

Asymmetrical development of biovulate cones resulting in uniovulate cones in *Ephedra rhytidosperma* (Ephedraceae)

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Abstract. Female cone morphology in *Ephedra*, including the number of initiated ovules and mature seeds per cone, provides important taxonomic characters used in sectional or species delimitation within *Ephedra*. Recent molecular phylogenies have indicated, however, that seed number per cone has changed repeatedly during the evolution of the genus. This study reports on the development of the female cone of *E. rhytidosperma*, based on a large sample of dissected cones studied under SEM. All cones were initially biovulate, and in the majority of cases, both female reproductive units (FRUs) developed a micropylar tube and formed mature seeds. In a few cases, the FRU pair developed asymmetrically in a cone, with one of them eventually aborting. There was no evidence of fusion of the FRU pair. Phylogenetically, *E. rhytidosperma* is in a clade with *E. equisetina*, which has uniovulate cones, and *E. gerardiana* and *E. minuta*, which have biovulate cones that also become unispermous via abortion. The biovulate condition may thus be ancestral in this clade.

Key words: Cone development, *Ephedra rhytidosperma*, Ephedraceae, morphological evolution, ovule abortion.

Introduction

The Ephedraceae comprise a single genus, *Ephedra* L., which consists of ca. 50 species

of shrubby plants with photosynthetic branches and reduced leaves that are widely distributed in temperate to arid areas of Eurasia, northern Africa, southwestern North America and western South America (Price 1996; Y. Yang and S. S. Renner, personal data on *Ephedra*). Morphological and molecular data indicate that *Ephedra* is sister to *Gnetum* and *Welwitschia*, the only other two genera of the gymnosperm order Gnetales (Crane 1985, Bowe et al. 2000, Chaw et al. 2000, Burleigh and Mathews 2004, for a review). Molecular phylogenetic analyses of *Ephedra* (Huang and Price 2003, Ickert-Bond and Wojciechowski 2004, Long et al. 2004, Rydin et al. 2004, Huang et al. 2005, Wang et al. 2005) together have sampled the majority of species in the genus, but suffer from insufficient phylogenetic signal in loci so far sequenced, resulting in poorly supported species relationships. Morphological character evolution in *Ephedra* has received uneven attention, with the Asian and African species much less well known than the European and North American ones, partly because of the difficulty of obtaining well-preserved (pickled) material. An effort is being made to redress this imbalance, which should also help in the interpretation and more

confident placement of recently discovered Cretaceous *Ephedra* cones from China (Yang 2001, 2004; Yang et al. 2005).

Female cone morphology in *Ephedra* contributes important taxonomic characters that have been used for infrageneric classification (together with other characters). For example, Soskov (1968) grouped species according to whether they had smooth twigs and monospermous cones or rough twigs and dispermous cones, while Pachomova (1971) recognized a group of five species with monospermous cones as section *Monospermae*. In a morphological study of representative species of *Ephedra*, Mussayev (1978) confirmed that seed number was relatively stable and hence could be used in species delimitation. Among the species he studied were *E. equisetina* Bunge and *E. monosperma* Gmel. ex C. A. Meyer, which are always monospermous (Cheng 1978; Yang, personal observation), while *E. distachya* L., *E. regeliana* Florin, and *E. sinica* Stapf are always dispermous (Pachomova 1971, Mussayev 1978, Yang 2001). Other species, for example, *E. intermedia* Schrenk ex C. A. Meyer and *E. przewalskii* Stapf, can have triovulate or biovulate cones (Yang 2004).

To explain the origin of uniovulate cones, Thoday and Berridge (1912) suggested that they arose via the fusion of the two female reproductive units (FRUs) of a biovulate cone, while Eames (1952) held that uniovulate cones resulted from the suppression or early abortion of one of the two FRUs. A recent study of *E. intermedia* suggested that in this species, biovulate cones result from triovulate cones via abortion of one of the three FRUs (Yang 2004). All three studies (Thoday and Berridge 1912, Eames 1952, Yang 2004) demonstrated an ontogenetic reduction of seed number, but this of course need not imply an evolutionary direction from many seeds per cone to fewer seeds. The evolutionary trajectory of seed number per cone can be inferred from the topologies of molecular trees for *Ephedra* (Ickert-Bond and Wojciechowski 2004, Long et al. 2004, Rydin et al. 2004, Huang et al. 2005, Wang et al. 2005). Taken together, these

studies have sampled ten of the 15 species occurring in China, the center of diversity of *Ephedra*.

