

MICROMORPHOLOGY OF THE SEED ENVELOPE OF *EPHEDRA* L. (GNETALES) AND ITS RELEVANCE FOR THE TIMING OF EVOLUTIONARY EVENTS

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Micromorphology of the seed envelope of *Ephedra* (Gnetales) is known to be variable, but variation patterns have never been systematically documented. We test the usefulness of this feature for species determination and subclade delimitation in *Ephedra* and investigate the relationship of this character to infrageneric evolutionary patterns. Most species have a basically smooth seed envelope, which in some species appears slightly striate or reticulate due to convex or depressed outer periclinal cell walls. *Ephedra rhytidosperma* from China and *Ephedra torreyana* from North America have transverse lamellae formed by the epidermis. A papillate surface is found in respective close relatives of these two species. Micromorphology of the seed envelope is generally not useful for species identification or subclade delineation. The amount of variation is low, and intraspecific variation, which in some cases seems to be correlated with hybridization and/or introgression, complicates species recognition. Furthermore, parallel evolution of similar micromorphological patterns in unrelated subclades of *Ephedra* is evident and cannot be explained by similar seed dispersal mechanisms. The Asian species with transverse lamellae or papillae on the seed are dispersed by frugivores whereas similar American species are anemochoric. Transverse ridges occur in several Early Cretaceous fossil seeds with affinity to *Ephedra*. However, our results indicate that the resemblance between these fossils and extant taxa with similar features is superficial and convergent. In line with other recent studies, we find that Cretaceous ephedroids are extinct stem relatives to the extant clade.

Keywords: Early Cretaceous, *Ephedra*, fossils, Gnetales, micromorphology, scanning electron microscopy, seed envelope, systematics.

Introduction

Ephedra L. (Gnetales) comprises ~50 species inhabiting dry, often sandy or rocky, subtropical to temperate areas of the Northern Hemisphere and South America (Kubitzki 1990). They are shrubs or herbs, similar in gross morphology, with opposite or whorled phyllotaxis. *Ephedra* species are usually dioecious, and ovulate cones have fleshy or dry bracts. The ovules are surrounded by an outer structure unique to Gnetales, the seed envelope. The apical part of the integument forms a micro-pylar tube that extends beyond the seed envelope and serves as the pollen-receiving area. Both morphological variation and molecular sequence divergence have been of limited value in understanding evolution in *Ephedra*, and phylogenetic relationships in the clade have long remained elusive. However, recent studies using additional molecular data (Ickert-Bond and Wojciechowski 2004; Rydin and Korall 2009) provide a robust framework for investigations of character evolution.

Several recent discoveries document a surprisingly rich diversity of ephedran species in the Early Cretaceous. Well-preserved

reproductive shoots bearing ovulate (and probably also pollen) cones are recorded, for example, from the Yixian Formation of western Liaoning, China (Guo and Wu 2000; Sun et al. 2001; Yang et al. 2005; Rydin et al. 2006b), the Manlaj locality in Mongolia (Krassilov 1982), and the Crato Formation, Brazil (Mohr et al. 2004). Furthermore, well-preserved coalified seeds are described from Buarcos, Portugal, and the Patuxent Formation of Virginia (Rydin et al. 2004; Rydin et al. 2006a).

Ephedra archaeorhytidosperma (Yang et al. 2005) is a compression fossil collected from the Jianshangou Bed in the lower part of the Yixian Formation and is of Early Cretaceous age (Aptian; see summary in Zhou 2006). The fossils from the Yixian Formation are of particular value in showing interconnections between vegetative and reproductive parts. However, they generally lack anatomical preservation, and it is often difficult to assess their phylogenetic position in detail. Features diagnostic for extant *Ephedra* (i.e., *Ephedra*-type pollen and papillae on the seed envelope; see also Rydin et al. 2006a), are typically not preserved, but the general habit of *E. archaeorhytidosperma* clearly indicates that it is an ephedroid plant.

One distinctive feature of this fossil is the presence of numerous small transverse ridges on the surface of the seed envelope (Yang et al. 2005). A similar seed surface pattern is also found in the extant Chinese species *Ephedra rhytidosperma*

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(Yang et al. 2005; Yang 2007), which is endemic to the Helan Mountains between Nei Mongol and Ningxia in China (Fu et al. 1999). Yang et al. (2005) suggested an affinity between *E. rhytidosperma* and *E. archaeorhytidosperma* for several reasons, among them the transverse laminar ridges. However, variation patterns in seed surface micromorphology have not been documented for extant species of *Ephedra*, and the distribution of this feature is thus unknown.

Here we document variation in micromorphological patterns of the seed envelope of extant *Ephedra*. We test the taxonomic usefulness of seed envelope patterns for species determination and subclade delineation in *Ephedra* and investigate evolutionary patterns of this feature. We also assess the phylogenetic position of the Early Cretaceous fossil *E. archaeorhytidosperma*.

Material and Methods

We investigated 117 specimens representing 48 living taxa from both the Old World and the New World (table 1). In general, two to five (or more) accessions of each species were examined, but for 13 species, only one specimen of each was available to us (table 1). Material at the pollination stage of development and mature seeds were studied. Ovulate cones were fixed in 70% ethanol or in FAA and dehydrated under vacuum in an ethanol series (70%, 80%, 96%, 100%). Herbarium material was softened using bis(2-ethylhexyl) sulphosuccinate sodium salt (following Erbar 1995) for 7–10 d at room temperature and then placed in 70% ethanol and dehydrated under vacuum as described above.

For SEM studies, specimens were postfixed in 2% osmium tetroxide (Fluka), dehydrated in an ethanol series (70%, 80%, 90%, 100%) and acetone, critical-point dried, mounted on stubs, and sputter-coated with gold. Seeds were examined with an SEM, either an Amray 1000A (Arizona State University), an ISI-SR-50 (University of Alaska Fairbanks), or a Hitachi S-4000 (University of Zurich).

For serial sections, specimens were embedded in Kulzer's Technovit 7100 (2-hydroethyl methacrylate), following the procedures outlined by Igersheim and Cichocki (1996) and sectioned on a Microm HM 355 rotary microtome with a conventional knife D. The sections were 6–7 μm thick and were stained with ruthenium red and toluidine blue for 2 + 2 minutes and mounted in Histomount. Mature, highly sclerified seeds were embedded in Bio-Plastic resin (Ward's Natural Science, Rochester, NY) and sectioned on a lapidary saw. The sections were mounted on slides, ground down to a minimal thickness, and photographed using reflective and transmitted light microscopy (Benedict et al. 2008). Permanent slides are deposited at the Department of Systematic Botany, Stockholm University (SUNIV), and the ASU Fossil Plant Collections, Arizona State University.

Results

General Morphology

The shape of *Ephedra* seeds varies between species and sometimes also within species (table 1). In longitudinal section, they may be lanceolate (fig. 1A; e.g., *E. torreyana*), ellip-

tic (fig. 1B; *E. foeminea*), ovate (fig. 1C; *E. equisetina*), or oblong (fig. 1D; *E. transitoria*). In transverse section, they are rounded or angled and the angles are sometimes prominent (e.g., in *E. rhytidosperma*). In one-seeded ovulate cones, the adaxial side of the seed is convex at midlength of the ovule/seed (e.g., *E. equisetina*, *E. californica*); in two-seeded cones, it is generally flat (e.g., *E. sinica*, *E. pedunculata*); and in three-seeded cones it has a median longitudinal ridge (e.g., *E. multiflora*, *E. przewalskii*). Two abaxial, lateral furrows may be present in some species (e.g., *E. sinica*, *E. pachyclada*, *E. triandra*). Mature seeds are typically larger than those at the pollination stage of development, and their seed envelopes have longer epidermal cells, but no other differences in shape or surface patterns are observed (table 1).

