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Pollen morphology of *Ephedra* (Gnetales) and its evolutionary implications

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Abstract
The *Ephedra* lineage can be traced at least to the Early Cretaceous. Its characteristically polyplicate pollen is well-represented in the fossil record and is frequently used as an indicator of paleoclimate. However, despite previous efforts, knowledge about variation and evolution of ephedroid pollen traits is poor. Here, we document pollen morphology of nearly all extant species of *Ephedra*, using a combination of scanning electron microscopy (SEM) and light microscopy (LM), and reconstruct ancestral states of key pollen traits. Our results indicate that the ancestral *Ephedra* pollen type has numerous plicae interspaced by unbranched pseudosulci, while the derived pollen type has branched pseudosulci and (generally) fewer plicae. The derived type is inferred to have evolved independently twice, once along the North American stem branch and once along the Asian stem branch. Pollen of the ancestral type is common in Mesozoic fossil records, especially from the Early Cretaceous, but it is less commonly reported from the Cenozoic. The earliest documentation of the derived pollen type is from the latest Cretaceous, after which it increases strongly in abundance during the Paleogene. The results of the present study have implications for the age of crown group *Ephedra* as well as for understanding evolution of pollination syndromes in the genus.

Keywords: *character evolution, light microscopy, phylogeny, polyplicate, pseudosulci, scanning electron microscopy,* Welwitschia

Pollen plays an important role in the lifecycle of all seed plants. Consequently, pollen characters have shown to be informative in studies of plant evolution and for resolving phylogenies (e.g. Doyle & Le Thomas 1994; Doyle & Endress 2000; Sauquet & Le Thomas 2003), for calibrating molecular dating analyses (Thornhill et al. 2012), as well as for studying plant reproductive biology (Ferguson & Skvarla 1982; Grayum 1986; Osborn et al. 1991; Bolinder et al. 2015). In addition, fossil pollen data are also frequently used for reconstructing past vegetation types and for inferring paleoclimates (Hoorn et al. 2012). Ephedroid pollen (i.e. pollen inferred to have been produced by *Ephedra* (Gnetales) or *Ephedra*-like extinct plants) is characteristically polyplicate, well known from the fossil record, and considered a good indicator of a very dry paleoclimate (Li et al. 2005; Hoorn et al. 2012).

The earliest reported pollen of probable ephedroid affinity dates to the Permian (Wilson 1962; Wang 2004). By the Early Cretaceous, ephedroid pollen had rapidly increased in abundance and distribution

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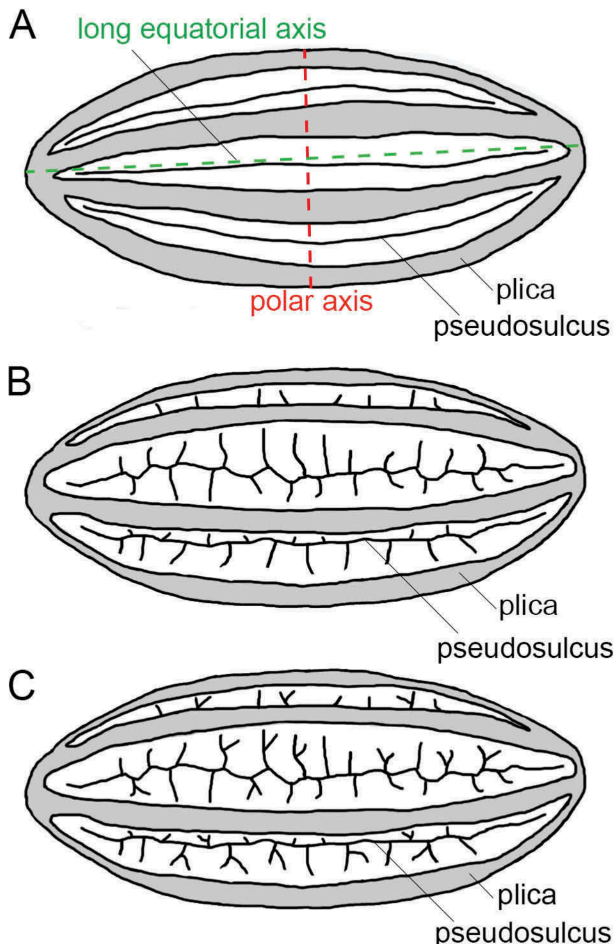


Figure 1. Schematic drawing of the polarity and different types of pseudosulci branching in *Ephedra* pollen. The long equatorial axis is equal to the longest axis and the polar axis is equal to one of the shortest axis. **A.** Pollen with unbranched pseudosulci; the ancestral type. **B.** Pollen with pseudosulci with first-order branching; the derived type. **C.** Pollen with pseudosulci with first- and second-order branching; the derived type.

at low palaeo-latitudes (Crane & Lidgard 1989), and a relatively large diversity of ephedroid megafossils is described from this time (e.g. Krassilov 1986; Yang et al. 2005; Rydin et al. 2006b; Wang & Zheng 2010). Towards the latter part of the Cretaceous, however, the abundance of ephedroid pollen appears to have decreased substantially (Crane & Lidgard 1989). The fossil record after the K-Pg boundary is perhaps less well studied, but there are currently no reported megafossils of ephedroid affinity after the Early Cretaceous. Current knowledge of the Cenozoic diversity of *Ephedra* (before the present) is thus based on the palaeo-palynological record, which is extensive and present also at higher palaeo-latitudes than are Cretaceous records (Akkiraz et al. 2008; Wodehouse 1933; Cookson 1956; Gray 1960; Ghosh et al. 1963; Nagy 1963; Shaw 1998; Hoorn et al. 2012). Today, *Ephedra* comprises about 50 species with remarkably low genetic (Ickert-Bond & Wojciechowski 2004; Rydin & Korall 2009) and morphological (Rydin et al. 2010) diversity, occupying arid regions of the world (Kubitzki 1990). Molecular analyses imply that the clade of extant species dates to the earliest Oligocene (Ickert-Bond et al. 2009; Rydin et al. 2010), and it has been suggested that most of this diversity might be the result of radiation caused by a shift from insect-pollination to wind-pollination early in the evolution of the crown group (Bolinder et al. 2014).

Ephedra pollen is large, between 34 and 81 µm in its longest (equatorial) diameter (Steeves & Barghoorn 1959), and, as in remaining Gnetales, the pollen wall consists of a homogenous tectum, a granular infratectum of varying density, and a thin foot layer adnate to a distinct lamellar endexine (Gullvåg 1966; Van Campo & Lugardon 1973; Hesse 1984; Zavada 1984; Kurmann 1992; Rowley 1995; El-Ghazaly & Rowley 1997; Osborn 2000; Tekleva & Krassilov 2009; Bolinder et al. 2015). Based on developmental studies, Huynh (1975) and El-Ghazaly et al. (1998) concluded that the longest axis in *Ephedra* pollen is equatorial and the polar axis is equal to one of the shortest axes (Huynh 1975; El-Ghazaly et al. 1998) (Figure 1). Although *Ephedra* pollen is typically described as inaperturate (Erdtman 1952; Huynh 1975; Kurmann & Zavada 1994; El-Ghazaly et al. 1998; Ickert-Bond et al. 2003; Doores et al. 2007), some authors have interpreted *Ephedra* pollen as polyaperturate (Steeves & Barghoorn 1959; Bharadwaj 1963), referring to the furrows that run between the plicae parallel to the long equatorial axis. In these furrows, which have been called hyaline lines (Woodhouse 1935; Steeves & Barghoorn 1959; Kedves 1987; Kurmann & Zavada 1994; El-Ghazaly et al. 1998), pseudosulci (Huynh 1975; Bolinder et al. 2015) and colpi (Steeves & Barghoorn 1959; Zhang & Xi 1983), the exine is much thinner than over the ridges and neither the tectum nor the infratectum is present (Osborn 2000; Tekleva & Krassilov 2009; Bolinder et al. 2015). When the pollen germinates, the exine splits open in two of these furrows and detaches from the intine (Land 1907; Mehra 1938; El-Ghazaly et al. 1998), and, based on the polarity described by Huynh (1975) and El-Ghazaly et al. (1998), we will hereafter refer to the furrows as pseudosulci (Figure 1) (following Huynh 1975; Bolinder et al. 2015).

Woodhouse (1935) classified *Ephedra* pollen into two types based on the number of ridges and the appearance of the 'hyaline line' in the grooves (i.e. the pseudosulcus). Later, Steeves and Barghoorn (1959) divided *Ephedra* pollen into four groups (Type A–D) based on the number and appearance of the ridges as well as the presence or absence of 'colpi'. Their type A has an average of five to nine plicae, which are triangular in the transverse section and interspaced by narrowly serpentine, sometimes laterally branched colpi. Pollen of type B has indistinct colpi and an average of 10 to 13 plicae, and type C is similar to B but with higher plicae as seen in the transverse section. Type D shows numerous plicae, up to 20, which are wide and rounded in the transverse section and not interspaced by colpi (Steeves & Barghoorn 1959). Zhang and Xi (1983) merged types B and C of Steeves and Barghoorn (1959), thus recognising three pollen types. In line with Woodhouse (1935), Kedves (1987) and Freitag and Maier-Stolte (1994) recognised only two pollen forms, based on the presence or absence of a 'hyaline line'.

Extensive intraspecific variation and dimorphism in pollen morphology have been reported (El-Ghazaly & Rowley 1997; Ickert-Bond et al. 2003; Doores et al. 2007). It has also been shown recently that the morphology and ultrastructure differ between *Ephedra* pollen of anemophilous and entomophilous species and that these differences influence the aerodynamic properties of the pollen grains (Bolinder et al. 2015). Although pollen of living species of *Ephedra* has been studied previously, few studies have aimed to assess pollen morphology across the entire genus. More importantly, *Ephedra* pollen morphology has never been studied in an evolutionary context. We use a combination of scanning electron microscopy (SEM) and light microscopy (LM) and a much larger sample than previously utilised to study variation and evolution of pollen morphology in extant *Ephedra* and compare the results with available information from ephedroid fossil pollen. We also investigate whether it is possible to assign extant and fossil pollen to specific subclades or species of extant *Ephedra*.

Material and methods

Taxon sampling

We selected 45 species for the present study, spanning the phylogenetic and geographical diversity of the genus and representing about 85% of the species. Pollen from two to five specimens of each species was studied, except for a few species (*Ephedra alata, E. aspera, E. boelckei, E. compacta* and *E. trifurcata*), where limited access to material prevented study of more than one specimen per species. The specimens studied by Steeves and Barghoorn (1959) are currently deposited at the herbaria (A) and (GH) and all specimens still available were included in the present study. For a full list of herbarium accessions, see the 'Specimens investigated' section.

Sample preparation

Both the size and morphology of Gnetales pollen are affected by conventional preparation methods (i.e. acetolysis and staining; Kedves 1987). Therefore, we tested if treatment in alcohol can have the same effect, and if pollen extracted directly from herbarium sheets, without further treatment, is a suitable way of studying natural variation in *Ephedra* pollen. For this purpose, anthetic microsporangiate structures of *E. viridis* were harvested from living plants housed in the glasshouses at Stockholm University. Pollen grains obtained from these plants were treated in five different ways before study: (a) no treatment and examination within one hour of collection (n = 30), (b) air-dried in an envelope for a week to approximate herbarium-dried material (n = 30), (c) placed in 70% ethanol (n = 30), (d) placed in 70% ethanol followed by dehydration with a conventional ethanol series (n = 30), and (e) air-dried for a week to approximate herbarium-dried material, washed with phosphate-buffered saline (PBS) and dehydrated in an ethanol series prior to investigation (n = 30). Following this preparation, the size and morphology of pollen grains were compared among treatments using an analysis of variance (ANOVA) and Tukey's Honestly Significant Differences (HSD) test in R version 3.1. (R Development Core Team 2014). Having established the best way to study natural variation in pollen morphology, anthetic structures were obtained from herbarium material and pollen grains were studied using SEM and LM without any treatment or preparation prior to the investigations.

Scanning electron microscopy and light microscopy

A minimum of 20 pollen grains per species were obtained from herbarium material and mounted on aluminium stubs using double-sided tape, sputter-coated with gold (40 s at 10 mA) and studied under SEM. Abnormal and seemingly aborted pollen was carefully avoided. In addition, one representative specimen of each species was selected for comparative studies using LM. For this purpose, 15 pollen grains of each species were mounted in glycerine and studied under LM with a 40× objective.

The lengths of polar and longest equatorial axes were measured during the SEM studies, and a shape estimate (polar axis/equatorial axis, P/E-ratio) was obtained. The number of plicae was counted on the visible side of the grain and multiplied by two to obtain the total number of plicae. Presence or absence of first- and second-order branches on the pseudosulci (Figure 1) was scored for each pollen grain.

Statistical comparison of pollen characters

Appropriate sample size for each species was controlled according to Van Emden (2008) at 95% power to limit the risk of Type I errors. To determine the best way to analyse the pollen data, the phylogenetic signal in each of the variables was first estimated using the branch length transformation parameter Pagel's lambda, λ (Pagel 1999a), a robust index with low Type I error rates (Freckleton et al. 2002). This allowed assessment of the extent to which interspecific differences in pollen traits are correlated with phylogenetic relatedness. The parameter λ may vary between 1 (if trait variance is perfectly correlated with phylogenetic distance, equivalent to a Brownian Motion model (BM; Schluter et al. 1997) and 0 (there is no relationship between trait variance and phylogenetic distance). Alternatively, λ may assume an intermediate value if there is some degree of phylogenetic dependence in the trait ($0 < \lambda < 1$). The value of λ was determined by comparing the likelihood fit of three different models to each pollen variable (1: $\lambda = 1$, 2: $\lambda = 0$ and 3: λ is estimated during model fitting) as implemented in the R package motmot (Thomas & Freckleton 2012). Model fit was assessed using the Akaike Information Criterion (AIC; Akaike 1974). The pollen variables were absolute size, as assessed by the long equatorial diameter, pollen shape, as gauged by the P/E ratio, and the number of plicae. Phylogenetic information was obtained from Rydin and Korall (2009).

In characters for which no correlation with the phylogeny was established (i.e. the best model is when $\lambda = 0$), variation was compared within and among species and clades (as defined in Figures 13 and 14 later) using a conventional ANOVA, Tukey's HSD and model selection using AIC in R (R Development Core Team 2014). For characters, where variance is correlated with the phylogeny (i.e. λ is significantly different from 0), variation was not compared any further.

Parsimony ancestral state reconstruction of pollen characters

Parsimony reconstruction of ancestral states for each pollen character was conducted based on to date the most well-sampled phylogeny of *Ephedra* (Rydin & Korall 2009), using the Trace Character History command in Mesquite version 2.75 (Maddison & Maddison 2011). These analyses were performed in order to assess the relative amount of evolutionary information in pollen characters. Parsimony was considered appropriate for this because, although it is appropriate for detailed analyses only when transition rates are low (Harvey & Pagel 1991; Pagel 1999b; Pirie et al. 2012), it will still allow determination of which pollen characters display a more conserved phylogenetic pattern. Ancestral states were estimated for the presence or absence of side branches on the pseudosulci using two alternative codings: (a) a binary state option: 0, absence of side branches on the pseudosulci (Figure 1A); or 1, presence of side branches on the pseudosulci (Figure 1B, C), and (b) a multistate approach: 0, complete absence of side branches on the pseudosulci (Figure 1A); 1, presence of first-order side branches (Figure 1B); and 2, presence of first- and second-order side branches on pseudosulci (Figure 1C). To compare observed estimates of gains and losses to those expected by chance, the terminals were shuffled 999 times using the Reshuffle Character command and the character history of each reshuffled character was traced. This allowed a distribution of parsimony steps needed for random characters to be compared with the observed number of steps.

For the continuous characters (number of plicae, length of the polar axis and the P/E ratio), the correlation between minimum, maximum and mean values was tested for, using simple regression with a linear model in R (R Development Core Team 2014). Minimum, maximum and mean values are strongly correlated (number of plicae: $r^2 = 0.96$, $p \ll 0.05$; length of the long equatorial axis: $r^2 = 0.87$, $p \ll 0.05$; P/E ratio: $r^2 = 0.77$, $p \ll 0.05$); therefore, mean values were used to reconstruct ancestral states using parsimony as earlier.

