arber E. and Parkin J. 1908. The relationship of the angiosperms to the Gnetales. *Annals of Botany* 22 489-515.

Bolinder K. 2011. Pollination mechanisms in *Ephedra* (Gnetales) in north-eastern Greece. Degree project in biology, Master of Science, Uppsala University.

Brewbaker J. L. and Kwack B. H. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany* 50 859-865.

Chase M. W., Soltis D. E., Olmstead R. G., Morgan D., Les D. H., Mishler B. D., Duvall M. R., Price R. A., Hills H. G., Qui Y-L., Kron K. A., Rettig J. H., Conti E., Palmer J. D., Manhart J. R., Sytsma K. J., Michaels H. J., Kress W. J., Karol K. G., Clark W. D., Hedrén M., Gaut B. S., Jansen R. K., Kim K-J., Wimpee C. F., Smith J. F., Furnier G. R., Strauss S. H., Xiang Q-Y., Plunkett G. M., Soltis P. S., Swensen S. M., Williams S. E., Gadek P. A., Quinn C. J., Eguiarte L. E., Golenberg E., Learn G. H., Graham S. W., Barrett S. C. H., Dayanandan S., and Albert V. 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.-M., Parkinson C. L., Cheng Y., Vincent T. M. and Palmer J. D. 2000. Seed plant phylogeny inferred from all three plant genomes: monophyly of extant gymnosperms and origin of Gnetales and conifers. *Proceedings of the National Academy of Sciences of the United States of America* 97 4086-4091.
- Christopherson R. W. 2011. *Geosystems*. The United States, Pearson Education Inc, 623 pp.
- Crane P. R. 1985. Phylogenetic analyses of seed plants and the origin of angiosperms. *Annals of the Missouri Botanical Garden* 72 716-793.
- Crane P. R. and Upchurch G. R. 1987. *Drewria potomacensis* gen. et sp. nov., an early Cretaceous member of Gnetales from the Potomac group of Virginia. *American Journal of Botany* 74 1722-1736.
- Crane P. R. and Lidgard S. 1989. Angiosperm Diversification and Paleolatitudinal Gradients in Cretaceous Floristic Diversity. *Science* 246 675-678.
- Crane P. R. 1996. The fossil history of the Gnetales. *International Journal of Plant Sciences* 157 50-57.
- El-Ghazaly G. and Rowley J. R. 1997. Pollen wall of *Ephedra foliata*. *Palynology* 21 7-18.
- El-Ghazaly G., Rowley R. and Hesse M. 1998. Polarity, aperture condition and germination in pollen grains of *Ephedra* (Gnetales). *Plant Systematics and Evolution* 213 217-231.
- Farris J. S. 1970. Methods for computing Wagner trees. *Systematic Biology* 19 82-92.
- Freitag H. and Maier-Stolte M. 1994. Ephedraceae. In Browicz (Ed) *Chorology of Trees and Shrubs in South-West Asia and Adjacent regions*. Volume 10. Pages 5-38. Warszawa, Polish Scientific Publishers.
- Friis E. M., Crane P. and Raunsgaard Pedersen K. 2011. *Early Flowers and Angiosperm Evolution*. Cambridge, Cambridge University press, 585 pp.
- Hesse M. 2009. *Pollen Terminology: an illustrated handbook*. Wien, Springer, 261 pp.
- Hooker J. D. 1863. I. On *Welwitschia*, a new genus of Gnetaceae. *Transactions of the Linnean Society of London* 24 1-48.
- Hoorn C., Straathof J., Abels H. A., Xu Y., Utescher T. and Dupont-Nivet G. 2012. A late Eocene palynological record of climate change and Tibetan Plateau uplift (Xining Basin, China). *Palaeogeography, Palaeoclimatology, Palaeoecology* 344-345 16-38.
- Horton B. K., Dupont-Nivet G., Zhou J., Waanders G. L., Butler R. F. and Wang J. 2004. Mesozoic-Cenozoic evolution of the Xining-Minhe and Dangchang basins, northeastern Tibetan Plateau: Magnetostratigraphic and biostratigraphic results. *Journal of Geophysical Research* 109 B04402.
- Huang J. and Price R. A. 2003. Estimation of the age of extant *Ephedra* using chloroplast *rbcl* sequence data. *Molecular Biology and Evolution* 20 435-440.