SEM Observations of the Seed Envelope

Epidermal cells are rectangular and arranged parallel to the longitudinal axis of the ovule or seed. The cells are usually ~20–30 μm wide. Cell length is variable within and between species (table 1), ranging in mature seeds from 50 to 500 μm at midlength of the seed. Cells are shorter in the apical region of the seed and in the seed envelope of ovules at the pollination stage of development.

Most species have a basically smooth seed envelope surface (figs. 1B, 1D, 3A). Epidermal cell boundaries are usually clearly visible, and periclinal walls are flat (fig. 1E), depressed (fig. 1F), or convex (fig. 1G). Sometimes (e.g., in *E. pachyclada*), cell boundaries are less obvious. Features are usually distinct but may be variable within species or even within a single seed (table 1). For example, some but not all specimens of *E. aspera* and *E. fasciculata* consistently have an indistinctly coarse surface pattern, which obscures cell boundaries (not shown). Variation is also observed in the shape of the end walls of cells; they are either straight or oblique and variously raised above the general epidermal cell surface (fig. 1E–1G). The shape of end walls of cells is usually highly variable within a single seed (e.g., fig. 1G).

In two unrelated species, *E. rhytidosperma* from East Asia (fig. 2C, 2G, 2K) and *E. torreyana* from North America (figs. 2B, 2F, 2J, 3C, 4), periclinal walls of adjacent epidermal cells grow outward and are united to form transverse lamellae on both the adaxial and the abaxial sides of the seed envelope. The outgrowths are typically 100–500 μm wide in both species and are formed by a few cells to up to 20 adjacent cells in *E. torreyana* and up to 40 cells in *E. rhytidosperma*. The lamellae are formed by the cells of the epidermis; the mesophyll is not involved in forming the lamellar outgrowths (fig. 3C). In two specimens of *E. torreyana*, the lamellae are “papillate,” that is, less prominent and formed by one to several adjacent papillae (fig. 1J). *Ephedra multiflora* has transverse ridges (fig. 1I) of the same width as those of *E. torreyana* and *E. rhytidosperma*, but the ridges are less prominently lamellar, which gives an overall wavy pattern rather than a lamellar one. In addition, weak tendencies to form tiny transverse ridges on at least parts of the seed envelope were observed in several unrelated species, for example, in one specimen of *E. trifurca* and in *E. pachyclada* and *E. alata*.

A few species in two unrelated clades have a prominently papillate seed surface (figs. 1H, 2I, 2L, 4). The papillae are

Table 1

Voucher Information of *Ephedra* Specimens Examined and Seed Characters Studied (Based on 10 Measurements)

Clade, specimen ID	Species	Locality	Voucher	Seed shape in longitudinal section; apex shape	General epidermal pattern	Sculpt size (μm)	Cell length (μm)	Periclinal cell walls	ITS ^a
Mediterranean:									
UAF	<i>E. foeminea</i> Forssk.	Italy	1526: 1983 (PE seed bank) ^b	Elliptic; acute	Smooth	...	80–200	Variable	...
130	<i>E. foeminea</i> Forssk.	Greece	Rydin 130 (Z) ^b	Narrowly elliptic; acute	Smooth	...	120–200	Convex	GU968546
152	<i>E. foeminea</i> Forssk.	Dalmatia	Freitag 19.807 (KAS) ^c	Elliptic; acute	Smooth	...	100–180	Convex	GU968551
159	<i>E. foeminea</i> Forssk.	Greece	Fries C-7619 (S) ^c	Narrowly elliptic; acute	Smooth	...	100–180	Convex	...
UAF	<i>E. alata</i> Decne.	Algeria	Cosson s.n. (MO) ^b	Ovate; acuminate	Smooth (partly weakly transversely lamellar)	...	Unclear	Flat	...
128	<i>E. alata</i> Decne.	Algeria	Anderberg 481 (S) ^c	Ovate; acuminate	Smooth (partly weakly transversely lamellar)	100–300	100–180	Flat or convex	...
147	<i>E. alata</i> Decne.	Algeria	Cosson C-311 (S) ^b	Ovate; acuminate	Smooth (partly weakly transversely lamellar)	~300	Unclear	Flat	...
082	<i>E. altissima</i> Desf.	Tunisia	Botan SU 18 (S) ^c	Elliptic; acute	Smooth	...	40–150	Convex	AY755773
132	<i>E. altissima</i> Desf.	Morocco	Freitag 35.001 (KAS) ^b	Ovate; acute	Smooth	...	150–200	Convex	...
080	<i>E. aphylla</i> Forssk.	Libya	Anderberg 853 (S) ^c	Elliptic; acute	Smooth	...	30–60	Convex	AY755771
124	<i>E. aphylla</i> Forssk.	Palestine	Kramer 4727 (Z) ^c	Narrowly elliptic; acute	Smooth	...	100–200	Convex	GU968544
154	<i>E. aphylla</i> Forssk.	Israel	Amdursky 402 (S) ^c	Ovate; acute	Smooth	...	150–300	Variable	GU968552
101	<i>E. fragilis</i> Desf.	Morocco	Jonsell 5412 (UPS) ^c	Narrowly oblong; acute	Smooth	...	50–100	Variable	FJ958014
109	<i>E. fragilis</i> Desf.	Morocco	Denk s.n. (S) ^c	Narrowly elliptic; acute	Smooth	...	70–140	Convex	FJ958019
120	<i>E. fragilis</i> Desf.	Hispaniola	Freitag 328–40 (Z) ^c	Narrowly elliptic; acute	Smooth	...	50–150	Convex	...
162	<i>E. major</i> Host ssp. <i>major</i>	Spain	Ipse 71/677E (Z) ^c	Narrowly oblong; acute	Smooth	...	120–220	Convex	GU968553
166	<i>E. major</i> Host ssp. <i>major</i>	Algeria	Hofmann 013–1971 (Z) ^c	Narrowly oblong; acute	Smooth	...	120–150	Convex	GU968557
167	<i>E. major</i> Host ssp. <i>major</i>	Algeria	Juillet 94 (Z) ^c	Narrowly oblong; acute	Smooth	...	80–180	Convex	GU968558
Clade A:									
146	<i>E. ciliata</i> Fisch. et C.A. Mey.	Morocco	Balls B2487 (S) ^c	Ovate; acute	Smooth	...	70–180	Variable	GU968548
153	<i>E. ciliata</i> Fisch. et C.A. Mey.	Turkmenistan	Androssov 3367 (S) ^c	Elliptic; acute	Smooth	...	80–200	Flat or depressed	...
096	<i>E. foliata</i> Boiss. et C.A. Mey.	Somalia	Thulin 10745 (UPS) ^c	Elliptic; acute	Smooth	...	80–180	Flat or convex	FJ958010
North America:									
ASU	<i>E. antisiphilitica</i> Berl. ex C.A. Mey.	Texas	Ickert-Bond 900 (ASU) ^b	Elliptic; obtuse	Smooth	...	110–140	Depressed	AY599148
UAF	<i>E. aspera</i> Engelm. ex S. Watson	Arizona	Rose 40086 (MO) ^b	Ovate; acute	Smooth	...	50–100	Depressed	...
ASU	<i>E. aspera</i> Engelm. ex S. Watson	Texas	Correll 23971 (NY) ^b	Ovate; acute	Indistinctly coarse	...	Unclear	Convex	...
UAF	<i>E. aspera</i> Engelm. ex S. Watson	California	Faulkner 545 (UCR) ^b	Ovate; acute	Indistinctly coarse	...	200–280	Convex	...