Results

Sample preparation

Size (length of the long equatorial axis). — There is a significant difference in the length of the long equatorial axis among pollen grains treated in different ways prior to investigation ($F_{145,\,4} = 65.4$ $p \ll 0.05$). However, there is no significant difference between freshly collected and air-dried pollen grains (Tukey's HSD; $p = 0.16$). Pollen obtained from herbarium material therefore captures the natural variation in pollen size in *Ephedra* (Figure 2A). There is a significant difference between pollen grains treated with ethanol in various ways compared to fresh and air-dried (Tukey's HSD; fresh versus ethanol $p \ll 0.05$; fresh versus air-dried + dehydration series $p \ll 0.05$; fresh versus ethanol + dehydration series $p \ll 0.05$;

air-dried versus ethanol + dehydration series $p \ll 0.05$; fresh versus air-dried + dehydration series $p \ll 0.05$; air-dried versus air-dried + dehydration series $p \ll 0.05$).

Shape (P/E ratio). — There is a significant difference in the P/E ratio among pollen grains treated in different ways ($F_{145,\ 4} = 27.6$ $p \ll 0.05$). However, there is no significant difference between fresh and air-dried pollen grains (Tukey's HSD; $p = 0.46$; Figure 2B). Further, there is no difference between pollen grains subjected to the different ethanol treatments (Tukey's HSD; ethanol + dehydration series versus ethanol $p = 0.06$; air-dried + dehydration series versus ethanol $p = 0.75$; ethanol + dehydration series versus air-dried + dehydration series $p = 0.55$). A significant difference in P/E ratio between pollen grains treated with ethanol in various ways compared to fresh and air-dried pollen grains (Tukey's HSD; fresh versus ethanol $p \ll 0.05$; air-dried + dehydration series versus fresh $p \ll 0.05$; fresh versus ethanol + dehydration series $p \ll 0.05$; air-dried versus ethanol + dehydration series $p \ll 0.05$; fresh versus air-dried + dehydration series $p \ll 0.05$; air-dried versus air-dried + dehydration series $p \ll 0.05$).

Pollen morphology

Mediterranean species (Figures 3, 7; Table I). — Pollen of Mediterranean species has 10 to 22 plicae extending parallel to the long equatorial axis and fusing at the tips. The plicae are psilate, wide and rounded in transverse section. Between adjacent plicae, there is a distinct or indistinct, unbranched pseudosulcus.

North American species (Figures 4, 8; Table I). — Pollen of *Ephedra californica*, *E. trifurca*, *E. pedunculata* and *E. torreyana* has 10 to 22 plicae that extend parallel to the long equatorial axis and fuse at the tips. The plicae are psilate, wide and rounded in transverse section. Between adjacent plicae there is a distinct or indistinct, unbranched pseudosulcus. Pollen of *E. antisyphilitica* and *E. compacta* has distinct pseudosulci that occasionally have first-order branches. Pollen of *E. aspera*, *E. fasciculata* and *E. funerea* has 5 to 18 narrow plicae that are psilate and triangular in transverse section and the pseudosulci have first-order branching. Pollen of *E. coryi*, *E. cutleri*, *E. nevadensis* and *E. viridis* has 4 to 12 plicae and the pseudosulci are always branched, often with both first- and second-order branches.

South American species (Figures 5, 9; Table I). — Pollen of South American species has 10 to 22 plicae that extend parallel to the long equatorial axis and fuse

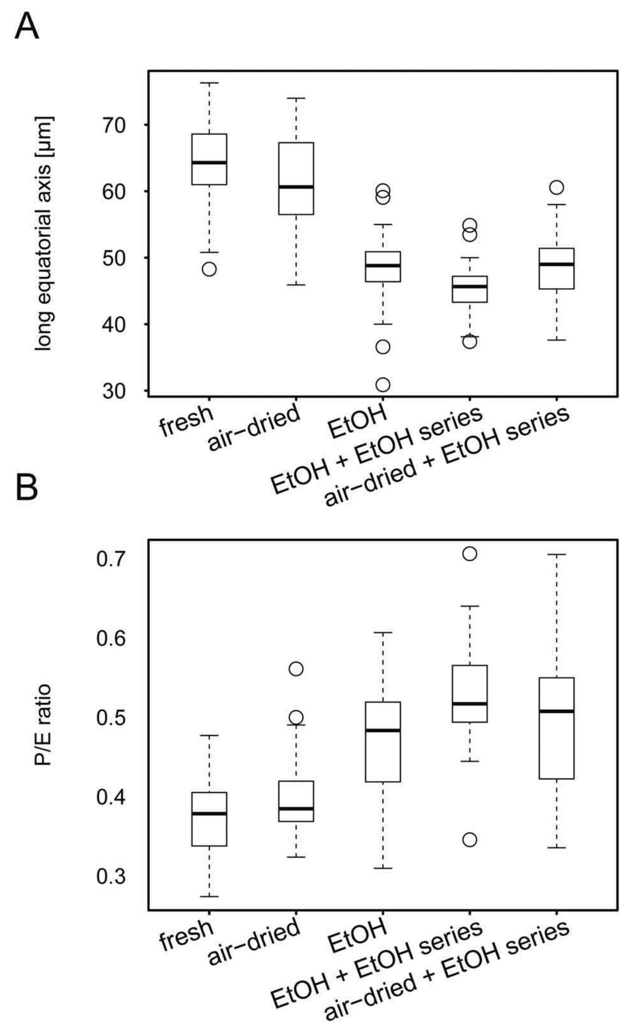


Figure 2. Effect of preparation method on two pollen traits. **A.** Length of the long equatorial axis (in micrometres). There are significant differences between pollen grains treated with ethanol in various ways and untreated pollen. There is no significant difference between fresh and air-dried pollen grains. **B.** P/E ratio. There are significant differences between pollen grains treated with ethanol and untreated pollen. There is no significant difference between fresh and air-dried pollen grains.

at the tips. The plicae are psilate, wide and rounded in transverse section. Between adjacent plicae, there is a distinct or indistinct, unbranched pseudosulcus.

Asian species (Figures 6, 10; Table I). — Pollen of Asian species generally has 4 to 12 plicae that extend parallel to the long equatorial axis and fuse at the tips. The plicae are psilate, narrow and triangular in transverse section. Between adjacent plicae there is a distinct, branched pseudosulcus that often shows first- and second-order branches. Pollen of *Ephedra likangensis*, *E. lomatolepis*, *E. minuta* and *E. saxatilis*

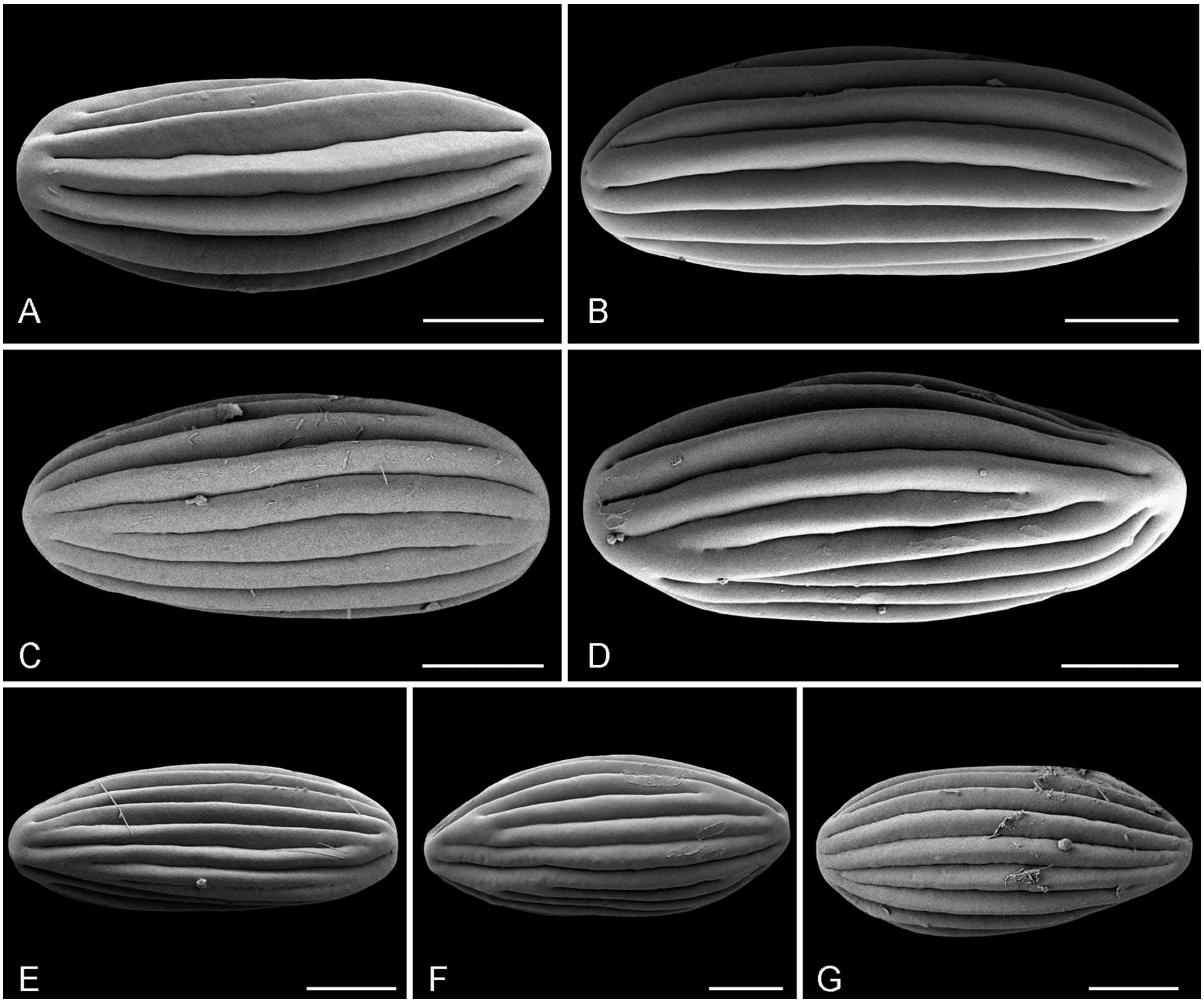


Figure 3. Pollen of *Ephedra*, Mediterranean species, scanning electron micrographs. **A.** *E. foeminea*, R Pampino & R Pichi-Sermolli 139 (L). **B.** *E. alata* AA Anderberg 480 (S). **C.** *E. altissima* H Freitag 35035 (KAS). **D.** *E. aphylla* G Samuelsoson 2696 (S). **E.** *E. ciliata* Handel-Mazetti 973 (WU). **F.** *E. foliata* Hedberg & Hedberg 92019A (UPS). **G.** *E. milleri* Miller 7667A (E). Scale bars – 10 μm.

has 10 to 20 plicae and branched pseudosulci (first-order branches only), deviating from the general pattern. The same holds for pollen of *E. sarcocarpa, E. strobilacea* and *E. transitoria*, which has 10 to 22 plicae and pseudosulci that are never branched.

Statistical comparison of pollen characters among species

In *Ephedra*, variance in the number of plicae is correlated with phylogenetic distance, i.e. the lambda model showed the best fit ($\lambda = 0.61$; 95% confidence interval: 0.23–0.86; ΔAIC = 30.4 compared to BM and ΔAIC = 11.7 compared to the model with $\lambda = 0$). It is clear that number of plicae varies considerably among species (Figure 12A) as well as among the different clades and pollen types (Figures 11A, B).

Variation among species in both pollen size (as gauged by the long equatorial diameter) and pollen shape (estimated by the P/E ratio) is independent of phylogeny. For both variables, this model ($\lambda = 0$) was much better than BM (ΔAIC = 24.4 [pollen size], ΔAIC = 33.4 [pollen shape]) and indistinguishable from the lambda model (ΔAIC = 0.0 [pollen size], ΔAIC = 1.21 [pollen shape]). Consequently, we used a conventional one-way ANOVA of independent groups, Tukey's HSD and model selection using AIC to compare pollen size and pollen shape within and among species and clades.

The size varies significantly among the different clades $F_{3,4018} = 32.5$, $p \ll 0.05$; only the North American clade (hereafter referred to as defined in Figures 13 and 14, excluding *Ephedra pedunculata*) do not differ significantly from Mediterranean

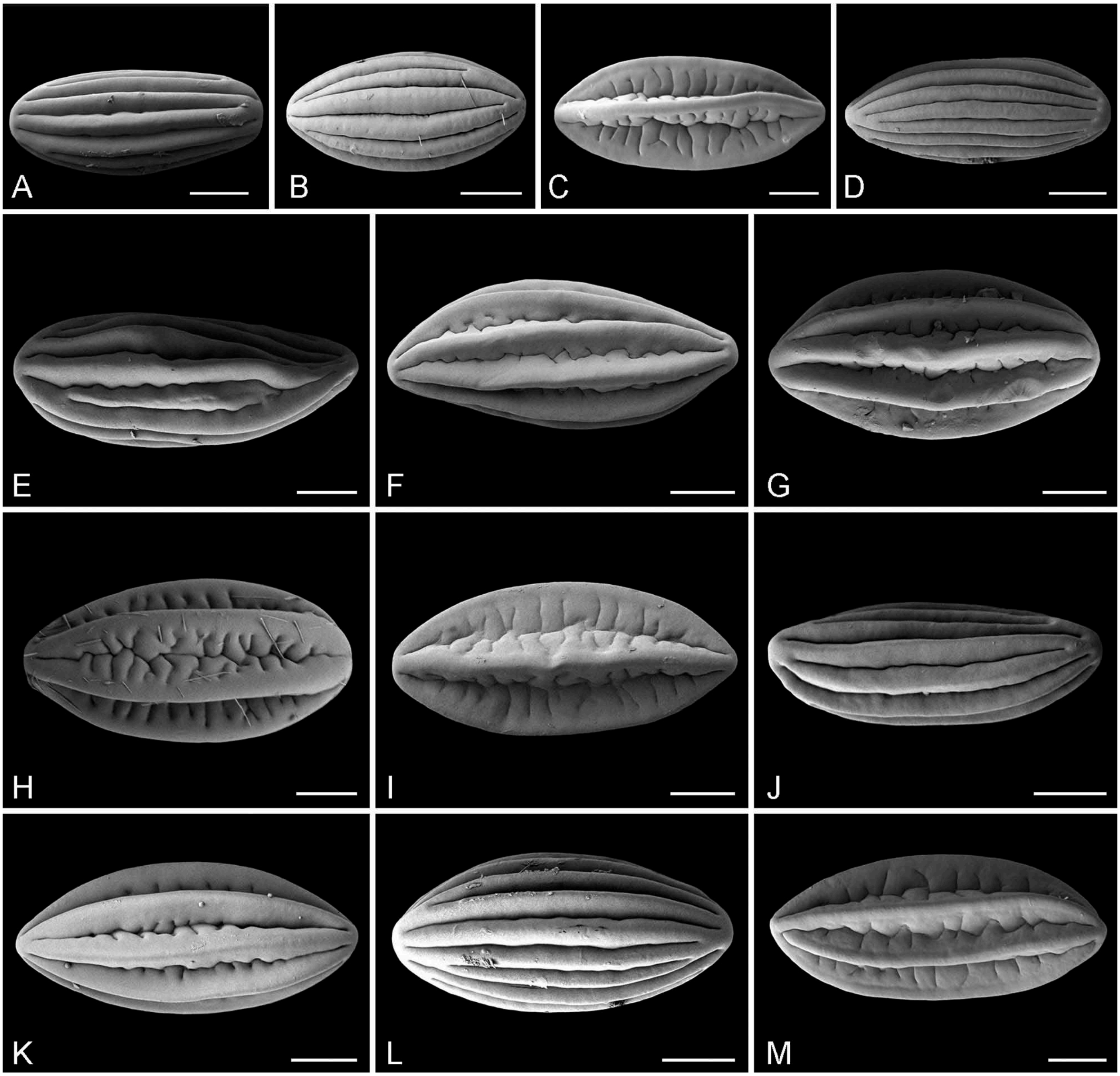


Figure 4. Pollen of *Ephedra*, North American species, scanning electron micrographs. **A.** *E. peduculata* RM Stewart 2265 (GH). **B.** *E. compacta* DS Corell & IM Johnston 20233 (NY). **C.** *E. viridis* LS Rose 58080 (S). **D.** *E. trifurca* CV Hartman 642 (GH). **E.** *E. californica* A Carter 3667 (L). **F.** *E. fasciculata* JH Lehr 2309 (NY). **G.** *E. aspera* BA Stein 31 (RSA). **H.** *E. nevadensis* P Raven 14251 (WU). **I.** *E. cutleri* CT Mason Jr. 2192 (TEX). **J.** *E. torreyana* Spellenberg 10204 (TEX). **K.** *E. funerea* CL Hitchcock 329 (GH). **L.** *E. antisyphilitica* Henderson 62-02a (BR). **M.** *E. coryi* DS Corell 32785 (S). Scale bars – 10 µm.

species ($p = 0.41$) (Figure 11B). Pollen size also differs significantly among species $F_{3,3977} = 46.46$, $p \ll 0.05$, as well as within some of the species (Figure 12B). However, the variation in size among species is significantly larger than among clades or within species (ΔAIC = 1489 [among clades], ΔAIC = 0 [among species], ΔAIC = 1572 [within species]). Also, pollen shape differs significantly among the different clades $F_{3,4018} = 48.43$, $p \ll 0.05$; but North American species do not differ significantly from South American species ($p = 0.41$) or Mediterranean species ($p = 0.09$) (Figure 11E). Pollen shape varies significantly among species $F_{44,3977} = 26.06$, $p \ll 0.05$ (Figure 12C), but the variation in shape is greater among species than among clades or within species (ΔAIC = 794 [among clades], ΔAIC = 0 [among species], ΔAIC = 930 [within species]).