- Ickert-Bond S. M., Skvarla J. J. and Chissoe W. F. 2003. Pollen dimorphism in *Ephedra* L. (Ephedraceae). *Review of Palaeobotany and Palynology* 124 325-334.
- Ickert-Bond S. M. and Wojciechowski M. F. 2004. Phylogenetic relationships in *Ephedra* (Gnetales): Evidence from nuclear and chloroplast DNA sequence data. *Systematic Botany* 29 834-849.
- Ickert-Bond S., Rydin C. and Renner S. S. 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.
- Krassilov V. A. New floral structure from the Lower Cretaceous of Lake Baikal area. *Review of Palaeobotany and Palynology* 47 9-16.
- Kubitzki K. 1990. Ephedraceae. In: Kubitzki K., Kramer K. U. and Green P. S. (Eds.) *The families and genera of vascular plants. Vol.1, Pteridophytes and Gymnosperms*, Berlin, Springer-Verlag 404 pp.
- Källersjö M., Farris J. S., Chase M., Bremer B., Fay M. F., Humphries C. J. Petersen G., Seberg O. and 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.
- Maheshwari P. 1935. Contributions to the morphology of *Ephedra foliata*, Boiss. I. The development of the male and female gametophytes. *Proceedings of the Indian Academy of Sciences* 1 586-606.
- Mathews S. 2009. Phylogenetic relationships among seed plants: Persistent questions and the limits of molecular data. *American Journal of Botany* 96 228-236.
- Mathews S., Clements M. D. and Beilstein M. A. 2010. A duplicate gene rooting of seed plants and the phylogenetic position of flowering plants. *Philosophical Transactions of the Royal Society* 365 383-395.
- Mehra P. N. 1938. The germination of pollen grains in artificial cultures in *Ephedra foliata* Boiss and *Ephedra gerardiana* Wall. *Proceedings of the Indian Academy of Sciences* 8 212-230.
- Moore P. D., Webb J. A. and Collinson M. E. 1991. *Pollen analysis*. Oxford, Blackwell Scientific. 216 pp.
- Niklas K. J. and Buchmann S. L. 1987. The aerodynamics of pollen capture in two sympatric *Ephedra* species. *Evolution* 41 104-123.
- Osborn J. M., Taylor T. N. and de Lima M. R. 1993. The ultrastructure of fossil ephedroid pollen with gnetalean affinities from the Lower Cretaceous of Brazil. *Review of Palaeobotany and Palynology* 77 171-184.

- Punt W., Hoen P. P., Blackmore S., Nilsson S. and Le Thomas A. 2007. Glossary of pollen and spore terminology. *Review of Palaeobotany and Palynology* 143 1-81.
- Raubeson L. A., McCoy S. K. R., Müller K., Wall P. K., Leebens-Mack J., Boore J. L. Jansen R. K. and dePamphilis C. W. 2006. Seed plant phylogeny based on sequences from 61 (mostly) shared plastid genes. *Botany 2006, California, USA, Abstract* 515.
- Rydin C., Källersjö M. and Friis E. M. 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* 170 1031-1043.
- Rydin C., Pedersen K. R. and Friis E. M. 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. and Friis E. M. 2005. Pollen germination in *Welwitschia mirabilis* Hook. f.: differences between the polyplicate pollen producing genera of the Gnetales. *Grana* 44 137-141.
- Rydin C., Raunsgaard Pedersen K., Crane P. and Friis E. M. 2006. Former diversity of *Ephedra* (Gnetales): evidence from Early Cretaceous seeds from Portugal and North America. *Annals of Botany* 98 123-140.
- Rydin C. and Korall P. 2009. Evolutionary relationships in *Ephedra* (Gnetales), with implications for seed plant phylogeny. *International Journal of Plant Sciences* 170 1031-1043.
- Rydin C., Khodabandeh A. and Endress P. K. 2010. The female reproductive unit of *Ephedra* (Gnetales): comparative morphology and evolutionary perspectives. *Botanical Journal of the Linnean Society* 163 387-430.
- Schmidt M. and Schneider-Poetsch H. A. W. 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.
- Scott R. A. 1960. Pollen of *Ephedra* from the Chinle formation (Upper Triassic) and the genus *Equisetosporites*. *Micropaleontology* 6 271-276.
- Steeves M. W. and Barghoorn E. S. 1959. The Pollen of *Ephedra*. *Journal of the Arnold Arboretum Harvard University* 40 221-255.
- Stefanovic S., Jager M., Deutsch J., Broutin J. and Masselot M. 1998. Phylogenetic relationships of conifers inferred from partial 28S rRNA gene sequences. *American Journal of Botany* 85 688-697.
- Wang Z.-Q. 2004. A new Permian gnetalean cone as fossil evidence for supporting current molecular phylogeny. *Annals of Botany* 94 281-288.
- Wilson L. R. 1962. Permian plant microfossils from the flowerpot formation, Oklahoma. *Oklahoma Geological Survey Bulletin* 49 5-50.