UAF	<i>E. aspera</i> Engelm. ex S. Watson	Big Bend Basin Road, Texas	Ickert-Bond 895 (ASU) ^b	Ovate; acute	Papillate	5–10	160–270	Flat; papillate	...
UAF	<i>E. californica</i> S. Watson	California	Hendrickson 8616 (ASU) ^b	Ovate; acute	Smooth	...	Unclear	Convex	...
ASU	<i>E. californica</i> S. Watson	California	Jepson 20690 (NY) ^b	Ovate; obtuse	Smooth	...	130–300	Convex	...
161	<i>E. californica</i> S. Watson	California	Tidestrom 9692 (S) ^c	Ovate; acute	Smooth	...	80–170	Convex	...
UAF	<i>E. clokeyi</i> Cutler	Arizona	Jones s.n. (MO) ^b	Narrowly elliptic; acute	Smooth	...	100–180	Flat	...
ASU	<i>E. compacta</i> Rose	Mexico	Correll & Johnson 19900 (NY) ^b	Oblong; acute	Smooth	...	70–180	Depressed	...
155	<i>E. compacta</i> Rose	Mexico	Purpus s.n. (S) ^c	Oblong; acute	Smooth	...	60–120	Depressed	...
UAF	<i>E. coryi</i> E.L. Reed	Texas	Warnock 10713 (TEX) ^b	Elliptic; obtuse	Smooth	...	90–150	Convex	...
UAF	<i>E. coryi</i> E.L. Reed	Texas	Correll 32762 (TEX) ^c	Elliptic; obtuse	Smooth	...	60–130	Convex	...
UAF	<i>E. cutleri</i> Peebles	Utah	Holmgren 12744 (ASU) ^b	Ovate; acute	Smooth	...	60–130	Flat	...
ASU	<i>E. cutleri</i> Peebles	Arizona	Ickert-Bond 692 (ASU) ^b	Oblong-unclear; acute	Smooth	...	120–260	Flat	...
UAF	<i>E. cutleri</i> Peebles	Arizona	Ickert-Bond 996 (ASU) ^b	Oblong-ovate; acute	Smooth	...	130–240	Flat or convex	...
UAF	<i>E. fasciculata</i> A. Nelson	Arizona	Ickert-Bond 513 (ASU) ^b	Ovate; acuminate	Indistinctly coarse	...	70–140	Flat or convex	AY599180
ASU		Arizona	Ickert-Bond 813 (ASU) ^b	Ovate; acuminate	Smooth	...	120–160	Flat or convex	...
UAF	<i>E. fasciculata</i> A. Nelson	Arizona	Ickert-Bond 541 (ASU) ^b	Ovate; acuminate	Indistinctly coarse	...	50–120	Convex	...
UAF		Arizona	Ickert-Bond s.n. (ASU) ^b	Ovate; acuminate	Smooth	...	170–240	Convex	...
UAF	<i>E. fasciculata</i> A. Nelson	Arizona	Ickert-Bond 573 (ASU) ^b	Lanceolate; acuminate	Papillate	10–25	Unclear	Flat; papillate	...
UAF	<i>E. funerea</i> Coville et Morton	Arizona	Baker 13971 (ASU) ^b	Lanceolate; acuminate	Papillate	5–10	Unclear	Flat; papillate	...
ASU	<i>E. funerea</i> Coville et Morton	California	Wolf 10599 (RSA, NY) ^b	Lanceolate; acuminate	Papillate	5–10	150–360	Flat; papillate	...
UAF	<i>E. funerea</i> Coville et Morton	California	Sanders 9049 (UCR) ^b	Lanceolate; acuminate	Papillate	5–10	Unclear	Flat; papillate	...
UAF	<i>E. nevadensis</i> S. Watson	Nevada	Gierisch 4722 (ARIZ) ^b	Ovate; acute	Smooth	...	160–410	Flat or convex	...
122	<i>E. nevadensis</i> S. Watson	n.a.	Polovado s.n. (Z) ^c	Ovate; acute	Smooth	...	50–120	Convex	...
UAF	<i>E. pedunculata</i> Engelm. ex S. Watson	Texas	Parks 3199 (MO) ^b	Oblong; acute	Smooth	...	100–250	Variable	...
ASU	<i>E. pedunculata</i> Engelm. ex S. Watson	Texas	Palmer 1291 (NY) ^b	Oblong; acute	Smooth	...	180–350	Variable	...
UAF	<i>E. torreyana</i> S. Watson	Arizona	Ickert-Bond 666 (ASU) ^b	Lanceolate; acuminate	Transversely lamellar	50–520	150–350	Flat or convex	...
ASU	<i>E. torreyana</i> S. Watson	Colorado	Franklin 3368 (NY) ^b	Lanceolate; acuminate	Transversely lamellar	120–580	150–450	Flat or convex	...
S	<i>E. torreyana</i> S. Watson	Nevada	Clokey 8224 (S) ^c	Lanceolate; acuminate	Transversely lamellar	100–500	100–300	Flat or convex	...
UAF	<i>E. torreyana</i> S. Watson	Arizona	Ickert-Bond 998 (ASU) ^b	Lanceolate; acuminate	Papillate	30–50	600–670	Convex; papillate	...
126	<i>E. torreyana</i> S. Watson	New Mexico	Porter & Porter 8998 (S) ^c	Lanceolate; acuminate	Papillate	20–30	100–500	Convex; papillate	...
UAF	<i>E. trifurca</i> Torrey ex S. Watson	Arizona	Skjot-Pedersen s.n. (PE) ^b	Lanceolate; acuminate	Smooth	...	120–200	Convex	...
UAF	<i>E. trifurca</i> Torrey ex S. Watson	Arizona	Ickert-Bond 577 (ASU) ^b	Lanceolate; acuminate	Smooth	...	140–220	Convex	...
ASU	<i>E. trifurca</i> Torrey ex S. Watson	Arizona	Ickert-Bond 753 (ASU) ^b	Lanceolate; acuminate	Smooth (partly weakly transversely lamellar)	...	130–170	Convex	AY599164

Table 1

(Continued)