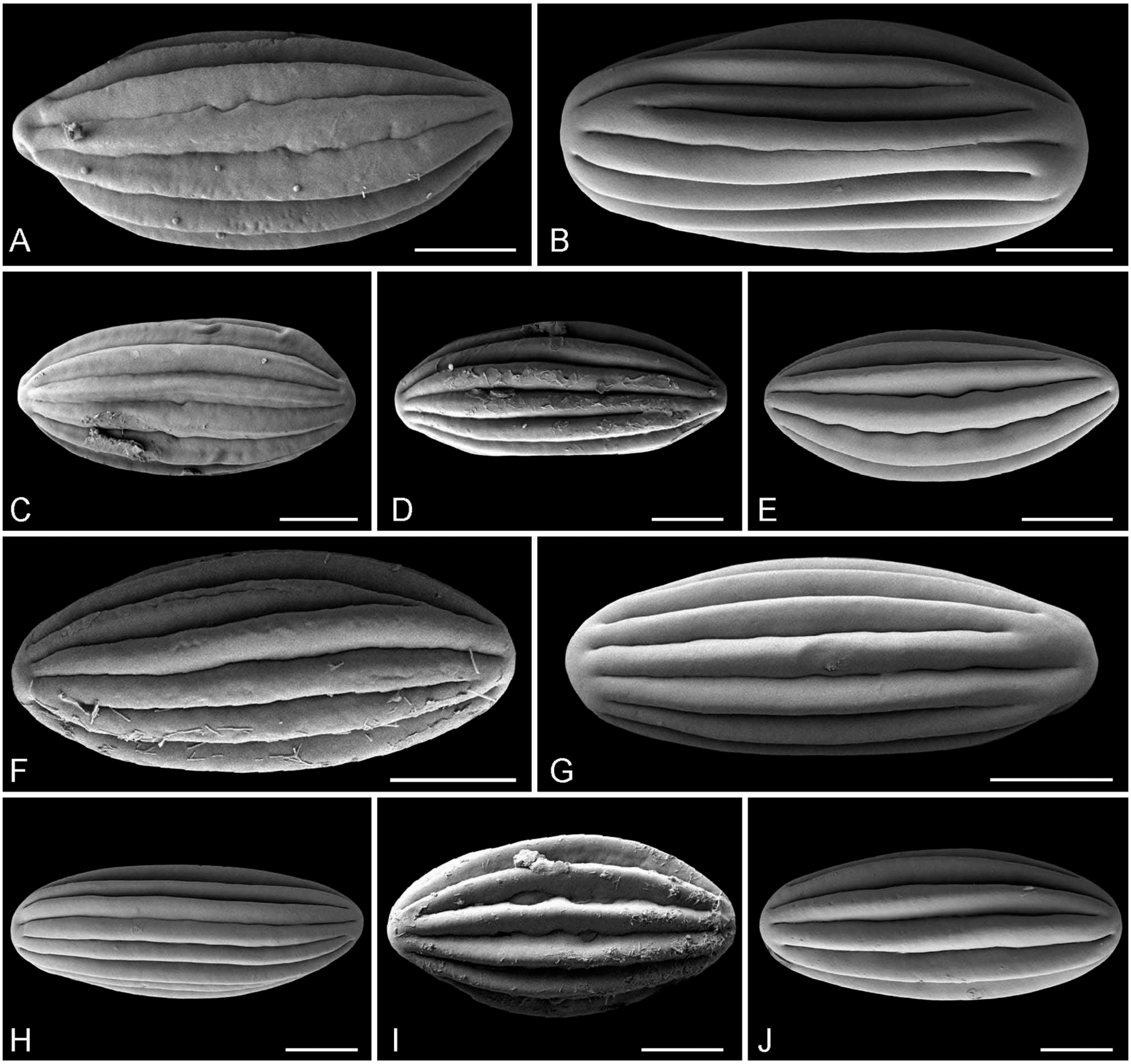


Figure 5. Pollen of *Ephedra*, South American clade, scanning electron micrographs. **A.** *E. americana* Gerth s.n. (L). **B.** *E. breana* KH & W Rechinger 63547 (W). **C.** *E. chilensis* C. Skottsberg 987 (F). **D.** *E. frustillata* MP Moreno 236 (NY). **E.** *E. multiflora* J Chiapella 2344 & E Vitek 09–0359 (W). **F.** *E. boelckei* Maas et al. 8184 (GB). **G.** *E. ochreata* RH Fortunato 5413 (NY). **H.** *E. triandra* J Chiapella 2505 & E Vitek 09–0520 (W). **I.** *E. trifurcata* O Zöllner 7928 (L). **J.** *E. tweediana* JH Hunzinker 1648 (S). Scale bars – 10 µm.

Ancestral state reconstruction of discrete pollen characters

Presence of side branches on pseudosulci. — There are five parsimony steps in the observed data compared to 7–20 for randomised data. Thus, the observed estimate does not overlap with the distribution of estimates for the random traits. Side branches are inferred to have evolved twice, once along the stem branch of the Asian clade and once along the stem branch of the North American species except *Ephedra pedunculata*. Side branches have subsequently been lost once among Asian species and twice among North American species (Figure 13A) and some species show a reversal to the ancestral state of having no side branches. Dividing the character further, and discriminating between having first-order branching only and first- and second-order branching of pseudosulci, 12 steps are inferred for the observed data compared to 12–20 for the randomised data. Thus, the observed estimate overlaps with the distribution of estimates for the random traits at the

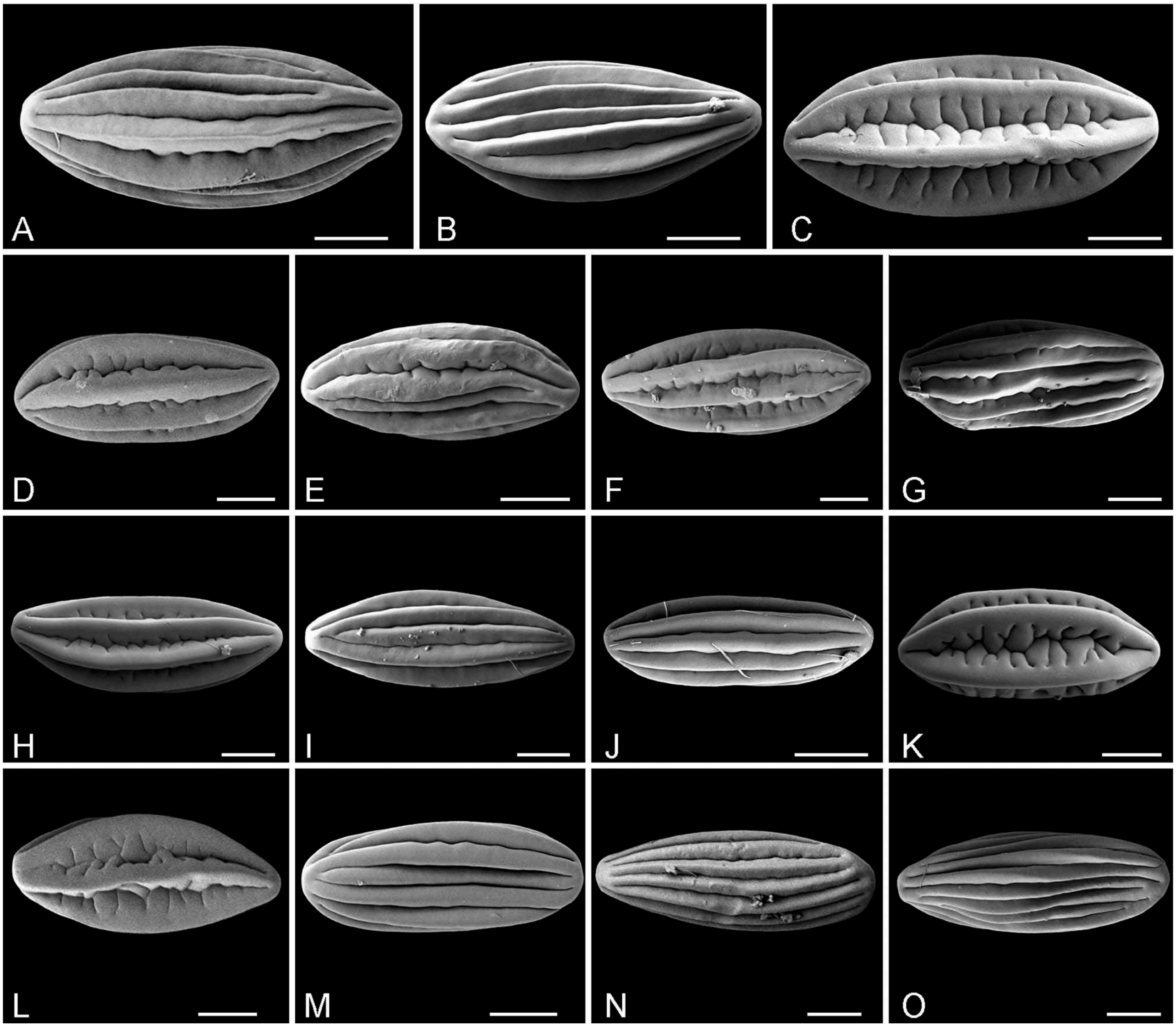


Figure 6. Pollen of *Ephedra*, Asian clade, scanning electron micrographs. **A.** *E. likiangensis* G Forrest 5564 (BM). **B.** *E. minuta* B Dikoré 8457 (MSB). **C.** *E. equisetina* QR Wu (MO). **D.** *E. gerardiana* Walter Koelz 5310 (S). **E.** *E. monosperma* H Freitag 33068 (KAS). **F.** *E. pachyclada* KH & F. Rechinger 3676 (W). **G.** *E. saxatilis* Parkinson 7077 (S). **H.** *E. intermedia* Lindberg 117–1947 (W). **I.** *E. lomatolepis* II Rusanovick & LA Krai 56 (NY). **J.** *E. regeliana* H Hastman 26 (MSB). **K.** *E. sinica* Y Yang 99531 (PE) **L.** *E. distachya* J Prudhomme 89 (WU). **M.** *E. sarcocarpa* KH Rechinger 46054b (W). **N.** *E. strobilacea* KH & F Rechinger 2703 (S). **O.** *E. transitoria* Doppelbaur 190 (M). Scale bars – 10 μm.

99th percentile. Second-order branching is inferred to have evolved once in the Asian clade and to have been lost twice. In addition, side branches have been entirely lost in a small clade, comprising *E. strobilacea*, *E. sarcocarpa* and *E. transitoria*. In the clade comprising the North American species except *E. pedunculata*, second-order branching is inferred to have originated at least once and to have been lost again at least once. In addition, and as in the Asian clade, reversals back to unbranched pseudosulci have occurred several times among the North American species (Figure 13B).

Ancestral state reconstruction of continuous pollen characters

Mean number of plicae. — The ancestral state in *Ephedra* is numerous plicae (14.8–16.14). During the course of evolution, there has been a general trend towards fewer plicae in all clades, but a few species are inferred to have evolved an even greater number of plicae (up to about 20; Figure 14A).

Mean length of the long equatorial axis (size). — There is ample size variation within and among species (Figure 12B) and most species have a mean equatorial

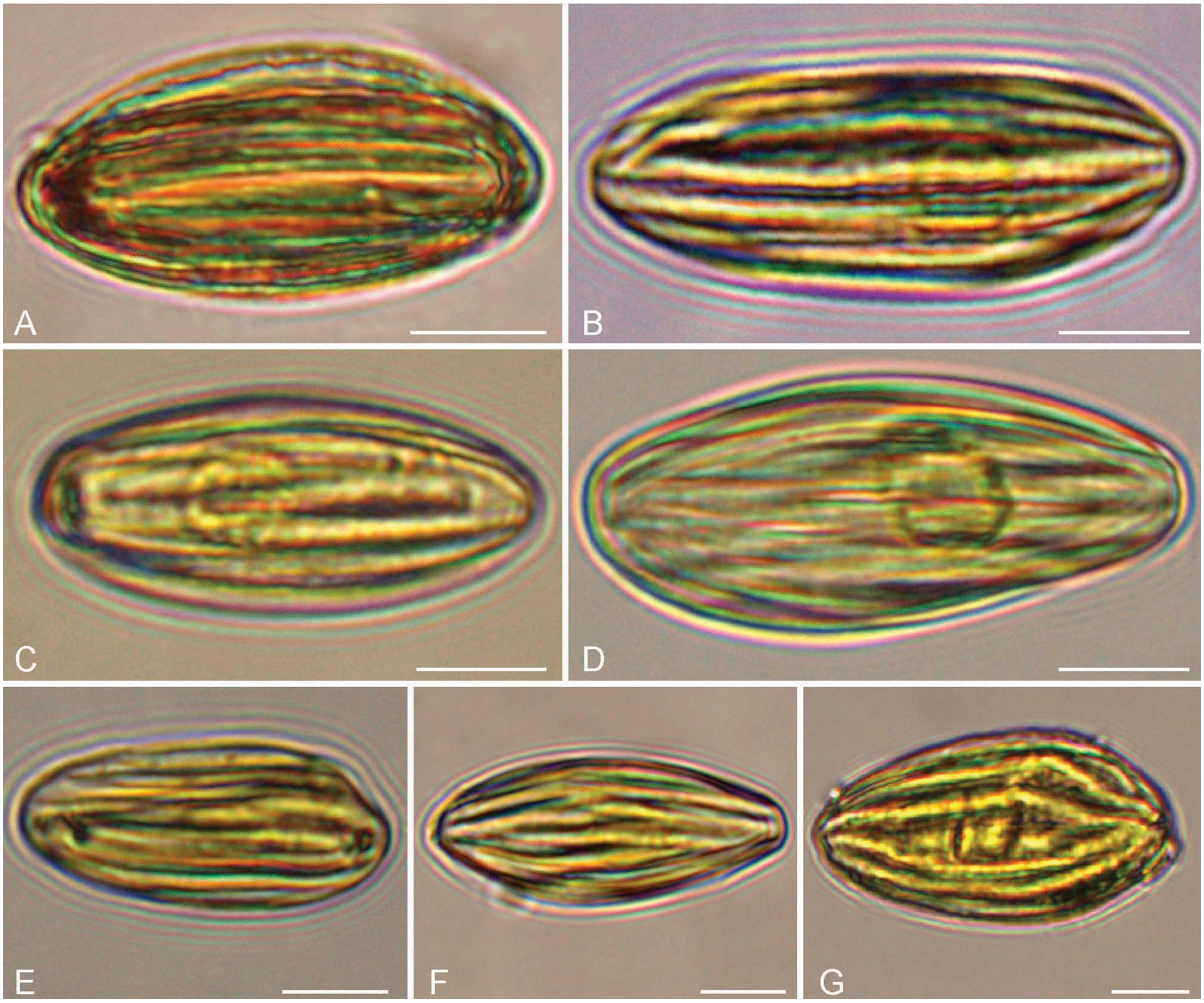


Figure 7. Pollen of *Ephedra*, Mediterranean species, light micrographs. **A.** *E. foeminea* R Pampino & R Pichi-Sermolli 139 (L). **B.** *E. alata* AA Anderberg 480 (S). **C.** *E. altissima* H Freitag 35035 (KAS). **D.** *E. aphylla* G Samuelsoson 2696 (S). **E.** *E. ciliata* Handel-Mazetti 973 (WU). **F.** *E. foliata* Hedberg & Hedberg 92019A (UPS). **G.** *E. milleri* Miller 7667A (E). Scale bars – 10 µm.

diameter ranging between 32.8 and 49.8 µm, although a few unrelated species stand out as having smaller (*Ephedra lomatolepis*, *E. boelckei* and *E. funerea*) or larger (*E. alata*, *E. sarcocarpa*, *E. viridis* and *E. nevadensis*) pollen grains.