Yang Y. 2010. A Review of Gnetalean Megafossils: Problems and Perspectives. *Taiwania* 55 346-354.

Yao Y.-F., Xi Y.-Z., Geng B.-Y. and Li C.-S. 2004. The exine ultrastructure of pollen in *Gnetum* (Gnetaceae) from China and its bearing on the relationship with the ANITA Group. *Botanical Journal of the Linnean Society* 146 415-425.

## Appendixes

### Appendix 1. Collections made during the field trip to Croatia (1-10 July 2013).

Voucher	Date	Location	Coordinates	Taxon
Lena Norbäck Ivarsson and Olle Thureborn 101	2/7-2013	Dalmatia, close to Slime, along smaller road. On cliffs.	43°24.521'N 16°53.370'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 102	2/7 – 2013	Dalmatia, close to Slime, along smaller road. On cliffs.	43°24.521'N 16°53.370'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 103	3/7 – 2013	Dalmatia, along D8, in the village Pisak. On stone wall.	43°24.135'N 16°51.376'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 104	3/7 – 2013	Dalmatia, along D8, in Ruskamen, next to exit from camping	43°24.605'N 16°44.682'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 105	4/7 – 2013	Dalmatia, Omiš, on the castle ruins.	43°26.695'N 16°41.595'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 106	4/7 – 2013	Dalmatia, Omiš, on the castle ruins.	43°26.699'N 16°41.589'E	<i>Ephedra foeminea</i> , female
Lena Norbäck Ivarsson and Olle Thureborn 107	4/7 – 2013	Dalmatia, Omiš, on the castle ruins	43°26.675'N 16°41.583'E	<i>Ephedra foeminea</i> , female
Lena Norbäck Ivarsson and Olle Thureborn 108	4/7 – 2013	Dalmatia, Omiš, on the castle ruins	43°26.673'N 16°41.593'E	<i>Ephedra foeminea</i> , female
Lena Norbäck Ivarsson and Olle Thureborn 109	5/7 – 2013	Dalmatia, Split, south side of Mont Marjan, along road Šetalište vana Meštrovića.	43°30.505'N 16°23.932'E	<i>Ephedra</i> sp. Vegetative
Lena Norbäck Ivarsson and Olle Thureborn 110	5/7 – 2013	Dalmatia, Split, on Mont Marjan, along Marangunićevo šetalište.	43°30.520'N 16°23.674'E	<i>Ephedra foeminea</i> , female
Lena Norbäck Ivarsson and Olle Thureborn 111	5/7 – 2013	Dalmatia, Split, on Mont Marjan, along Marangunićevo šetalište.	43°30.527'N 16°23.715'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 112	5/7 – 2013	Dalmatia, Split, on Mont Marjan, along Marangunićevo šetalište.	43°30.527'N 16°23.707'E	<i>Ephedra</i> sp. Vegetative
Lena Norbäck Ivarsson and Olle Thureborn 113	5/7 – 2013	Dalmatia, Split, on Mont Marjan, along Marangunićevo šetalište.	43°30.539'N 16°23.909'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 114	5/7 – 2013	Dalmatia, Split, on Mont Marjan, by the chapel “St Jeronim” (S. Girolamo)	43°30.550'N 16°24.077'E	<i>Ephedra foeminea</i> , male
Lena Norbäck Ivarsson and Olle Thureborn 115	5/7 – 2013	Dalmatia, Split, on Mont Marjan, by the chapel “St Jeronim” (S. Girolamo)	43°30.551'N 16°24.098'E	<i>Ephedra</i> sp. Vegetative
Lena Norbäck Ivarsson and Olle Thureborn 116	7/7 - 2013	Dalmatia, along D8, in Ruskamen, next to exit from camping	43°24.608'N 16°44.656'E	<i>Ephedra foeminea</i> , male