Clade, specimen ID	Species	Locality	Voucher	Seed shape in longitudinal section; apex shape	General epidermal pattern	Sculpt size (μm)	Cell length (μm)	Periclinal cell walls	ITS ^a
253	<i>E. trifurca</i> Torrey ex S. Watson	Arizona	Goodding 2268 (S) ^b	Lanceolate; acuminate	Smooth	...	150–320	Convex	...
254	<i>E. trifurca</i> Torrey ex S. Watson	Arizona	Nelson & Nelson 1290 (S) ^b	Lanceolate; acuminate	Smooth	...	170–240	Convex	...
ASU	<i>E. viridis</i> Coville	California	Parish 2975 (NY) ^b	Oblong-ovate; acute	Smooth	...	190–280	Convex	...
091	<i>E. viridis</i> Coville	Utah	Holmgren et al. 1826 (UPS) ^c	Ovate; acute	Smooth	...	25–60	Convex	FJ958005
South America: ASU	<i>E. americana</i> Humb. et Bonpl. ex Willd.	Ecuador	Juncosa 2257 (NY) ^b	Elliptic; acute	Smooth	...	190–400	Depressed	...
127	<i>E. americana</i> Humb. et Bonpl. ex Willd.	Argentina	Novara 8219 (S) ^c	Elliptic; acute	Smooth	...	120–300	Convex	GU968545
UAF	<i>E. boelkei</i> Roeg.	Argentina	Ickert-Bond 1252 (ASU) ^b	Ovate; acute	Smooth	...	100–250	Convex	AY599175
ASU	<i>E. breana</i> Phil.	Chile	Ickert-Bond 1233 (ASU) ^b	Elliptic; rounded	Smooth	...	140–240	Depressed	...
025	<i>E. chilensis</i> K. Presl	n.a.	Chase 10140 (K) ^c	Elliptic; acute	Smooth	...	40–80	Convex	AY755744
ASU	<i>E. chilensis</i> K. Presl.	Argentina	Jostasato 4333 (NY, ARIZ) ^b	Elliptic; acute	Smooth	...	120–220	Convex	...
075	<i>E. chilensis</i> K. Presl.	Chile	Forbes 49.0542 (UC) ^c	Elliptic; acute	Smooth	...	30–100	Convex	AY755767
123	<i>E. chilensis</i> K. Presl.	Chile	Gay 400 (Z) ^c	Elliptic; acute	Smooth	...	40–140	Convex	GU968543
UAF	<i>E. multiflora</i> Phil. ex Stapf	Chile	Ickert-Bond 1211 (ASU) ^b	Ovate; acuminate	Transversely wavy	120–480	120–470	Flat	AY599173
40 UAF	<i>E. multiflora</i> Phil. ex Stapf	Chile	Ickert-Bond 1231 (ASU) ^b	Ovate; acuminate	Transversely wavy	100–500	100–500	Flat	...
UAF	<i>E. ochreate</i> Miers	Argentina	Ickert-Bond 1253 (ASU) ^b	Narrowly ovate; obtuse	Smooth	...	110–220	Flat or convex	...
ASU	<i>E. ochreate</i> Miers	Argentina	Ickert-Bond 1257 (ASU) ^b	Narrowly ovate; obtuse	Smooth	...	100–180	Depressed	...
UAF	<i>E. rupestris</i> Benth.	Ecuador	Ickert-Bond 1100 (ASU) ^b	Elliptic; obtuse	Smooth	...	50–150	Depressed	AY599167
073	<i>E. rupestris</i> Benth.	Ecuador	Ornduff 9675 (UC) ^c	Elliptic; obtuse	Smooth	...	25–60	Depressed	AY755765
ASU	<i>E. triandra</i> Tul.	Argentina	Capitanelli 584 (ASU) ^b	Oblong; narrowly acute	Smooth	...	80–190	Depressed	...
UAF	<i>E. triandra</i> Tul.	Argentina	R. Leal 15981 (ASU) ^b	Oblong; narrowly acute	Smooth	...	150–400	Depressed	...
UAF	<i>E. tweediana</i> Fisch. ex C.A. Mey.	Uruguay	Herter 1010 (MO) ^b	Elliptic; obtuse	Smooth	...	120–200	Depressed	...
076	<i>E. tweediana</i> Fisch. ex C.A. Mey.	Argentina	Forbes 66.0742 (UC) ^c	Elliptic; obtuse	Smooth	...	30–100	Variable	AY755768
Asia H: 002	<i>E. likiangensis</i> Florin	n.a.	Rydin 03–926 (S) ^c	Elliptic; acute	Smooth	...	20–45	Convex	AY755739
157	<i>E. likiangensis</i> Florin	n.a.	Cult. 1988–844 (K) ^c	Oblong; obtuse	Smooth	...	40–110	Convex	...
158	<i>E. likiangensis</i> Florin	n.a.	Cult. 18480.000 (K) ^c	Oblong; acute	Smooth	...	90–150	Convex	...
007	<i>E. minuta</i> Florin	Sikang, China	Rydin 03–930 (S) ^c	Elliptic; acute	Smooth	...	40–80	Convex	AY755742
063	<i>E. minuta</i> Florin	n.a.	Rydin 04–486 (S) ^c	Elliptic; acute-obtuse	Smooth	...	30–50	Convex	AY755756
Asia K: UAF	<i>E. equisetina</i> Bunge	Mount Helan, China	Yang 2004003 (PE) ^b	Ovate; acute	Papillate and transversely lamellar	5–10; 0–200	Unclear	Flat or convex; papillate	...
071	<i>E. equisetina</i> Bunge	Georgia	Merello et al. 2241 (MO) ^c	Elliptic; acute	Papillate	2–4	20–80	Flat or convex; papillate	AY755763

125	<i>E. equisetina</i> Bunge	Turkmenistan	Sintensis 666 (S) ^c	Narrowly ovate; acute	Smooth	...	80–200	Convex	...
142	<i>E. equisetina</i> Bunge	Russian Altai	Freitag 05.2008 (KAS) ^c	Ovate; acute	Smooth	...	100–200	Convex	...
234	<i>E. equisetina</i> Bunge	Turkmenistan	Lipsky 2610 (S) ^c	Ovate; acute	Smooth	...	40–100	Convex	GU968572
249	<i>E. equisetina</i> Bunge	Turkestan	Moldengauer 22 (S) ^c	Ovate; acute	Smooth	...	75–200	Convex	...
250	<i>E. equisetina</i> Bunge	Turkmenistan	Lipsky 3653 (S) ^b	Ovate; acute	Smooth	...	100–180	Convex	...
251	<i>E. equisetina</i> Bunge	Turkmenistan	Lipsky 2587 (S) ^c	Ovate; acute	Smooth	...	100–200	Convex	...
252	<i>E. equisetina</i> Bunge	Kopet Dag Mountain	Lipsky 2124 (S) ^c	Ovate; acute	Papillate	4–10	70–150	Convex	...
148	<i>E. gerardiana</i> Wall. ex Florin	Almora, India	Parker 2099 (S) ^c	Oblong; acute	Smooth	...	50–150	Depressed	...
UAF	<i>E. major</i> Host	Morocco	Lewalle 9642 (MO) ^b	Oblong; acute	Papillate	4–8	40–100	Flat; papillate	...
163	<i>E. major</i> Host	Spain	Montserrat 319171 (Z) ^c	Elliptic; acute	Papillate	4–10	100–130	Convex; papillate	GU968554
164	<i>E. major</i> Host	Transcaucasia	Grossheim s.n. (Z) ^c	Elliptic; acute	Papillate	2–8	90–150	Convex; papillate	GU968555
165	<i>E. major</i> Host	Herzegovina	Baenitz s.n. (Z) ^c	Elliptic; acute	Papillate	2–8	100–175	Convex; papillate; with apical warty projections	GU968556
169	<i>E. major</i> Host	France	Zogg & Gassner 8388 (Z) ^c	Elliptic; acute	Papillate	2–8	120–180	Convex; papillate	GU968559
156	<i>E. pachyclada</i> Boiss.	Hissar, Turkestan	Regel s.n. (S) ^c	Elliptic; rounded	Smooth (at mid-length with weak transverse lamellae)	10–35	Unclear	Flat or convex; papillate; with apical warty projections	...
UAF	<i>E. rhytidosperra</i> Pachom.	Mount Helan, China	Yang 20060620 (PE) ^b	Obovate-elliptic; acute	Transversely lamellar	100–500	Unclear	Convex	...
138	<i>E. saxatilis</i> (Stapf) Royle ex Florin	n.a.	Cult. 1947–2603 (K) ^c	Oblong; obtuse	Smooth	...	50–130	Flat or convex	...
144	<i>E. saxatilis</i> (Stapf) Royle ex Florin	Nepal	Freitag 098–38–74–84 (KAS) ^c	Oblong; obtuse	Smooth	...	100–200	Flat or convex	...
Asia M:									
140	<i>E. distachya</i> L.	n.a.	Cult. 46126.000 (K) ^c	Elliptic; acute	Smooth	...	15–40	Convex	...
143	<i>E. sarcocarpa</i> Aitch. et Hemsl.	Iran	Freitag 13.988 (KAS) ^c	Ovate; acute	Smooth	...	100–170	Flat	...
UAF	<i>E. strobilacea</i> Bunge	Asia Media	Collector unknown (PE seed bank no. 0679: 1961) ^b	Lanceolate; narrowly acute	Smooth	...	40–100	Depressed	...
150	<i>E. strobilacea</i> Bunge	Turkmenistan	Androssov 1900 (S) ^c	Lanceolate; narrowly acute	Smooth	...	100–240	Flat or convex	GU968549
111	<i>E. transitoria</i> Riedl	Saudi Arabia	Collenette 9095 B (E) ^c	Narrowly oblong; acute	Smooth	...	70–130	Flat	FJ958021
Asia N:									
173	<i>E. fedtschenkoae</i> Paulsen	Xinjiang, China	Zhu Taiyan 650764 (N) ^c	Narrow elliptic; acute	Smooth	...	60–100	Flat or convex	...
UAF	<i>E. intermedia</i> Schrenk ex C.A. Mey.	Gansu, China	Yang LZ060707 (PE) ^b	Ovate; acute	Smooth	...	100–200	Flat	...
006	<i>E. intermedia</i> Schrenk ex C.A. Mey.	Tien-Shan Mountains, Asia Media	Rydin 03–925 (S) ^c	Elliptic; acute	Smooth	...	60–170	Flat or convex	AY755741
092	<i>E. lomatolepis</i> Schrenk	Kazakhstan	Baitulin et al. s.n. (UPS) ^c	Ovate; acute	Smooth	10–40	Unclear	Flat or convex; with rare warty projections	FJ958006
102	<i>E. lomatolepis</i> Schrenk	Tschu-Ili Mountains, Turkestan	Titow 488 (S) ^c	Ovate; acute	Smooth	10–20	100–170	Flat; with rare warty projections	FJ958015