Mean P/E ratio (shape). — The ancestral state in *Ephedra* is a P/E ratio of 0.42 to 0.44, a state shared by most Mediterranean, South American and North American species. Among Asian species, a somewhat smaller P/E ratio (0.39–0.41) is more common (Figure 14C).

Discussion

Pollen morphology

Pseudosulci. — The appearance of the pseudosulci is the most important pollen morphological difference among species of *Ephedra*, and perhaps also the most important character from an ecological and evolutionary perspective. The ancestral pollen type in *Ephedra* lacks side branches of the pseudosulci, whereas pollen with branched pseudosulci represents a derived pollen type. The only *Ephedra* species known to be insect-pollinated (*E. foeminea*, Bolinder et al. 2014, and perhaps also *E. aphylla*, Bino et al.

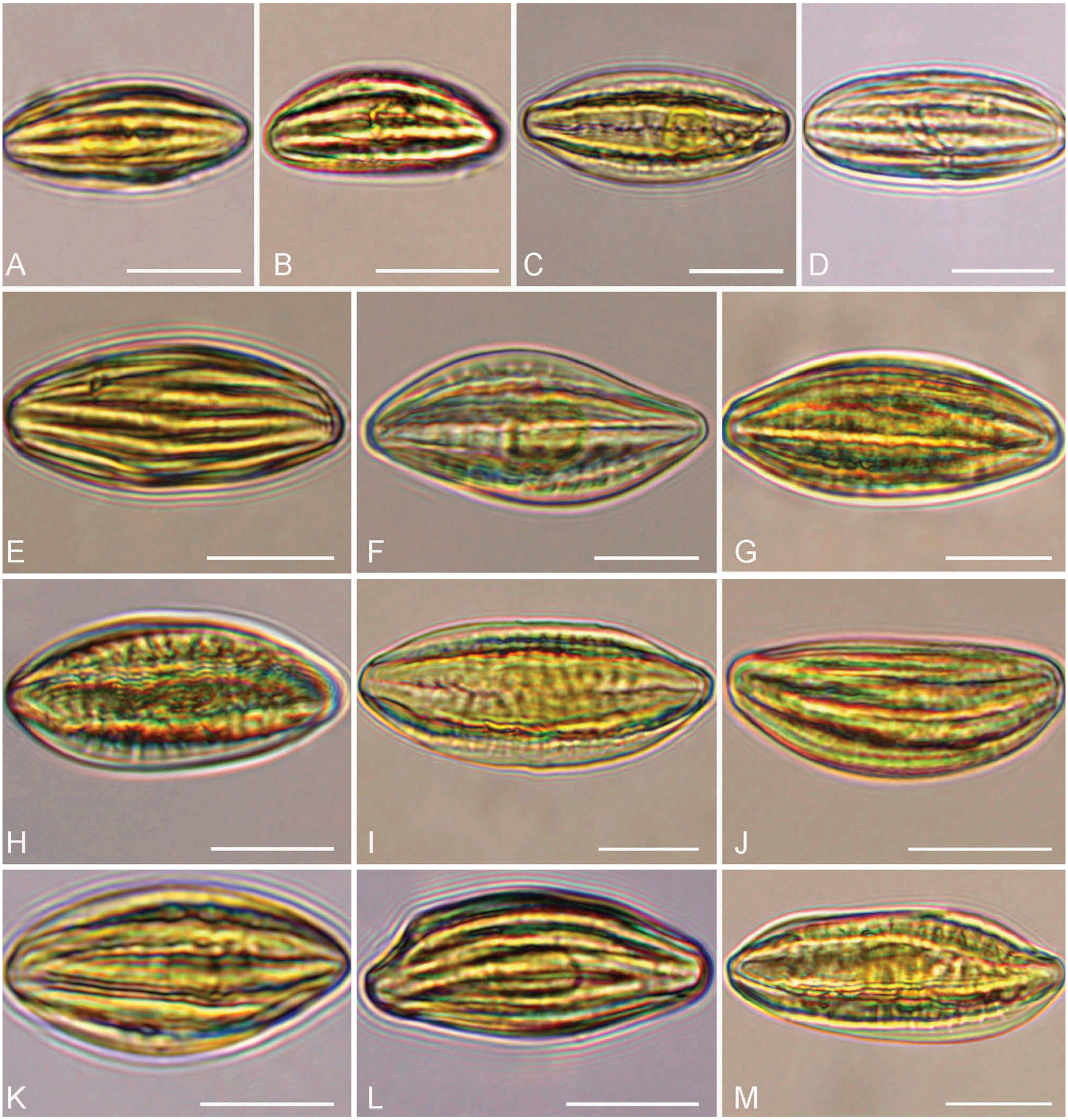


Figure 8. Pollen of *Ephedra*, North American species, light micrographs. **A.** *E. pedunculata* RM Stewart 2265 (GH). **B.** *E. compacta* DS Corell & I. M. Johnston 20233 (NY) **C.** *E. viridis* LS Rose 58080 (S). **D.** *E. trifurca* CV Hartman 642 (GH) **E.** *E. californica* A Carter 3667 (L). **F.** *E. fasciculata* JH Lehar 2309 (NY). **G.** *E. aspera* BA Stein 31 (RSA). **H.** *E. nevadensis* Raven 14251 (WU). **I.** *E. cutleri* CT Mason jr. 2192 (TEX). **J.** *E. torreyana* Spellenberg 10204 (TEX). **K.** *E. funerea* CL Hitchcock 329 (GH). **L.** *E. antisyphilitica* Henderson 62-02a (BR). **M.** *E. coryi* DS Corell 32785 (S). Scale bars – 10 µm.

1984) show the ancestral pollen type (Bolinder et al. 2014, 2015; Rydin & Bolinder 2015) and has a denser ultrastructure and therefore a reduced flight capacity compared to the derived pollen type (Bolinder et al. 2015). However, the correlation between pollination syndrome and pollen morphology is ambiguous, because pollen of some putative anemophilous species (i.e. *E. trifurca*; Niklas et al. 1986; Niklas & Kerchner 1986; Buchmann et al. 1989; Niklas 2015) is also of the ancestral pollen type.

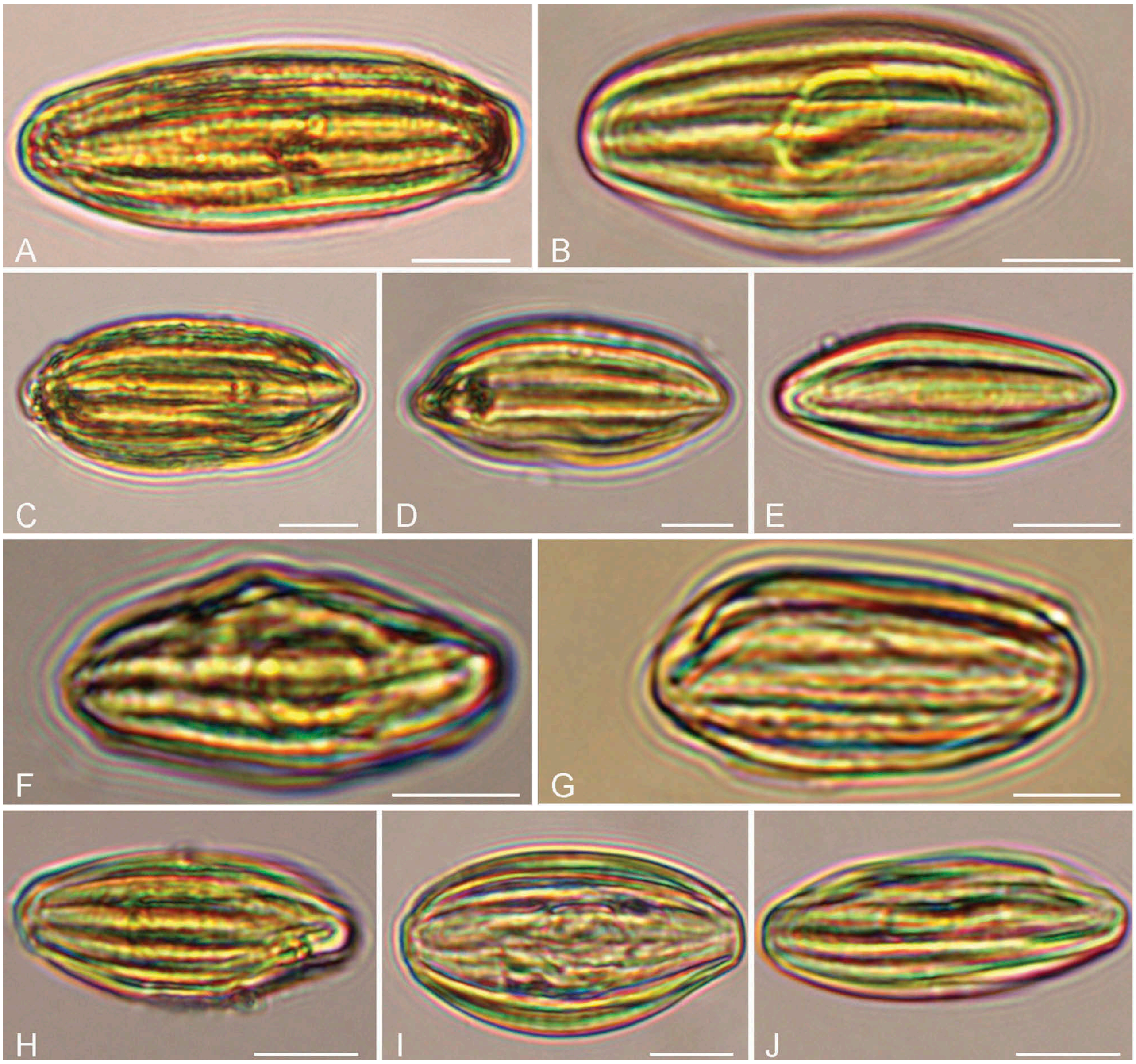


Figure 9. Pollen of *Ephedra*, South American clade, light micrographs. **A.** *E. americana* Gerth s.n. (L). **B.** *E. breana* KH & W Rechinger 63547 (W). **C.** *E. chilensis* C. Skottsberg 987 (F). **D.** *E. frustillata* MP Moreno 236 (NY). **E.** *E. multiflora* J Chiapella 2344 & E Vitek 09–0359 (W). **F.** *E. boelckei* Maas et al. 8184 (GB). **G.** *E. ochreata* RH Fortunato 5413 (NY). **H.** *E. triandra* J Chiapella 2505 & E Vitek 09–0520 (W). **I.** *E. trifurcata* O Zöllner 7928 (L). **J.** *E. tweediana* JH Hunzinker 1648 (S). Scale bars – 10 μm.

Branched pseudosulci (the derived pollen type) appear to have evolved twice independently, once along the branch leading to the Asian clade and once along the branch leading to the North American clade (excluding *Ephedra pedunculata*; Figure 13). This indicates that there might, in fact, be two separate kinds of derived pollen types in *Ephedra*; however, we find it impossible to distinguish the derived pollen type of Asian species from that of North American species, suggesting, instead, convergent evolution of identical pollen types. South American species, as well as the North American *E. pedunculata*, appear to have retained the ancestral pollen type, and their pollen cannot be distinguished from that of the Mediterranean species or from a few Asian and other North American species. It is thus not possible to identify species based solely on pollen morphology; not even assignment to a particular subclade of *Ephedra* is possible. For example, although first-order side branches on the pseudosulci are nearly universally present among North American and Asian species, the feature has been lost once in the Asian clade and at least twice in the North American clade (Figure 13A). Second-order