Table 1

(Continued)

Clade, specimen ID	Species	Locality	Voucher	Seed shape in longitudinal section; apex shape	General epidermal pattern	Sculpt size (μm)	Cell length (μm)	Periclinal cell walls	ITS ^a
174	<i>E. lomatolepis</i> Schrenk	Pakistan	Bosshard et al. 803.24 (Z) ^c	Elliptic; acute	Smooth	...	70–190	Flat or depressed	GU968562
UAF	<i>E. regeliana</i> Florin	Xinjiang, China	K.C. Kuan 1067 (PE) ^b	Narrow elliptic; acute	Smooth	...	50–100	Flat or depressed	...
UAF	<i>E. simica</i> Stapf	Hebei, China	Unknown coll. s.n. (herb. no. 00015747, PE) ^b	Ovate; acute	Smooth	...	40–150	Flat	...
UAF	<i>E. simica</i> Stapf	Inner Mongolia, China	Chu 20060801 (PE) ^b	Ovate; acute	Smooth	...	70–150	Flat	...
151	<i>E. simica</i> Stapf	Inner Mongolia, China	Eriksson 05–9020 (S) ^c	Ovate; acute	Smooth	...	Unclear	Depressed	GU968550

^a The internal transcribed spacer of the nuclear ribosomal DNA (ITS) is available for some of the vouchers and has been included in phylogenetic analyses in published studies (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004, 2010; Rydin and Korall 2009). n.a. = information not available.

^b Material = mature seeds.

^c Material in pollination stage of development.

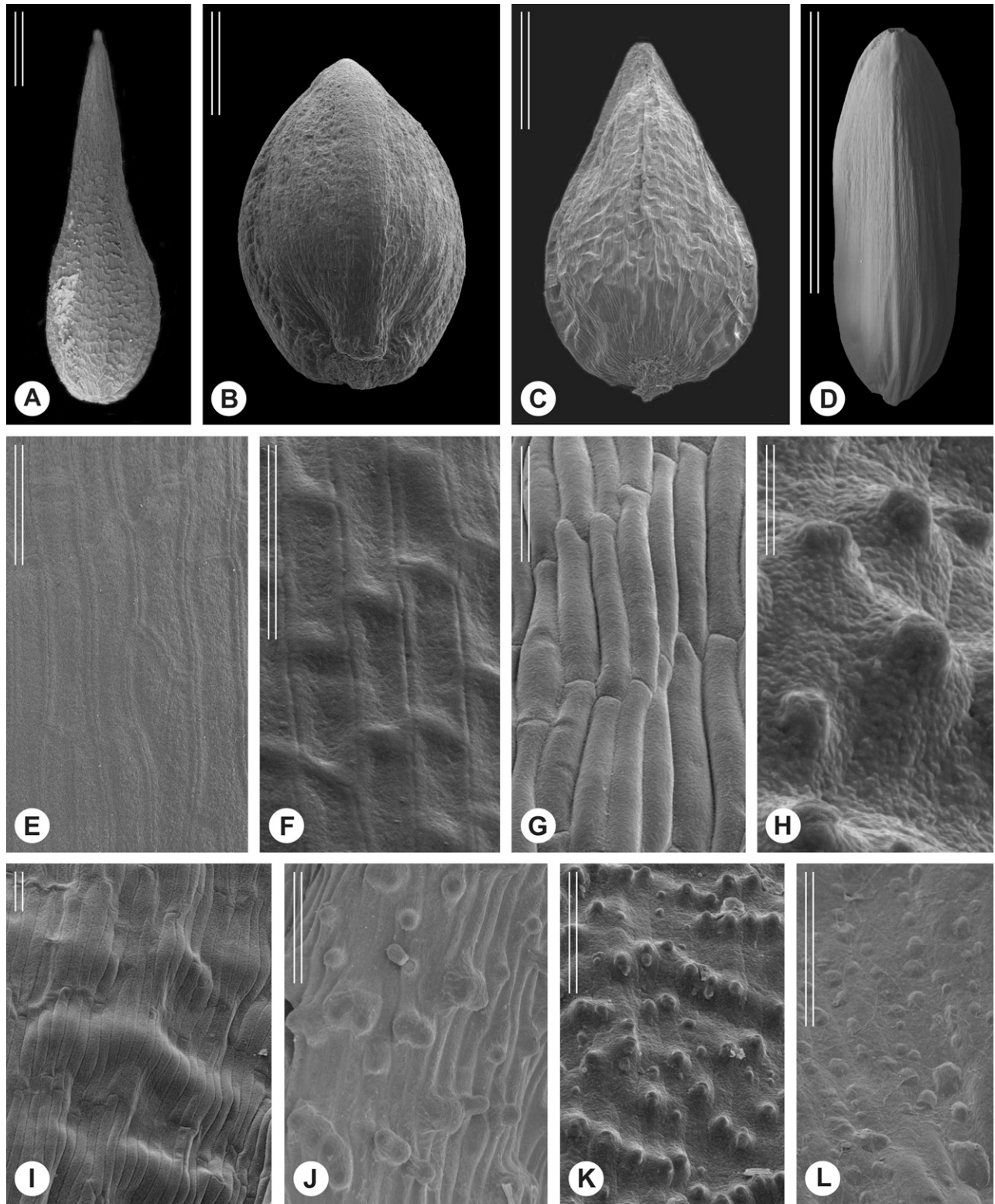


Fig. 1 Seed shapes in longitudinal outline (A–D) and surface patterns of the seed envelope (E–L). Scale bars: A–D, 2 mm; E–G, I–L, 100 μ m; H, 20 μ m. A, Seed lanceolate in *Ephedra torreyana* (Franklin 3368 [NY]); B, seed elliptic in *Ephedra foeminea* (1526 [PE seed bank]); C, seed ovate in *Ephedra multiflora* (Ickert-Bond 1211 [ASU]); D, seed oblong in *Ephedra transitoria* (Collenette 9095B [E], at pollination stage of development, i.e., smaller than the others); E, outer periclinal cell walls flat in *Ephedra sarcocarpa* (Freitag 13.988 [KAS]); F, outer periclinal cell walls depressed in *Ephedra regeliana* (K.C. Kuan 1067 [PE]); G, outer periclinal cell walls convex in *Ephedra trifurca* (Ickert-Bond 577 [ASU]); H,

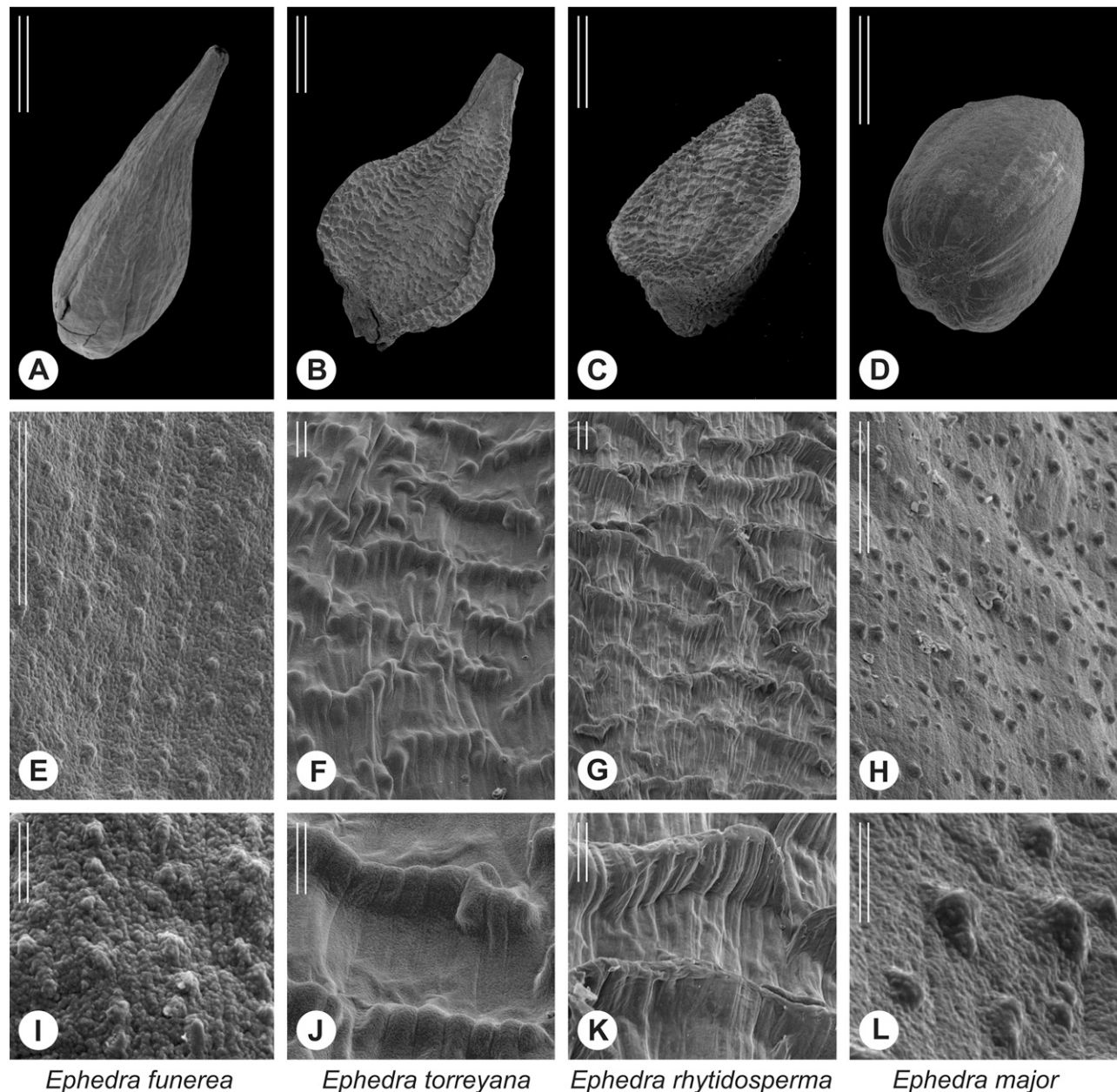


Fig. 2 Micromorphological patterns of the seed envelope: transverse lamellae (B–C, F–G, J–K) and papillae (A, D–E, H–I, L). Scale bars: A–D, 2 mm; E–H, J–K, 100 μ m; I, L, 20 μ m. A, E, I, Papillate surface of the North American species *Ephedra funerea* (Sanders 9049 [UCR]); B, F, J, transverse lamellar surface of the North American species *Ephedra torreyana* (Ickert-Bond 666 [ASU]); C, G, K, transverse lamellar surface of the Asian species *Ephedra rhytidosperra* (Yang 20060620 [PE]); D, H, L, papillate surface of the Eurasian species *Ephedra major* (Lewalle 9642 [MO]).

formed by the outer periclinal walls of the epidermis (fig. 3B). There are generally ~2–8 papillae per cell and each papilla is 2–10 μ m across. As with the transverse lamellae, the cells of the mesophyll are never involved in forming the papillae. A papillate seed surface is present in the Old World

species *E. equisetina* and *E. major* (fig. 2D, 2H, 2L) and in the New World species *E. funerea* (fig. 2A, 2E, 2I). Investigated specimens of *E. major* and *E. funerea* consistently have a papillate seed envelope, but in *E. equisetina* there is substantial intraspecific variation (table 1). Several investigated

outer periclinal cell walls papillate in *Ephedra equisetina* (Yang 2004003 [PE]); I, “wavy” transverse ridges in *E. multiflora* (Ickert-Bond 1231 [ASU]); J, “papillate lamellae” in *E. torreyana* (Porter & Porter 8998 [S]); K, “ridge-like papillae” in *E. equisetina* (Yang 2004003 [PE]); L, “wart-like projections” in *Ephedra pachyclada* (Regel s.n. [S]).

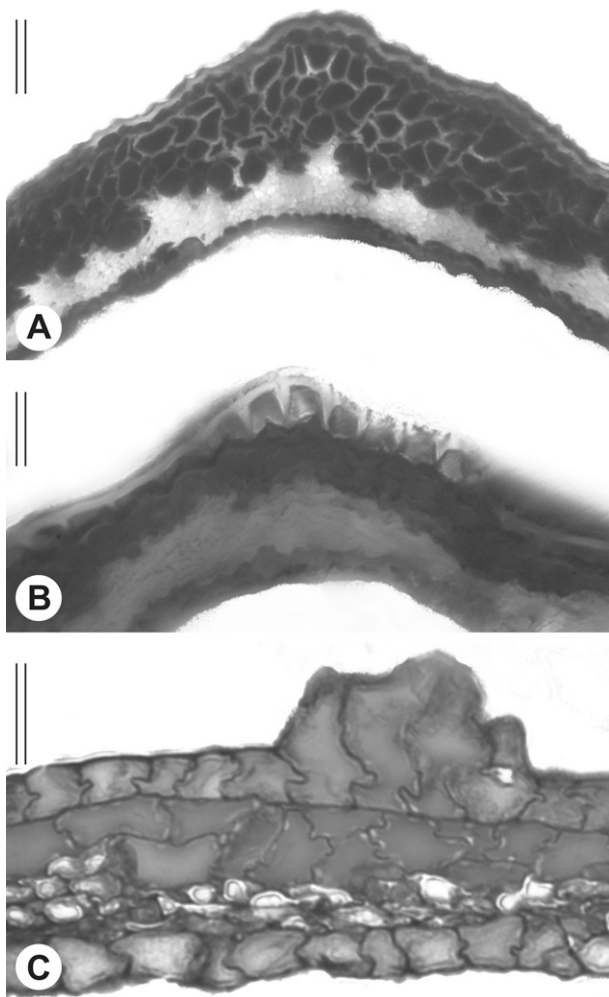


Fig. 3 Seed envelope in transverse section. Scale bars: A, 900 μm ; B, 300 μm ; C, 30 μm . A, Smooth envelope of *Ephedra ochreatea* (Ickert-Bond 1257 [ASU]); B, papillate envelope of *Ephedra funerea* (Baker 13971 [ASU]); C, transverse lamella in *Ephedra torreyana* (Porter and Porter 8998 [S]).