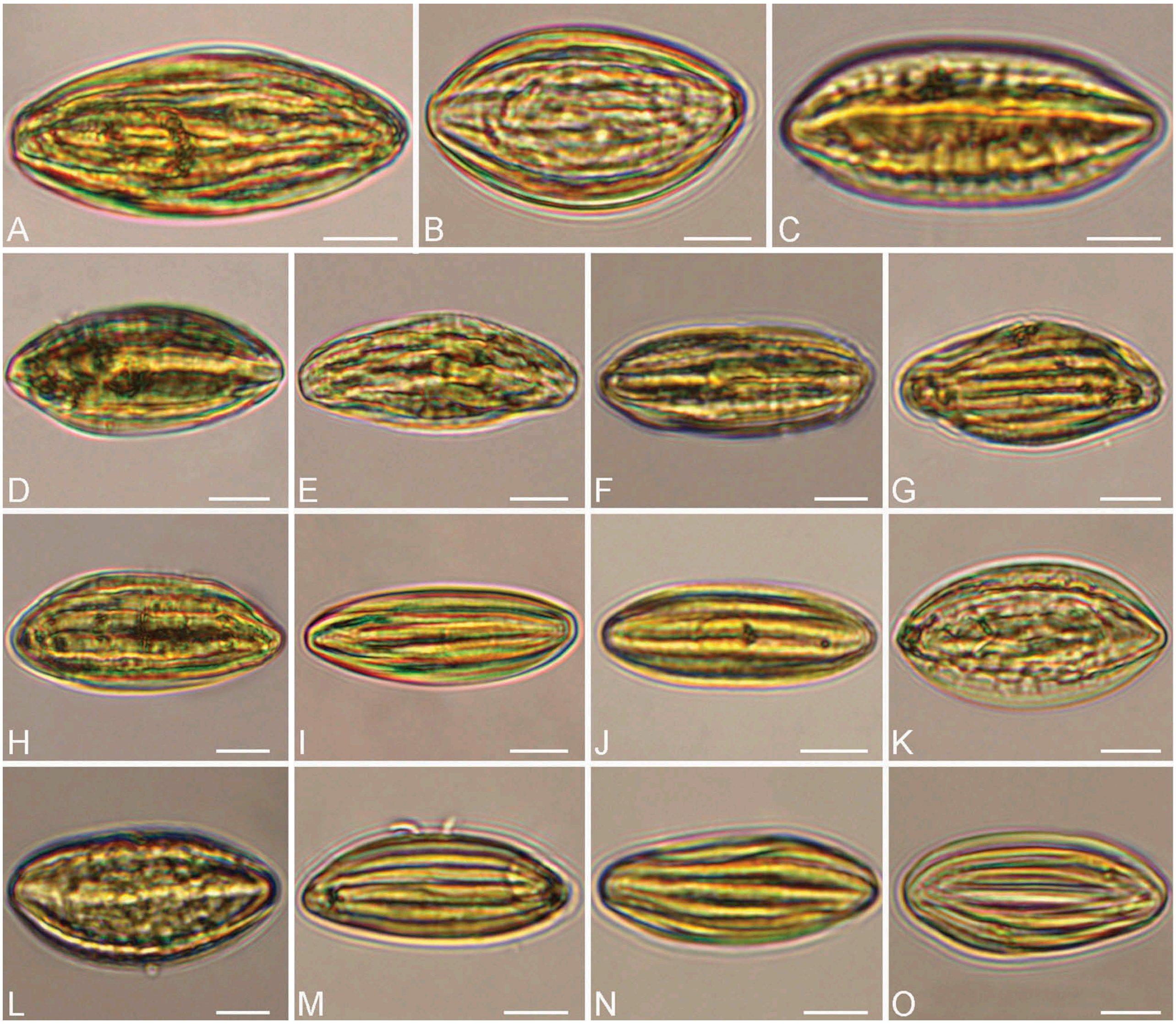
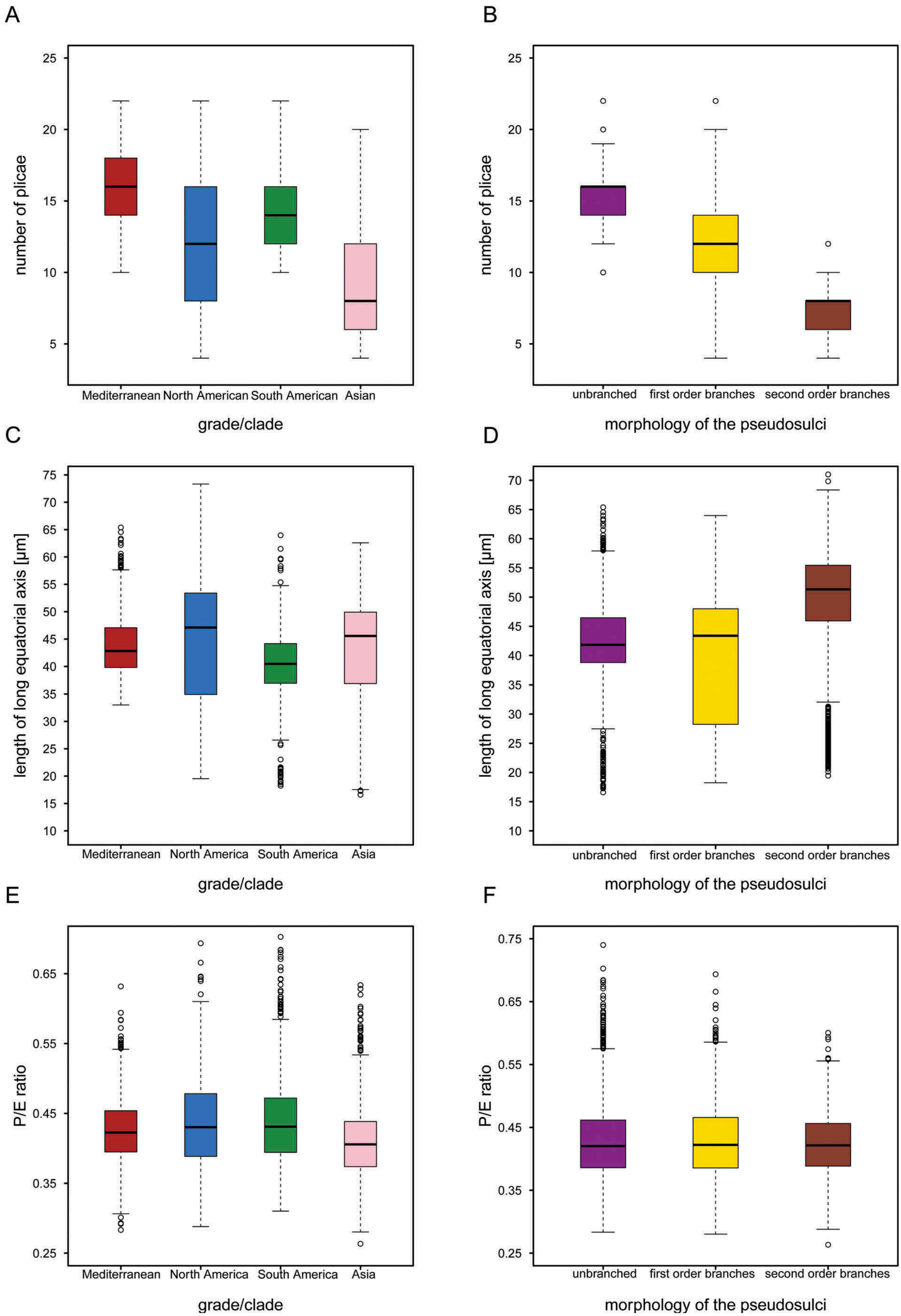


Figure 10. Pollen of *Ephedra*, Asian clade, light micrographs. **A.** *E. likiangensis* G Forrest 5564 (BM.) **B.** *E. minuta* B Dikoré 8457 (MSB). **C.** *E. equisetina* QR Wu (MO). **D.** *E. gerardiana* Walter Koelz 5310 (S). **E.** *E. monosperma* H Freitag 33068 (KAS). **F.** *E. pachyclada* KH. & F Rechinger 3676 (W). **G.** *E. saxatilis* Parkinson 7077 (S). **H.** *E. intermedia* Lindberg 117–1947 (W). **I.** *E. lomatolepis* II Rusanovick & LA Krai 56 (NY). **J.** *E. regeliana* H Hastman 26 (MSB). **K.** *E. sinica* Y Yang 99531 (PE). **L.** *E. distachya* J Prudhomme 89 (WU). **M.** *E. sarcocarpa* KH Rechinger 46054b (W). **N.** *E. strobilacea* KH & F Rechinger 2703 (S). **O.** *E. transitoria* Doppelbaur 190 (M). Scale bars – 10 μm.

branching of the pseudosulci is not a good characteristic to assign pollen to clades or identify monophyletic groups in *Ephedra* either: It is inferred to have evolved at least twice, once in the Asian clade and once among the North American species, and to have later been lost repeatedly (Figure 13B). It should be noted that the presented analyses do not accommodate for phylogenetic uncertainty, however, we find it impossible to distinguish among pollen types with second-order branching, regardless of whether the pollen comes from closely related species or from species of different clades and geographical regions, and the character cannot be used as a diagnostic feature.

The function of branched pseudosulci and/or second-order branches is not fully understood. In general, pollen morphology is not only related to pollination mode but also to the degree of dehydration at dispersal (Franchi et al. 2002). Pollen of species that release partly hydrated pollen has a thick pollen exine, more rapid germination of the male gametophyte and longer pollen viability (Franchi et al. 2002). In *Ephedra*, pollen with unbranched pseudosulci (the ancestral type) has a thick exine and

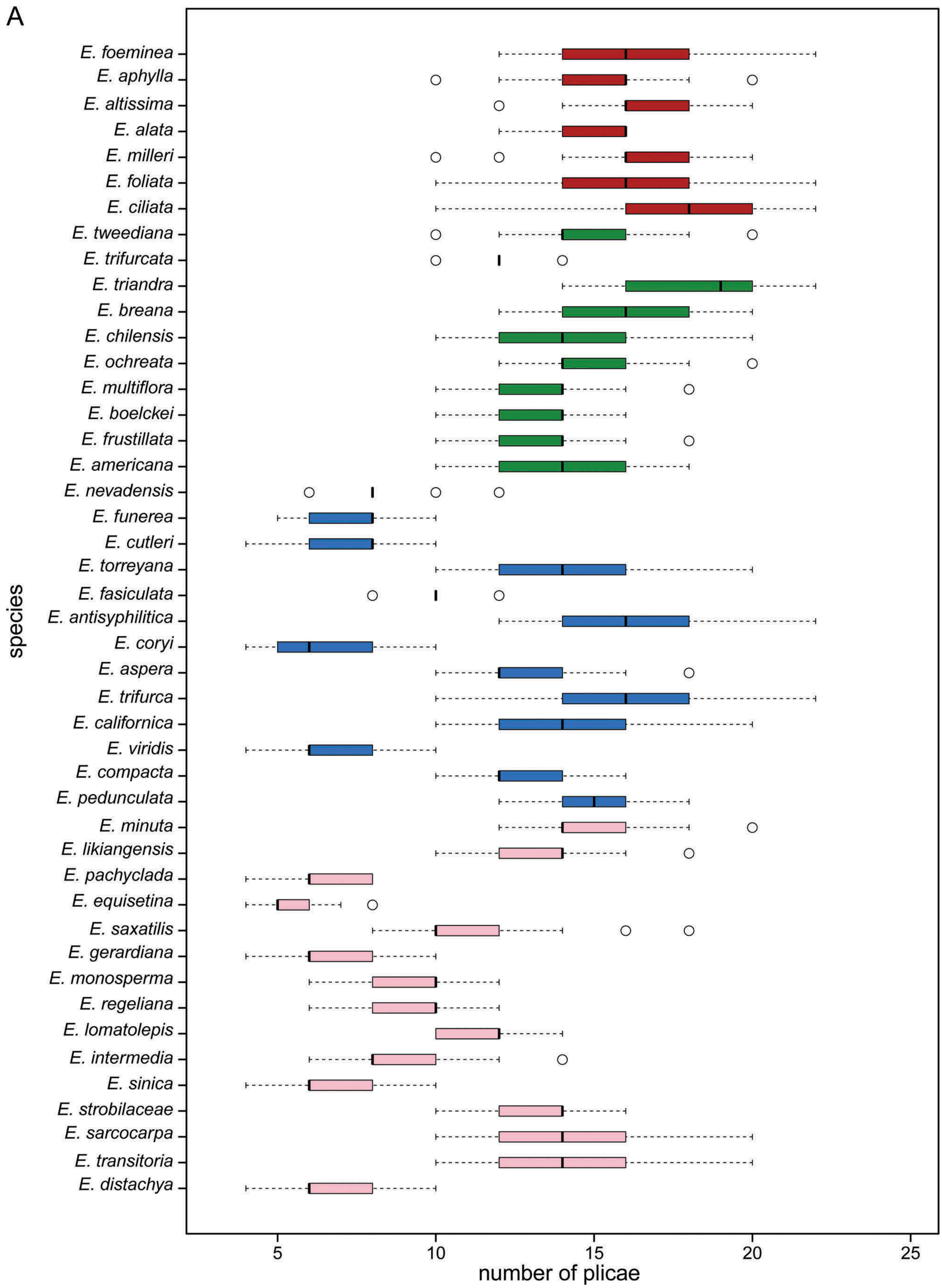

A
25
20
15
10
5
number of plicae
Mediterranean North American South American Asian
grade/clade
B
25
20
15
10
5
number of plicae
unbranched first order branches second order branches
morphology of the pseudosulci
C
75
70
65
60
55
50
45
40
35
30
25
20
15
10
length of long equatorial axis [µm]
Mediterranean North America South America Asia
grade/clade
D
70
65
60
55
50
45
40
35
30
25
20
15
10
length of long equatorial axis [µm]
unbranched first order branches second order branches
morphology of the pseudosulci
E
0.65
0.55
0.45
0.35
0.25
P/E ratio
Mediterranean North America South America Asia
grade/clade
F
0.75
0.65
0.55
0.45
0.35
0.25
P/E ratio
unbranched first order branches second order branches
morphology of the pseudosulci

germinates faster than pollen with branched pseudosulci (the derived types; Bolinder et al. 2015 and KB personal observation, June 2012). Although this has not been explicitly tested, we hypothesise that side branches on the pseudosulci (first- and second-order) facilitate dehydration and subsequent rehydration and together with the spacious ultrastructure facilitate long distance dispersal by air of the pollen grains. A consequence is, however, that pollen with branched pseudosulci (the derived type) germinates slower (KB personal observation, June 2012), perhaps because the pollen first needs to rehydrate on the female structure (or germination medium) before germination can take place. The feature may thus represent a trade-off between dispersal and germination ability. In passing, it is interesting to note that among the many extinct forms of ephedroid pollen, there is a type (common in the Eocene) with extensively branched pseudosulci, and side branches extending almost to the ridge of the plicae (*Ephedripites* (subgenus *Distachyapites*) *claricristatus*). This type, albeit with some modification, is also known from the Neogene, but not from extant species of *Ephedra*.

Number of plicae. — The number of plicae is mostly consistent within species although some intraspecific variation occurs (Figure 12A). It is further clear that there has been a trend towards reduction of the number of plicae during the course of evolution in *Ephedra* (Figure 14A). The morphology and structure of the exine are known to have substantial implications for the pollination biology of plants (Ferguson & Skvarla 1982; Grayum 1986; Osborn et al. 1991; Bolinder et al. 2015). For entomophilous pollen to be successfully transported from the microsporangiate structures to the ovulate structures (specifically in this case, to the pollination drop), the pollen needs to adhere to an insect vector. Insect-pollination is probably the ancestral mode of pollination in the Gnetales (Bolinder et al. 2014; Rydin & Bolinder 2015) and pollen of the entomophilous *Welwitschia* (Pearson 1907; Wetschnig & Depish 1999) and *Ephedra foeminea* is sticky and forms distinct clumps (Hesse 1984; Bolinder et al. 2014, 2015). However, since pollenkitt is lacking in the Gnetales (Hesse 1984), the means, by which this stickiness is accomplished is currently unknown. In addition to the observed stickiness (Hesse 1984; Bolinder et al. 2014, 2015), we suggest that the numerous plicae facilitate attachment to the setae on the body of an insect vector. Pollen of *Welwitschia* is similar to the ancestral *Ephedra* pollen type in many respects; it is also polyplicate and ellipsoid with the longest axis equal to one of the equatorial axes (Carafa et al. 1996; El-Ghazaly et al. 1998). But in contrast to that of *Ephedra, Welwitschia* pollen has a single broad sulcus extending parallel to the long equatorial axis, where the exine splits open at germination (Rydin & Friis 2005). Furthermore, the area between the plicae differs ultrastructurally between the two genera. In *Welwitschia* grains, both tectum and infratectum are present in the furrows between plicae, but in *Ephedra* grains these layers are absent in the furrow regions (Osborn 2000; Bolinder et al. 2015). The functional implication is that while the furrows of *Ephedra* are zones of weakness that can function as apertures, those of *Welwitschia* are not. The third member of the Gnetales, *Gnetum*, is also thought to be insect-pollinated (Kato & Inoue 1994;

←
Figure 11. Variation in pollen traits among clades (as defined in Figures 13 and 14) and pollen types. **A.** Number of plicae in the different grades/clades. There are considerably more plicae in pollen of the Mediterranean and South American species than in the Asian and North American species. **B.** Number of plicae in pollen with different pseudosulcus morphology. Pollen with unbranched pseudosulci has significantly more plicae than pollen with branched pseudosulci. Pollen with first- and second-order branched pseudosulci has fewer plicae than pollen with first-order branches only. **C.** Length of the long equatorial axis in different clades. There is no noteworthy difference among pollen of the species in the different grade/clades. **D.** Length of the long equatorial axis in pollen of different pseudosulcus morphology. Pollen with first- and second-order branches on the pseudosulci is slightly larger than pollen with unbranched pseudosulci or pseudosulci with first-order branching only. **E.** P/E ratio in different clades. Pollen of Asian species tends to have a smaller P/E ratio than pollen of remaining species. **F.** P/E ratio in pollen with different pseudosulcus morphology. There is no significant difference in P/E ratio among pollen with different pseudosulcus morphology.

→
Figure 12. Variation in pollen traits among species. Mediterranean species in red, North American species in blue, South American species in green and Asian species in pink. **A.** Number of plicae. Pollen of Mediterranean and South American species generally has a larger number of plicae than does pollen of North American and Asian species. There is relatively high intraspecific variation in this character and it overlaps extensively among species. **B.** Size as gauged by the length of the long equatorial axis. There is some variation among species but the large intraspecific variation, in particular in the North American and Asian species, makes the overlap extensive among species and clades. **C.** Shape as assessed by the P/E ratio. There is some variation among species but the large intraspecific variation makes the overlap extensive among species and clades.