specimens of *E. equisetina* lack papillae altogether and have smooth seed envelopes (not shown). Seeds of *E. equisetina* from Mount Helan, China, have transverse lamellae similar to those in *E. rhytidosperra* and *E. torreyana* but with papillae on the lamellae (fig. 1K).

The specimens of *E. pachyclada* and *E. lomatolepis* and one specimen of *E. major* (table 1) have wartlike projections on the surface (fig. 1L). In *E. pachyclada* and *E. major*, they are present only in the apical region of the seed envelope. In *E. lomatolepis*, they are rare but may occur over the entire seed surface. Each projection is $\sim 10\text{--}40\ \mu\text{m}$ across. Some overarch cell boundaries, and some appear to have collapsed.

In several unrelated species (e.g., *E. alata*, *E. californica*, *E. equisetina*, *E. major* ssp. *major*, *E. intermedia*, and *E. pachyclada*), we observed stomata in the apical region of the seed envelope. They have the same shape and structure as those of cone bracts and leaves; that is, there are no obvious subsidiary

cells, and the guard cells are sunken to the level of the base of the epidermal cell layer.

Discussion

Comparative Structural Evaluation and Systematic Implications

Generally, phyllotaxy determines the number of ovules/seeds per cone (Takaso 1985; Rydin et al. 2010), but a reduction in ovule number has taken place in one-seeded cones in some decussate-leaved (e.g., *Ephedra antisiphilitica*, *E. equisetina*) and trimerous-leaved taxa (e.g., *E. trifurca*). The shape of the ovule/seed in transverse section at its midlength is largely determined by pressure from other organs, that is, the number of ovules/seeds in the cone, whereas transverse shape in the apical region of the ovule/seed is determined by the number of vascular bundles in the seed envelope (Rydin et al. 2010).

The majority of *Ephedra* species have smooth seed envelopes with minor variation in the micromorphological patterns, detectable only under SEM. The outer periclinal cell wall of the epidermis can be convex or depressed, which results in striate or reticulate patterns of the seed envelope. Taxa of the Mediterranean species complex (fig. 4) are often striate, whereas many Asian species have flat periclinal walls, but variation is extensive within species.

Transverse lamellae on the surface of the seed envelope, visible with the naked eye, are present in two distantly related species: *E. rhytidosperra* from China and *E. torreyana* from North America. In both species, the lamellae are between 100 and 500 μm wide (variable in width within a single seed) and are formed by the outer epidermis. As seen in transverse sections of the seed envelopes of *E. torreyana* (fig. 3C) and of *E. rhytidosperra* (Yang 2002), the lamellae are formed by extensions of the outer tangential cell wall of one to several adjacent epidermal cells. The inner tangential cell wall of these cells is in line with that of the other epidermal cells, and the mesophyll is not involved in the formation of the lamellae (fig. 3C; Yang 2002; Rydin et al. 2010). A similar pattern, but one that is less prominently lamellar, is observed in *E. multiflora* from South America, and weak ridges are seen in parts of seeds of several other species (table 1).

From our survey it is evident that a similar lamellate micromorphological pattern has evolved independently in several clades in *Ephedra* (fig. 4). It does not appear to have evolved in response to a common dispersal syndrome or other ecological factors. *Ephedra rhytidosperra* has fleshy cone bracts at seed maturity, and seeds are probably dispersed by birds or lizards. In contrast, *E. torreyana* has dry winged bracts at seed maturity, and seeds are dispersed by the wind (Hollander et al. 2010). We do not find any other shared ecological trait that could explain this parallelism.

A papillate surface of the seed envelope has arisen (at least) twice in the Asian clade (*E. equisetina*, *E. major*) and once in the North American clade (*E. funerea*; fig. 4). Like the species with transversely lamellar seeds, there is no ecological correlation between species with papillate seeds. *Ephedra equisetina* and *E. major* have fleshy cone bracts, whereas *E. funerea* has dry winged bracts. Interestingly, however, there is some support for an evolutionary link between papillae and transverse