A
E. foeminea
E. aphylla
E. altissima
E. alata
E. milleri
E. foliata
E. ciliata
E. tweediana
E. trifurcata
E. triandra
E. breana
E. chilensis
E. ochreata
E. multiflora
E. boelckei
E. frustillata
E. americana
E. nevadensis
E. funerea
E. cutleri
E. torreyana
E. fasiculata
E. antisyphilitica
E. coryi
E. aspera
E. trifurca
E. californica
E. viridis
E. compacta
E. pedunculata
E. minuta
E. likiangensis
E. pachyclada
E. equisetina
E. saxatilis
E. gerardiana
E. monosperma
E. regeliana
E. lomatolepis
E. intermedia
E. sinica
E. strobilaceae
E. sarcocarpa
E. transitoria
E. distachya
species
5
10
15
20
25
number of plicae

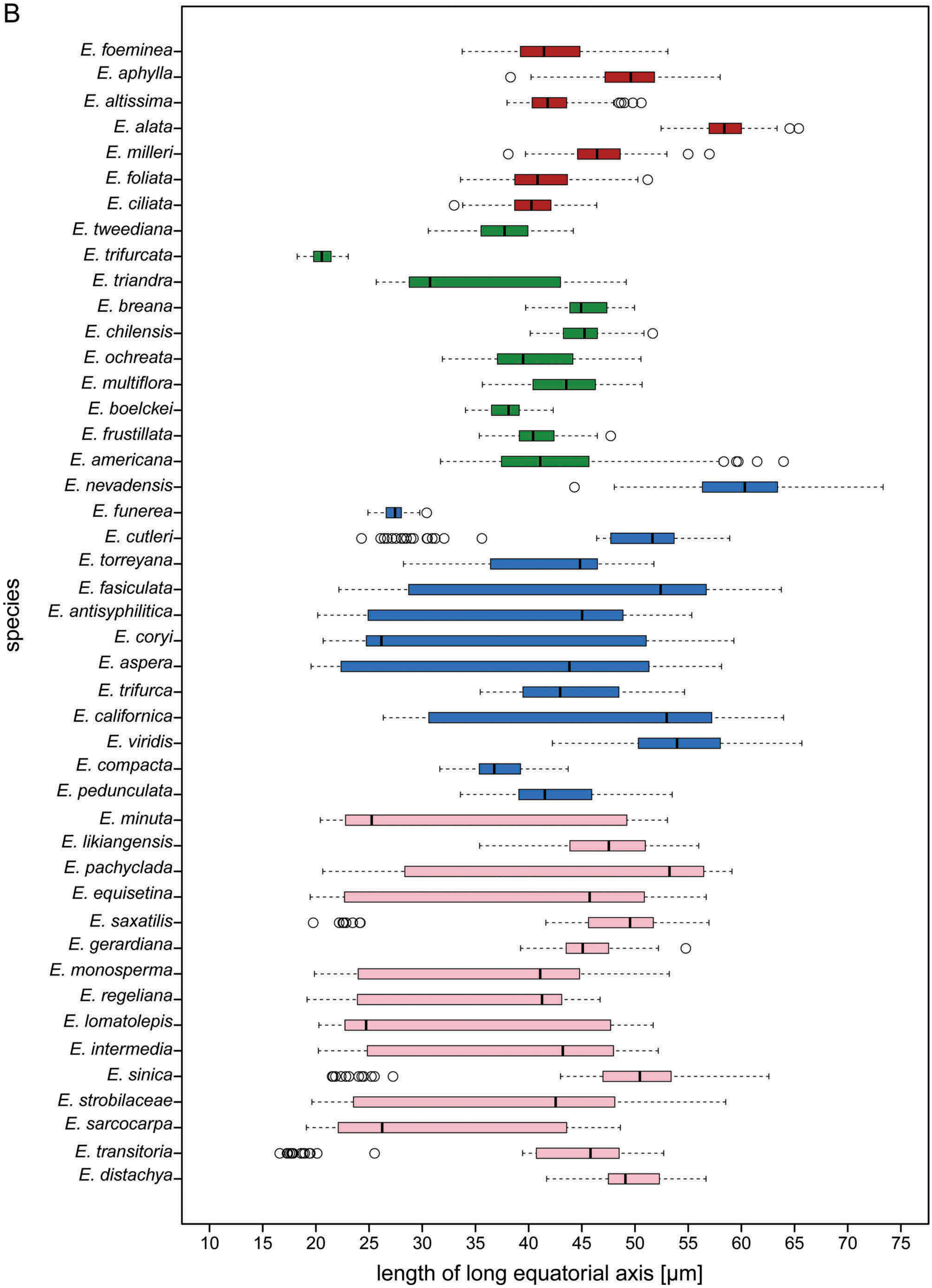


Figure 12. (*Continued*).

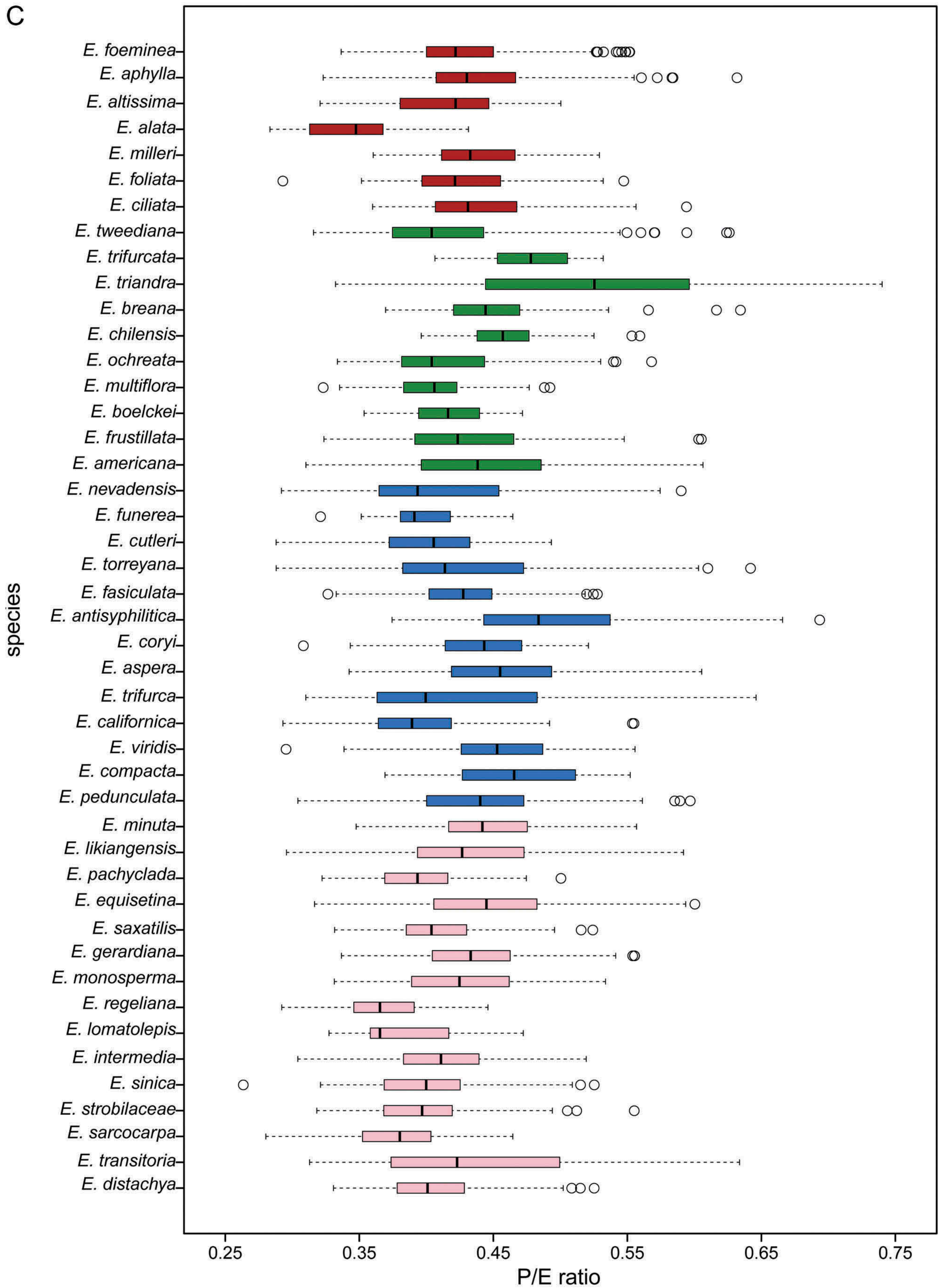
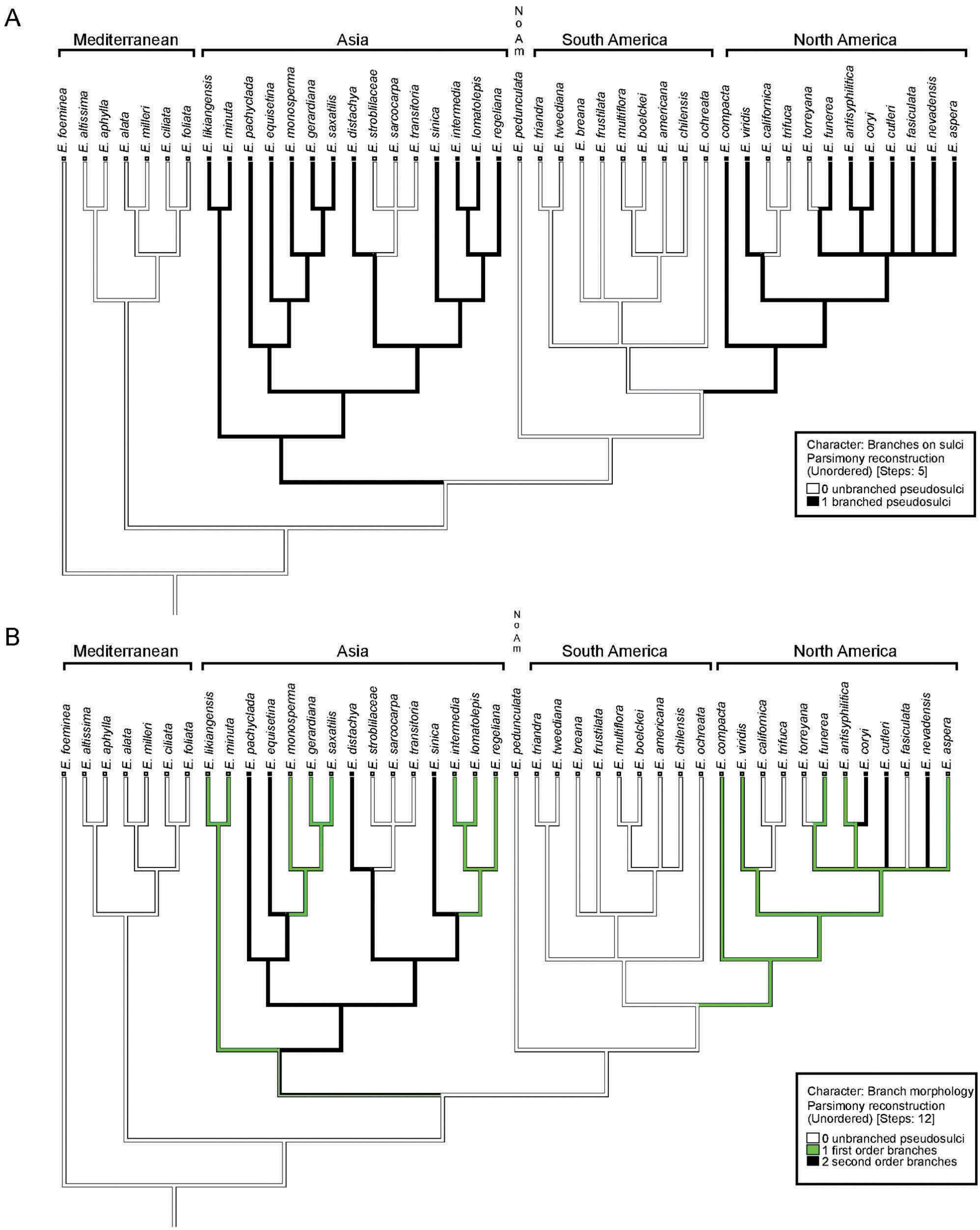


Figure 12. (*Continued*).


A
Mediterranean
Asia
NoAm
South America
North America
E. foeminea
E. altissima
E. aphylla
E. alata
E. milleri
E. ciliata
E. foliata
E. likiangensis
E. minuta
E. pachyclada
E. equisetina
E. monosperma
E. gerardiana
E. saxatilis
E. distachya
E. strobilaceae
E. sarcocarpa
E. transitoria
E. sinica
E. intermedia
E. lomatolepis
E. regeliana
E. pedunculata
E. triandra
E. tweediana
E. breana
E. frustilata
E. multiflora
E. boelckei
E. americana
E. chilensis
E. ochreata
E. compacta
E. viridis
E. californica
E. trifuca
E. torreyana
E. funerea
E. antisyphilitica
E. coryi
E. cutleri
E. fasiculata
E. nevadensis
E. aspera
Character: Branches on sulci
Parsimony reconstruction
(Unordered) [Steps: 5]
0 unbranched pseudosulci
1 branched pseudosulci
B
Character: Branch morphology
Parsimony reconstruction
(Unordered) [Steps: 12]
0 unbranched pseudosulci
1 first order branches
2 second order branches

Kato et al. 1995). Pollen of this genus is spherical with a large number of spines covering the exine surface (Woodhouse 1935; Osborn 2000; Yao et al. 2004), which probably also facilities adherence to the bodies of insects. It has further been suggested that the spines of *Gnetum* pollen are homologous with the plicae of *Ephedra* and *Welwitschia* pollen (Osborn 2000), which further supports numerous 'plicae' (modified into spines in *Gnetum*) as the ancestral state in the Gnetales.

Pollen size. — The size of pollen grains varies tremendously within species of *Ephedra*, and does in many cases not overlap among individuals of a single species. Even though the interspecific variation in size is greater than the intraspecific variation, we do not find the character useful for species identification due to the large overlap among species. For the same reason, it is problematic to assign dispersed fossil ephedroid pollen grains to a species (or even clade) based solely on size. Furthermore, the variation in size is not phylogenetically informative ($\lambda = 0$; Figure 14B) and the evolutionary conclusions that can be drawn from pollen size variation are therefore limited. Also the variation in shape (P/E ratio) is large within and among species. Again, the variation between species is larger than within species (Figure 12C), but there is a lot of overlap and the P/E ratio cannot be considered an informative character. The P/E ratio of North American species does not differ significantly from that of South American or Mediterranean species, indicating that North American species have retained the ancestral shape of pollen grains also present in Mediterranean and South American species. This is further supported by the estimate of phylogenetic signal and ancestral state reconstruction ($\lambda = 0$; Figure 14C). There is a tendency for species in the Asian clade to have smaller mean P/E ratios, meaning that pollen of Asian species have evolved a different shape compared to that of species in the other clades (Figures 12C, 14C).

Comparison with previous work. — The morphology of the pseudosulci has, together with number of plicae, traditionally been used to classify *Ephedra* pollen into 2–4 different pollen types (Woodhouse 1935; Steeves & Barghoorn 1959; Zhang & Xi 1983; Kedves 1987; Freitag & Maier-Stolte 1994). The ancestral type defined here is equivalent to the '*fragilis* type' described by Beug (1956) and Freitag and Maier-Stolte (1994), and to type D described by Steeves and Barghoorn (1959) and Zhang and Xi (1983). The derived type defined here is equivalent to the '*distachya* type' described by Beug (1956) and Freitag and Maier-Stolte (1994), and this type was divided into several subgroups (types A, B and C) by Steeves and Barghoorn (1959) and (types A and BC) by Zhang and Xi (1983). None of the previously described pollen types (A, B, C) corresponds to pollen of the Asian or North American species and we find no support for any of these previously suggested delimitations within the derived pollen type. Taken together, our results demonstrate that the taxonomic value of pollen morphology is limited.