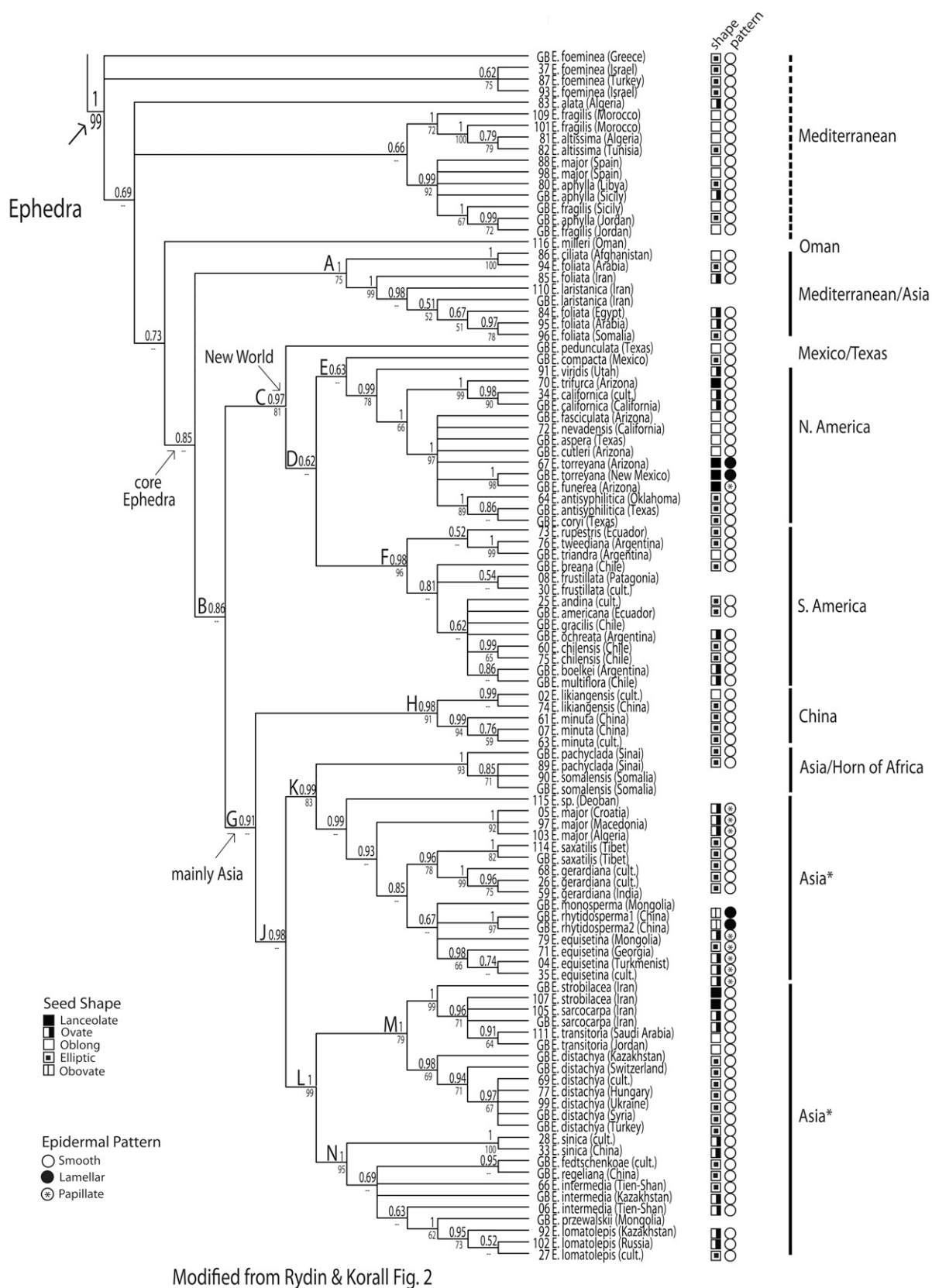


Fig. 4 Seed shape in longitudinal section (squares) and micromorphological patterns of the seed envelope (circles) mapped on a phylogeny of *Ephedra* (phylogeny redrawn, with permission, from work by Rydin and Korall [2009]).

lamellae. *Ephedra funerea* (papillate seeds) is sister to one of the two accessions assigned to *E. torreyana* (transverse lamellar), and similarly, *E. equisetina* and *E. major* (papillate seeds) belong in the same clade as *E. rhytidosperma* (transverse lamellar; fig. 4; Rydin and Korall 2009). The wartlike projections observed in a few species (*E. pachyclada* [fig. 1L], *E. lomatolepis*, and one specimen of *E. major*) are typically larger than the papillae of *E. equisetina*, *E. funerea*, and *E. major* but very thin, and some appear to have collapsed. The development and histology of these structures are not known to us; they are not observed in serial sections (Rydin et al. 2010). They may be the remains of collapsed papillae but could also potentially be an artifact caused, for example, by material degradation.

Further, there are intermediate forms of lamellae and papillae. Two specimens of *E. torreyana* have papillae-like lamellae as well as distinct papillae (fig. 1J), indicating that the lamellae are formed by fused papillae. Hybridization and/or introgression may also be responsible for some seed surface patterns. Wendt (1993) found clear indications of hybridization between *E. torreyana* var. *powelliorum* and *E. aspera* at the type locality of *E. torreyana* var. *powelliorum* in Big Bend National Park, Texas, and our data support this interpretation. Our specimen of *E. aspera* from the Big Bend Basin (see table 1) has a papillate seed envelope (not shown), whereas all other specimens of *E. aspera* investigated here lack papillae. Similarly, in one sample from Mount Helan, China, identified as *E. equisetina*, we found a pattern of transverse lamellae with papillae on the lamellae (fig. 1K), thus combining features otherwise characteristic of *E. rhytidosperma* and *E. equisetina*, respectively. The specimen could have originated from hybridization, since these two species occur in close proximity at Mount Helan.

Species determinations/delimitations in *Ephedra* are often uncertain or incorrect (e.g., Freitag and Maier-Stolte 1994; Ickert-Bond and Wojciechowski 2004; Rydin and Korall 2009). Morphological variation is limited, and variable characters, such as growth habit, pollen morphology, and leaf and cone morphology, show substantial parallelism and/or intra-specific variation (Foster 1972; El-Ghazaly and Rowley 1997; Ickert-Bond et al. 2003; Ickert-Bond and Wojciechowski 2004; Huang et al. 2005). Micromorphology of the seed envelope exhibits a similar pattern. Because of a relatively low amount of variation, evident parallel evolution of similar features, and intraspecific variation of traits, micromorphology of the seed envelope of *Ephedra* is generally not useful for species identification or subclade delimitation.

The Fossil Record and the Age of the Extant Clade

The surface sculpturing of the seed envelope of extant *E. rhytidosperma* is indeed very similar in size and shape to that described for the Early Cretaceous compression fossil *E. archaeorhytidosperma* (Yang et al. 2005). However, from our study it is clear that similar patterns of ridged seeds are not unique to these two species but have evolved independently at least twice in the extant clade. Moreover, the micromorphological patterns (e.g., the transverse lamellae) of extant species are formed by the epidermis alone (this study; Yang 2002; Rydin et al. 2010). In the fossil, however, it is unlikely that the ridged pattern of the seed envelope would have been preserved

if formed only by a thin and soft tissue, such as the epidermis. Although epidermal cell patterns can often be preserved in fossil cuticles, these are rarely present in plant fossils from the Yixian Formation (e.g., Zhou et al. 2003; Rydin et al. 2006b) due to rapid decay during the initial fossilization process and replacement of organic tissue with pyrite microcrystallines (Leng and Yang 2003).

A preserved “ridged” pattern, formed by sclerenchymatous tissue of the mesophyll, occurs in other Early Cretaceous fossils with a probable gnetalean affinity (Rydin et al. 2006a; Friis et al. 2007, 2009). The mesofossils *E. portugallica* Rydin, Pedersen, Crane et Friis and *E. drewriensis* Rydin, Pedersen, Crane et Friis (Rydin et al. 2006a) appear to have had smooth seed envelopes, but other seeds of uncertain affinity (e.g., fig. 7G, 7H in Rydin et al. 2006a) have transverse lamellae. The epidermis and perhaps also parts of the mesophyll are typically abraded in these fossils (but sometimes preserved in small areas in the apical-most region; C. Rydin, personal observation).

Based on comparative anatomy and histology, Rydin et al. (2010) found that the Cretaceous mesofossils *E. portugallica* and *E. drewriensis* (Rydin et al. 2004, 2006a) are extinct members of the stem lineage of *Ephedra*. They hypothesized a reduction of the vasculature of the seed envelope from probably four vascular bundles in the fossils to three in ancestral members of the crown group and to two in some extant forms. This morphological transformation series was thought to reflect the evolutionary origin of the ephedran seed envelope from a pair of cone bracts. Using the conclusions in Rydin et al. (2010) to assess the phylogenetic position of *E. archaeorhytidosperma* would also support the fossil being a stem relative of the extant clade. In *Ephedra*, the number of vascular bundles is typically strongly correlated with the apical, transverse shape of the seed envelope (Rydin et al. 2010). While the seeds of *E. archaeorhytidosperma* appear to have four angles (C. Rydin, personal observation), that is, four vascular bundles in the seed envelope, extant *E. rhytidosperma* is nested within the Asian clade, in which species have two vascular bundles in the seed envelope (Rydin et al. 2010). It is in fact even difficult to firmly assign *E. archaeorhytidosperma* to the ephedran lineage, since synapomorphies of the extant clade (*Ephedra*-type pollen and apical papillae on the seed envelope) have not been observed in the fossil.

Thus, in spite of a striking and indisputable similarity with extant *Ephedra*, the fossils appear to have been components of a Cretaceous diversity that became largely extinct toward the end of the period. Findings in several previous studies lend support to this hypothesis. Crane and Lidgard (1989) demonstrate a substantial decline in diversity and abundance of ephedroid pollen toward the early Late Cretaceous. Phylogenetic and temporal analyses (Ickert-Bond et al. 2009; Rydin and Korall 2009) and comparative anatomy/morphology (Rydin et al. 2010; and this study) concomitantly support that extant diversity is the result of a (second) radiation in *Ephedra*, which presumably began in the Paleogene.

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