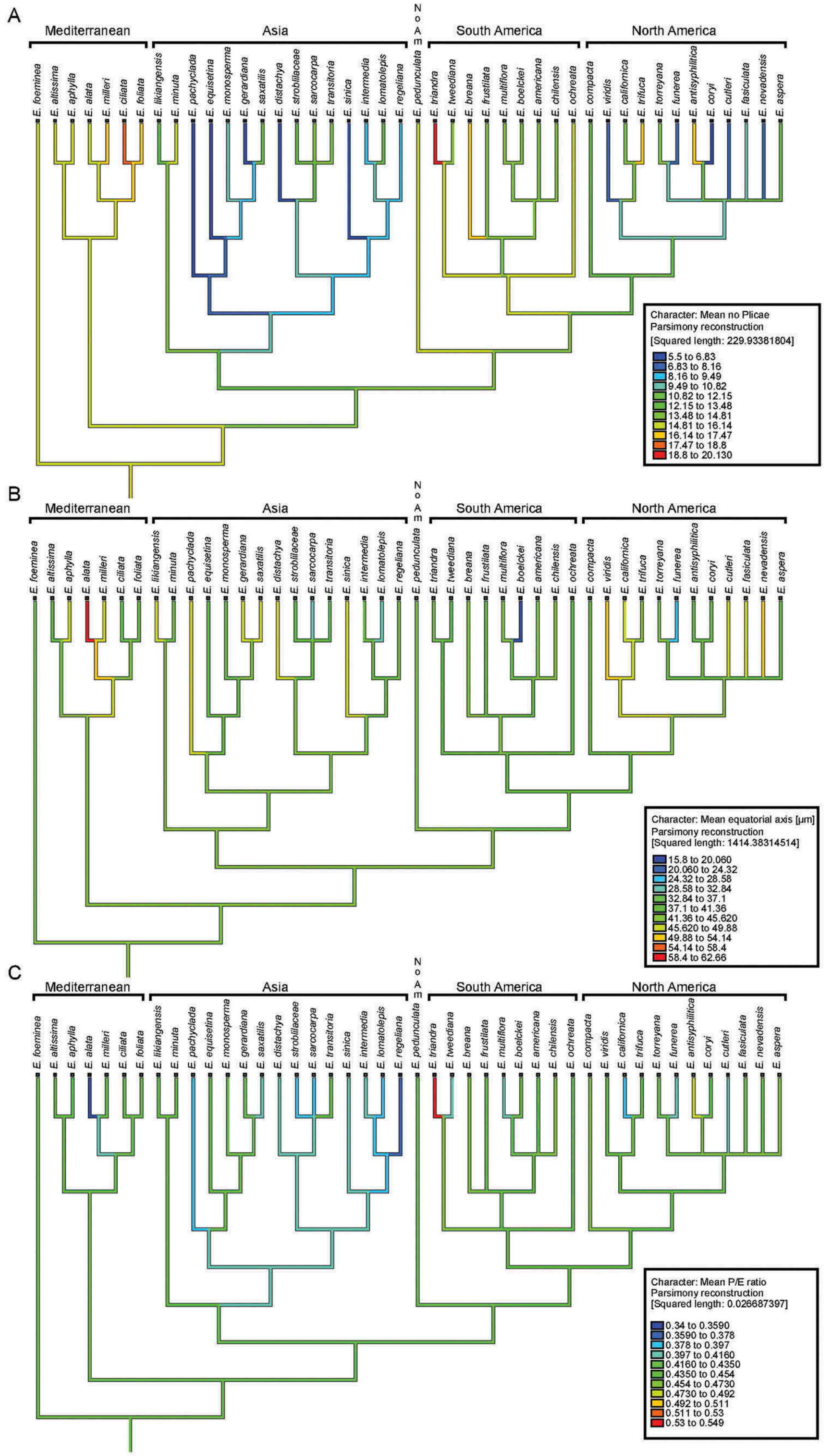
Pollen dimorphism. — Several kinds of intraspecific pollen dimorphism have been reported. Kedves (1987) reported a dimorphism in size in several *Ephedra* species and argues that variation in size, therefore, is not a valuable taxonomic character. We have not observed size dimorphism in our data. Ickert-Bond et al. (2003) reported dimorphism,

←

Figure 13. Ancestral state reconstruction of pseudosulci branching. **A.** Branches on the pseudosulci coded as a binary character: Absence of side branches (0) and presence of side branches (1). Absence of side branches on the pseudosulci is the ancestral state in *Ephedra*. Presence of branches on the pseudosulci is inferred to have evolved independently twice, once along the stem lineage of the Asian clade and once on along the stem lineage of the North American clade. There are reversals to the ancestral state in both clades. **B.** Side branches on the pseudosulci as a multistate character: Absence of branches (0), presence of first-order branches (1) and presence first- and second-order branches (2). First-order branches on the pseudosulci are inferred to have originated several times. The character has subsequently been lost several times among North American species and once in the Asian clade. Second-order branching of the pseudosulci has evolved at least once in both clades.

→

Figure 14. Ancestral state reconstruction of the mean of number of plicate, size and shape of pollen grains. **A.** Mean number of plicae. During the course of evolution in *Ephedra* there has been a general trend towards reduction of the number of plicae in the North American and Asian clades. Numerous plicae have, however, evolved at least twice among the North American species. Mediterranean and South American species generally have an intermediate number of plicae, and numerous plicae have evolved several times. **B.** Size (mean length of the long equatorial axis). The variation in size in the genus is not phylogenetically informative. **C.** Shape (mean P/E ratio). There is no difference in P/E ratio between Mediterranean, South American and North American species. Pollen of Asian species has, in general, a smaller P/E ratio than that of the other grade/clades.


A
Mediterranean
Asia
NoAm
South America
North America
E. foeminea
E. altissima
E. aphylla
E. alata
E. milleri
E. ciliata
E. foliata
E. likiangensis
E. minuta
E. pachyclada
E. equisetina
E. monosperma
E. gerardiana
E. saxatilis
E. distachya
E. strobilaceae
E. sarcocarpa
E. transitoria
E. sinica
E. intermedia
E. lomatolepis
E. regeliana
E. pedunculata
E. triandra
E. tweediana
E. breana
E. frustillata
E. multiflora
E. boelckei
E. americana
E. chilensis
E. ochreata
E. compacta
E. viridis
E. californica
E. trifuca
E. torreyana
E. funerea
E. antisyphilitica
E. coryi
E. cutleri
E. fasiculata
E. nevadensis
E. aspera
Character: Mean no Plicae
Parsimony reconstruction
[Squared length: 229.93381804]
5.5 to 6.83
6.83 to 8.16
8.16 to 9.49
9.49 to 10.82
10.82 to 12.15
12.15 to 13.48
13.48 to 14.81
14.81 to 16.14
16.14 to 17.47
17.47 to 18.8
18.8 to 20.130
B
Mediterranean
Asia
NoAm
South America
North America
E. foeminea
E. altissima
E. aphylla
E. alata
E. milleri
E. ciliata
E. foliata
E. likiangensis
E. minuta
E. pachyclada
E. equisetina
E. monosperma
E. gerardiana
E. saxatilis
E. distachya
E. strobilaceae
E. sarcocarpa
E. transitoria
E. sinica
E. intermedia
E. lomatolepis
E. regeliana
E. pedunculata
E. triandra
E. tweediana
E. breana
E. frustillata
E. multiflora
E. boelckei
E. americana
E. chilensis
E. ochreata
E. compacta
E. viridis
E. californica
E. trifuca
E. torreyana
E. funerea
E. antisyphilitica
E. coryi
E. cutleri
E. fasiculata
E. nevadensis
E. aspera
Character: Mean equatorial axis [µm]
Parsimony reconstruction
[Squared length: 1414.38314514]
15.8 to 20.060
20.060 to 24.32
24.32 to 28.58
28.58 to 32.84
32.84 to 37.1
37.1 to 41.36
41.36 to 45.620
45.620 to 49.88
49.88 to 54.14
54.14 to 58.4
58.4 to 62.66
C
Mediterranean
Asia
NoAm
South America
North America
E. foeminea
E. altissima
E. aphylla
E. alata
E. milleri
E. ciliata
E. foliata
E. likiangensis
E. minuta
E. pachyclada
E. equisetina
E. monosperma
E. gerardiana
E. saxatilis
E. distachya
E. strobilaceae
E. sarcocarpa
E. transitoria
E. sinica
E. intermedia
E. lomatolepis
E. regeliana
E. pedunculata
E. triandra
E. tweediana
E. breana
E. frustillata
E. multiflora
E. boelckei
E. americana
E. chilensis
E. ochreata
E. compacta
E. viridis
E. californica
E. trifuca
E. torreyana
E. funerea
E. antisyphilitica
E. coryi
E. cutleri
E. fasiculata
E. nevadensis
E. aspera
Character: Mean P/E ratio
Parsimony reconstruction
[Squared length: 0.026687397]
0.34 to 0.3590
0.3590 to 0.378
0.378 to 0.397
0.397 to 0.4160
0.4160 to 0.4350
0.4350 to 0.454
0.454 to 0.4730
0.4730 to 0.492
0.492 to 0.511
0.511 to 0.53
0.53 to 0.549

regarding presence and absence of first-order branches on the pseudosulci, for a probable hybrid between *E. funerea* and *E. torreyana*. This finding is indirectly supported by our results since *E. funerea* has the derived pollen type with side branches on the pseudosulci, whereas *E. torreyana* probably represents a reversal back to the ancestral state and has pollen without side branches. Ickert-Bond et al. (2003) also showed the presence of two types of pollen in the same microsporangium of *E. trifurca* (with and without first-order branches), but this observation is not supported by the data presented here. However, we have seen similar examples in some specimens of *E. alata* that are of probable hybrid origin, as indicated by our results and molecular data (CR personal observation, December 2014). In these specimens, a fraction of the pollen has side branches on the pseudosulci while the majority lacks the feature (as does all pollen in most specimens of *E. alata*; Figures 3B, 7B). Specimens of putative hybrid origin were, however, removed from the present study at an early stage.

The dimorphism described by El-Ghazaly and Rowley (1997), Ickert-Bond et al. (2003) and Doores et al. (2007), concerning a curvature of the ridges (plicae), is not supported by our data. These studies describe a 'normal' pollen form with straight ridges and a variant form with sinuous ridges. We have not observed such dimorphism in any pollen from any accession investigated in the present study. Instead, we have seen that treatment of pollen with alcohol alters the size and shape of *Ephedra* pollen considerably (Figure 2), as does preparation with acetolysis (Kedves 1987), which typically affects some but not all grains. In all studies reporting dimorphism of the ridges (El-Ghazaly & Rowley 1997; Ickert-Bond et al. 2003; Doores et al. 2007), pollen was treated with alcohol, acetolysed and/or critical-point dried, and reported intraspecific dimorphism concerning sinuous or straight ridges is most likely a consequence of specimen preparation.

Conclusions and evolutionary implications

Our results show that *Ephedra* pollen occurs in two distinct forms, an ancestral type (with unbranched pseudosulci) present in Mediterranean and South American species, and a derived type (with branched pseudosulci) that appears to have evolved independently twice, once in the Asian clade and once in the clade containing all North American species except *E. pedunculata*. There are repeated reversals back to the ancestral state within both these clades. Although there is phylogenetic information in several pollen features, the repeated reversals in combination with some degree of intraspecific variation make it difficult to assign individual pollen grains to species or subclades of the genus. Further, we find no clear correlation between the two pollen types and pollination syndrome. Species that have the ancestral type may be insect-pollinated (such as *E. foeminea*; Bolinder et al. 2014, 2015) or wind-pollinated (such as *E. trifurca*; e.g. Niklas et al. 1986). The derived pollen type is, however, only known from wind-pollinated species.

Polyplicate pollen is not unique to the Gnetales; it also occurs in several extant and extinct angiosperms, such as the Alismatales, Laurales and Zingiberales (Hesse et al. 2000; Friis et al. 2004). Furthermore, it is far from clear that all dispersed polyplicate pollen in Mesozoic strata referred to as 'ephedroids', was produced by a single group of plants. However, the gnetalean affinity of individual grains can be assessed based on ultrastructural studies of the pollen wall (Hesse et al. 2000; Tekleva & Krassilov 2009; Friis et al. 2011). Ultrastructural information is only available for a few Mesozoic ephedroids (Trevisan 1980; Osborn et al. 1993; Kedves 1994), and those are clearly of the ancestral ephedroid pollen type described here (see also Bolinder et al. 2015). Germinated pollen grains (i.e. shed exines) are also found *in situ* in *Ephedra* seeds from the Early Cretaceous (Rydin et al. 2004, 2006a) and, as assessed by their many plicae and the absence of side branches on the pseudosulci, this pollen is also clearly of the ancestral type. The same inference can be made for other dispersed ephedroid pollen grains found in Mesozoic strata (although the ultrastructure has not been studied for any of these grains) (Srivastava 1968; Scott 1960; Wilson 1962; Stover 1964; Muller 1968; Brenner 1976; de Lima 1980; Osborn et al. 1993; Takahashi 1995; Narváez & Sabino 2008; Abubakar et al. 2011).

Ephedroid pollen with branched pseudosulci (i.e. the derived type) has, to our knowledge, only been described twice from the Mesozoic: once from the lower Upper Cretaceous Raritan Formation in North America (Steeves & Barghoorn 1959) and once from the Xining Basin of the Tibetan Plateau (Norbäck Ivarsson 2014), found in a section from the Cenomanian–Maastrichtian (Horton et al. 2004). After the K-Pg boundary, pollen of the derived type gradually becomes much more common and dominates over the ancestral type in most palaeo-palynofloras from the Paleocene and onwards (Cookson 1956; Gray 1960; Ghosh et al. 1963; Nagy 1963; Shaw 1998; Hoorn et al. 2012). This increase of the derived pollen type in the Cenozoic probably represents an adaptation to climatic changes after the K-Pg boundary. Previous studies have indicated an early Oligocene (Ickert-Bond et al. 2009) or even younger (Huang & Price 2003) age of crown-group *Ephedra*. The results of the present study and a first occurrence of fossil pollen of the derived type in the Late Cretaceous indicate that the crown-group *Ephedra* is much older than previously estimated.

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Disclosure statement

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Specimens investigated

Mediterranean species. – Ephedra alata Decne., KH Rechinger 102 (S), AA Anderberg 480 (S), Ibrahim & Mahdi s.n. (S), AbdEaleem s.n. (CAI); *E. altissima* Desf., A Faure s.n. (S), M Staudinger 6714 (W), H Freitag 35035 (KAS); *E. aphylla* Forssk., G Samuelsson 2696 (S), F Wettstein 2751 (WU), J Bornmüller 1746 (WU), WM Amer (CAI); *E. ciliata* Fisch. et C.A.Mey., NT Yakovleva (W), EK Balls 2487 (S), H Handel-Mazetti 973 (WU), JET Aitchison 496537 (GH); *E. foeminea* Forssk., K Bolinder 542 (S), L Norbäck Ivarsson & O Thureborn s.n. (S), R Pamaninio & R Pichisermolli 139 (L), FS Meyers & JE Dinsmoore 8124 (L), JS Andersen et al. 2098 (S), Bornmüller 1746 (A), O Porch s.n. (WU); *E. foliata* Boiss et C.A.Mey., B Tiagi s.n. (S), Wendelbo & Assadi 16604 (W), Hedberg & Hedberg 92019A V-60888 (UPS), Thulin & Al-Gifri 9975 (V-096087) 183895 (UPS), PN Parker 3304 (A); *E. milleri* Freitag et Maier-St., P Hein et al. YP 1110 (B), Miller 7667A (E).

North American species. – Ephedra antisyphilitica Berlandier ex C.A.Mey., HC Hanson 344 (NY), ME Jones 3726 (BR), Hendersoin 62-02a (BR), E Palmer 1292 (GH); *E. aspera* Engelm., KF Parker 7799 (NY), NH Holmgren 6604 (NY), RD Worthington 13620 (NY), BA Stein 31 (RSA); *E. californica* S.Watson, FM Reed 5772 (L), C Epling & WM Robinsson s.n. (L), A Carter 3667 (L); *E. compacta* Rose, DS Corell & IM Johnston 20233 (NY); *E. coryi* E.L. Reed, SM Ickert-Bond 953 (ASU), DS Corell 32805 (S), DS Corell 32785 (S); *E. cutleri* Peebles, CT Mason Jr. 2192 (ASU), NH Holmgren 12744 (NY), DE Atha et al. s.n. (NY); *E. fasciculata* A.Nelson, ME Jones s.n. (RSA), FW Gould 1526 (GH), JH Lehr 2309 (NY), PA Munz 12053 (RSA); *E. funerea* Coville et C.V.Morton, J Wash & IW Clokey 8224 (NY), LS Rose 67021 (S), CL Hitchcock 12329 (A); *E. nevadensis* S.Watson, IW Clokey 6509 (S), C Epling & W Robinson (S), P Raven 14251 (WU), LS Rose 58108 (NY), *E. pedunculata* Engelm. ex S.Watson, Johnston 8847 (TEX, LL), Hendrickson 23183 (TEX, LL), RM Steward 2265 (GH); *E. torreyana* S.Watson, RW Spellenberg 10204 (NMC), IW Clokey 7816 (NY), E Neese & K Mutz 11414 (NY), Johnston et al. 10578 (F); *E. trifurca* Torr., RD Worthington 24587 (NY), A Nelson 1619 (NY), MC Johnston et al. 10573 (NY), P Allen (S), CV Hartman 642 (GH); *E. viridis* Coville, LS Rose 58080 (S), JL Reveal 100 (NY), Neely 4353 (NY), JT Howell 3824 (GH).

South American clade. – Ephedra americana Humb., Bonpl. ex Willd., Gerth s.n. (L), E Günter & O Buchtien (S), RE Fries 1044 (S), M Cardenas 4 (GH); *E. boelckei* F.A.Roig, Maas et al. 8184 (GB); *E. breana* Phil., KH Rechinger & W Rechinger 63547 (W), IM Johnston 3613 (GH); *E. chilensis* C.Presl., C Skottsberg 987 (F), Werdermann 138 (E), Jouffel 2751 (GH); *E. frustillata* Miers, MP Moreno 236 (NY), JB Hatcher s.n. (NY), LR Parodi 11858 (GH); *E. multiflora* Phil. ex Stapf, Baines et al. 233 (E), J Chiapella 2344 & E Vitek 09–0359 (W), Johnson 6286 (GH); *E. ochreata* Miers, A Donat 34 (GB), MP Moreno 433 (NY), RH Fortunato 5413 (NY), W Fischer 15 (GH); *E. triandra* Tul., J Chiapella 2505 & E Vitek 09–0520 (W), A Cuezzo 1954 (S), K Fiebrig 215 (GH); *E. trifurcata* Zöllner, O Zöllner 7928 (L); *E. tweediana* Fisch. et C.A.Mey., R Gallinal 5683 (GH), C Osten 22008 (S), S Venturi 348 (S), JH Hunziker 1648 (S).

Asian clade. – Ephedra distachya L., J Prudhomme 89 (WU), K Bolinder 764 (S), I Rácz et al 35308 (S), Rechinger 53066 (W), Flora Germanica Exsicatae 2325 (GH); *E. equisetina* Bunge, RC Ching 109 (MO), QR Wu s.n. (MO), KK Andrjoschchenko 4027 (S), E Mokeeva (A); *E. gerardiana* Wall. ex Florin, P Wendelbo s.n. (WU), Walter Koelz 5310 (S), P Wendelbo s. n. (S), Österreichische Karako Exp. 1039 (W), Stainton, Syes & Williams 813 (E); *E. intermedia* Schrenk et C.A.Mey., Lindberg 117–1947 (W), W Koeltz 2305e (NY), Y Yang 08070801 (PE), JET Atchison 1122 (GH); *E. likiangensis* Florin, JF Rock 3694 (NY), G Forrest 5564 (BR); *E. lomatolepis* Schrenk, II Rusanovick & LA Krai 56 (NY); *E. minuta* Florin, B Dichore 8457 (MSB), Long et al. 153 (W); *E. monosperma* J.G.Gmel. ex C.A. Mey., H Freitag 33068 (KAS), Potanin s.n. (BR), CG 81–0152 (BR); *E. pachyclada* Boiss., KH & F Rechinger 3676 (W), A Danin S-2455 (S), JP Mandaville s.n. (BM); *E. regeliana* Florin, H Hastman 26 (MSB), GL Webster & E Nasir 5959 (W), GL Webster & E Nasir 5950 (GH); *E. sarcocarpa* Aitch. et Hemsl., F Rechinger 46054b (W), Ruttner 594 (W), Tsanshakt & Riedel 15977 (W), F Rechinger 46295 (W), Aellen & Estafandri 2786 C-7840 (S); *E. saxatilis* (Stapf) Royle ex Florin, F Lobblichler 87 (M), CE Parkinson 7077 (S); *E. sinica* Stapf, E Licent 13523 (S), Y Yang 99531 (PE), W Qingru 9812 (MO); E Licewtak 13523 (A); *E. strobilacea* Bunge, Supra 3369a (NY), A Michelson 3369b (S), KH Rechinger & F Rechinger 2703 (S), Leonard 5582 (BR); *E. transitoria* Riedl, V Täckholm et al. 9166 (BR), Doppelbaur 190 (M), Rechinger 8990 (W).

Table I. Pollen properties and distribution for all investigated *Ephedra* species.

Clade, species	Distribution	Polar axis (μm)	Equatorial diameter (μm)	P/E ratio	Number of plicae	Pseudosulci
Mediterranean species						
E. foeminea Forssk.	Eastern Mediterranean	18.1 (13.6–23.6) ± 2.3	42.2 (33.8–53.1) ± 3.8	0.43 (0.34–0.55) ± 0.05	15.8 (12–22) ± 2.6	Not branched
E. alata Decne.	North Africa; Near East	20.0 (16.7–23.2) ± 1.6	58.4 (52.5–65.4) ± 3.2	0.34 (0.28–0.43) ± 0.03	14.9 (12–16) ± 1.5	Not branched
E. altissima Desf.	North Africa	17.5 (14.5–20.9) ± 1.5	42.5 (38.0–50.6) ± 3.2	0.41 (0.32–0.50) ± 0.05	16.5 (12–20) ± 1.6	Not branched
E. aphylla Forssk.	North Africa; Near East	21.6 (16.8–28.0) ± 2.2	49.4 (38.3–58.0) ± 3.7	0.44 (0.32–0.63) ± 0.06	15.5 (10–20) ± 2.2	Not branched
E. ciliata Fisch. et C.A.Mey.	Mediterranean to Central Asia	17.6 (14.2–22.8) ± 1.7	40.2 (33.0–46.4) ± 2.7	0.44 (0.35–0.59) ± 0.05	17.5 (10–22) ± 2.7	Not branched
E. foliata Boiss. et C.A.Mey.	Southeast Mediterranean to Central Asia	17.6 (13.3–22.8) ± 1.8	41.3 (33.6–51.2) ± 3.4	0.42 (0.29–0.55) ± 0.04	16.7 (10–22) ± 2.7	Not branched
E. milleri Freitag et Maier-St.	Endemic to Oman	20.5 (16.1–24.4) ± 1.9	46.8 (38.1–57.0) ± 3.5	0.44 (0.36–0.52) ± 0.04	16.3 (10–20) ± 2.0	Not branched
North American (including Mexico) species						
E. antisyphilitica Berlandier ex. C.A.Mey.	Southwest North America; North Mexico	19.4 (9.1–28.3) ± 4.5	40.2 (20.2–55.34) ± 11.4	0.49 (0.37–0.69) ± 0.07	16.6 (12–22) ± 2.7	Rarely short first-order branches, no secondary branches
E. aspera Engelm.	Southwest North America; North Mexico	17.7 (8.0–28.5) ± 5.5	39.5 (19.5–58.1) ± 13.8	0.46 (0.34–0.61) ± 0.06	13.0 (10–18) ± 2.0	Short first-order branches, no secondary branches
E. californica S. Watson	California; Mexico Baja	18.5 (9.4–29.1) ± 6.3	46.2 (26.3–64.0) ± 13.2	0.39 (0.29–0.55) ± 0.05	14.5 (10–20) ± 2.3	Not branched
E. compacta Rose	Central Mexico	17.3 (13.9–20.7) ± 1.7	37.3 (31.7–43.7) ± 2.9	0.46 (0.37–0.55) ± 0.05	12.5 (10–16) ± 1.7	Occasionally short first-order branches, no secondary branches
E. coryi E.L.Reed	New Mexico, Texas	14.9 (9.8–28.1) ± 6.0	33.9 (20.7–59.3) ± 13.4	0.44 (0.31–0.52) ± 0.04	6.5 (4–10) ± 1.5	Long first-order branches, often secondary branched
E. cutleri Peebles	Arizona, Colorado, New Mexico, Utah	19.4 (8.6–26.1) ± 4.3	47.9 (24.3–58.9) ± 9.6	0.41 (0.28–0.49) ± 0.04	7.0 (4–10) ± 1.3	First-order branches, occasionally secondary branched
E. fasiculata A. Nelson	Arizona, California, Nevada, Utah	20.0 (9.3–30.6) ± 6.1	46.7 (22.2–63.8) ± 13.4	0.43 (0.33–0.53) ± 0.05	10.1 (8–12) ± 1.1	First-order branches, no secondary branches
E. funerea Coville et C.V.Morton	California to Nevada (Death Valley area)	10.9 (9.3–12.2) ± 0.65	27.4 (24.9–30.4) ± 1.13	0.40 (0.32–0.46) ± 0.03	7.2 (5–10) ± 1.2	First-order branches, no secondary branches
E. nevadensis S.Watson	Southwest North America	21.9 (11.4–30.7) ± 5.5	52.8 (24.7–73.3) ± 14.2	0.42 (0.29–0.59) ± 0.07	7.7 (6–12) ± 1.4	First-order branches, occasionally secondary branched
E. pedunculata Engelm. ex S.Watson	Texas, North Mexico	18.4 (13.6–23.7) ± 1.9	42.6 (33.6–53.5) ± 4.6	0.43 (0.30–0.59) ± 0.06	15.0 (12–18) ± 1.4	Not branched
E. torreyana S.Watson	Southwest North America; North Mexico	17.9 (14.2–22.9) ± 1.4	42.3 (28.2–51.8) ± 6.3	0.43 (0.29–0.64) ± 0.07	14.5 (10–20) ± 2.1	Not branched
E. trifurca Torr. Ex S.Watson	Southwest North America; North Mexico	18.4 (11.7–24.7) ± 2.4	44.1 (35.5–54.7) ± 5.1	0.44 (0.31–0.64) ± 0.08	16.2 (10–22) ± 2.5	Not branched

(*Continued*)

Table I. (*Continued*).

Clade, species	Distribution	Polar axis (μm)	Equatorial diameter (μm)	P/E ratio	Number of plicae	Pseudosulci
E. viridis Coville	Southwest North America	24.4 (18.4–30.4) ± 2.7	54.1 (42.2–65.7) ± 5.2	0.45 (0.30–0.57) ± 0.05	6.9 (4–10) ± 1.2	First-order branches, occasionally secondary branched
South American species						
E. americana Humb. et Bonpl. ex Willd.	Andean South America + Argentina	18.5 (14.3–24.9) ± 2.1	42.8 (31.7–64.0) ± 7.1	0.44 (0.31–0.61) ± 0.06	13.9 (10–18) ± 1.7	Not branched
E. boelckei F.A.Roig	Argentina	15.8 (13.4–17.7) ± 1.0	38.0 (34.1–42.3) ± 2.0	0.42 (0.35–0.47) ± 0.03	13.5 (10–16) ± 1.4	Not branched
E. breana Phil.	Argentina & Chile	20.9 (16.5–25.6) ± 2.5	45.3 (39.7–50.0) ± 2.8	0.46 (0.37–0.63) ± 0.07	16.3 (12–20) ± 2.2	Not branched
E. chilensis C.Presl.	Andean South America	20.7 (17.9–24.1) ± 1.2	45.0 (10.2–51.7) ± 2.4.	0.46 (0.40–0.56) ± 0.03	14.2 (10–20) ± 2.3	Not branched
E. frustillata Miers	South Argentina; Chile	17.6 (12.7–23.9) ± 2.4	40.6 (35.4–47.7) ± 2.6	0.43 (0.32–0.60) ± 0.06	13.5 (10–18) ± 1.8	Not branched
E. multiflora Phil. ex. Stapf	North Chile; Argentina	17.4 (13.5–22.0) ± 2.0	43.2 (35.7–50.7) ± 3.6	0.40 (0.32–0.49) ± 0.03	13.6 (10–18) ± 2.0	Not branched
E. ochreata Miers	Argentina	16.9 (12.4–26.1) ± 3.3	40.4 (31.9–50.6) ± 4.7	0.42 (0.33–0.57) ± 0.05	15.1 (12–20) ± 1.9	Not branched
E. triandra Tul.	Argentina to South Brazil	18.2 (15.1–22.2) ± 1.6	35.8 (25.7–49.2) ± 7.7	0.53 (0.33–0.74) ± 0.09	18.8 (14–24) ± 2.6	Not branched
E. trifurcata Zöllner	Chile	9.8 (8.0–11.3) ± 0.7	20.5 (18.2–23.04) ± 1.22	0.48 (0.41–0.53) ± 0.03	12.3 (10–14) ± 1.0	Not branched
E. tweediana Fisch. et C.A.Mey.	Uruguay and Argentina	15.6 (12.2–19.8)± 1.6	37.6 (30.6–44.2)± 3.0	0.41 (0.32–0.62)± 0.06	14.2 (10–20)± 1.8	Not branched
Asian species						
E. distachya L.	Europe and Central Asia	20.0 (15.0–25.1) ± 1.8	49.6 (41.7–59.9) ± 3.7	0.41 (0.32–0.53) ± 0.04	6.6 (4–10) ± 1.2	First-order branches, occasionally secondary branched
E. equisetina Bunge	Central Asia; Russia; North China	17.3 (6.4–28.8) ± 6.8	38.5 (19.5–56.7) ± 13.8	0.44 (0.31–0.60) ± 0.06	5.5 (4–8) ± 1.1	First-order branches, often secondary branched
E. gerardiana Wall. ex Florin	Himalaya region to southwest China	19.7 (15.1–24.4) ± 1.8	45.7 (39.2–54.8) ± 2.9	0.43 (0.33–0.55) ± 0.05	6.4 (4–10) ± 1.3	First-order branches, no secondary branches
E. intermedia Schrenk et C.A.Mey.	Central Asia	15.1 (8.7–24.0) ± 4.9	37.0 (20.2–52.2) ± 11.4	0.41 (0.30–0.52) ± 0.04	9.2 (6–14) ± 1.5	First-order branches, no secondary branches
E. likiangensis Florin	Southwest China	20.3 (15.1–27.1) ± 2.4	46.9 (35.4–56.0) ± 5.0	0.44 (0.30–0.59) ± 0.06	13.0 (10–18) ± 2.2	Occasionally short first-order branches, no secondary branches
E. lomatolepis Schrenk	Central Asia	12.6 (7.7–21.7) ± 5.9	32.3 (20.3–51.7) ± 12.8	0.38 (0.32–0.47) ± 0.04	11.5 (10–14) ± 1.3	Not branched
E. minuta Florin	West China	15.9 (7.7–25.5) ± 6.6	35.4 (20.4–53.1) ± 13.4	0.45 (0.35–0.56) ± 0.05	15.2 (12–20) ± 2.4	Occasionally short first-order branches, no secondary branches
E. monosperma J.G.Gmel. ex C.A.Mey.	Siberia to northwest China	15.4 (8.2–21.9) ± 5.1	36.1 (19.9–53.2) ± 10.9	0.42 (0.33–0.53) ± 0.05	9.8 (6–12) ± 1.6	First-order branches, no secondary branches

E. pachyclada Boiss.	Iran to Afghanistan	18.0 (8.8–25.32) ± 5.1	46.0 (20.6–59.1) ± 13.8	0.39 (0.32–0.50) ± 0.04	6.7 (4–8) ± 1.2	First-order branches, occasionally secondary branched
E. regeliana Florin	Central to East Asia	13.3 (7.5–18.2) ± 3.4	36.4 (19.2–46.7) ± 9.5	0.37 (0.29–0.44) ± 0.03	9.4 (6–12) ± 1.3	Short first-order branches, no secondary branches
E. sarcocarpa Aitch. et Hemsl.	Afghanistan; Pakistan	12.0 (7.0–19.7) ± 4.4	31.3 (19.1–48.6) ± 10.5	0.38 (0.28–0.50) ± 0.04	14.4 (10–20) ± 2.9	First-order branches, no secondary branches
E. saxatilis (Stapf) Royle ex Florin	West China	18.8 (8.4–23.7) ± 3.9	46.0 (19.8–57.0) ± 9.9	0.41 (0.33–0.52) ± 0.04	10.9 (8–18) ± 2.4	First-order branches, no secondary branches
E. sinica Stapf	North China; Mongolia	19.0 (8.1–25.6) ± 4.4	47.5 (21.2–62.6) ± 10.7	0.40 (0.26–0.52) ± 0.05	6.8 (4–10) ± 1.4	First-order branches, often secondary branched
E. strobilacea Bunge	Central Asia to Afghanistan	15.6 (7.1–26.91) ± 5.2	39.1 (19.6–58.5) ± 12.0	0.39 (0.32–0.56) ± 0.04	13.4 (10–16) ± 1.6	Not branched
E. transitoria Riedl	West Asia	17.1 (9.2–26.2) ± 4.5	40.4 (16.6–52.7) ± 12.5	0.44 (0.31–0.63) ± 0.08	14.3 (10–20) ± 2.1	Not branched

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