The present study focuses on the ontogeny of female cones in *E. rhytidosperma* Pachomova, a species that exhibits variability in the number of seeds per cone, which can be two or one. I then use a published molecular phylogeny that includes *E. rhytidosperma* (Wang et al. 2005) to infer the evolutionary direction of seed number evolution. *Ephedra rhytidosperma* holds particular interest because well-preserved fossil cones from the Early Cretaceous of western Liaoning in China, *Ephedra archaeorhytidosperma* Yang et al. (2005), have traits resembling those of extant *E. rhytidosperma*. A better understanding of the ontogeny of the female cones of *E. rhytidosperma* thus also contributes to a fuller understanding of the fossils.

Materials and methods

Ephedra rhytidosperma is endemic to the lower lands of Mt. Helan and nearby of China (Pachomova 1967, Cheng 1978, Shen 1995), where it grows at altitudes between 1200 and 2050 m. I have repeatedly visited 8 populations on both sides of Mt. Helan over the past seven years (1999–2006). Material was collected in April, May, June, and early July in 1999, 2000, and 2001, and a voucher (Y. Yang NM99018) has been deposited in the herbarium of the Institute of Botany in Beijing (PE). At Mt. Helan, the range of *E. rhytidosperma* overlaps with that of the uniovulate species *E. equisetina* Bunge (Cheng 1978, Shen 1995, Fu et al. 1999, and personal observation), but the two species do not occur in the same habitats, and *E. equisetina* also has a much wider distribution, occurring from the Canary Islands through Mid-Asia into northern China.

Ephedra rhytidosperma grows as an erect shrub or a cushion, attaining heights of 5–60 cm. Its strongly lignified woody stems usually are stout and knotty. The twigs are usually clustered at the nodes of older woody branches, but are opposite and decussate at the nodes of this year's green twigs; annual sprouts are usually slender, measuring 0.8–1 mm in diameter. The leaves are opposite, ca. 1 mm long, and broadly triangular or scale-like.

Male cones are sessile and opposite of each other at nodes of non-woody green branches or twigs; they have two to three pairs of bracts. In each bract axil, two bracteoles are fused into a sheath from which a yellow antherophore protrudes. Each antherophore bears five to eight yellow synangia. Female cones are aggregated at the nodes of woody branches or opposite at nodes of non-woody green branches and twigs. Each bears two (rarely three) pairs of bracts that are red and fleshy at maturity. Only the uppermost pair of bracts, which is conspicuously longer than the lower pair(s), is fertile. Cones can be monospermous or dispermous, with both seeds potentially maturing. As in all species of *Ephedra*, ovulate units possess an outer envelope and an integument that forms the c. 1 mm-long micropylar tube. The mature yellowish-brown seeds protrude from the uppermost pair of bracts and measure 4–6 mm in length and ca. 3 mm in diameter. They bear a prominent longitudinal dorsal ridge and tiny dense transverse laminar protuberances on the outer surface of the outer envelope (Yang et al. 2005 includes SEM images of the seeds).

Female cones at various developmental stages were fixed in FAA (Formalin Alcohol Acid). For scanning electron microscopy (SEM), the fixed cones were dissected to expose the primordia of the female reproductive units, dehydrated in alcohol series, critical point dried, and coated with gold. Photographs were taken with a Hitachi S-800 scanning electron microscope operating at 30 KV in the Electron Microscopy Laboratory of the Institute of Botany, Chinese Academy of Sciences.

Results

Development of female reproductive units.

When apical buds (Fig. 1A) shift from vegetative to reproductive growth, one FRU appears in each axil of the uppermost pair of bracts (Fig. 1B). The apical bud is used up in the process, leaving a groove between the FRU primordia (Fig. 1B). Next, two adaxial protuberances arise that form the outer envelope surrounding the ovule primordium by further zonal growth (Fig. 1C). At this time, just next to the inner side of the outer envelope, the inner envelope of the ovule is initiated as an annular protuberance (Fig. 1D, E), eventually producing the micropylar tube when a longitudinal

ridge arises on the dorsal side of the female reproductive unit (Fig. 1F-H).

Asymmetric growth and abortion of female reproductive units. The two FRUs of a cone can develop symmetrically, resulting in biovulate cones, or asymmetrically, resulting in uniovulate cones (Figs. 1H and I, 2A-F). The abortion of FRUs can occur at different stages during ovule ontogeny (Fig. 2C-F).

Discussion

Seed cones in *Ephedra* can be triovulate, biovulate, or uniovulate, with abortion of ovules then leading to mature cones that have fewer seeds than they had ovules. Monospermous cones in *E. rhytidosperma* always start out as biovulate cones and become monospermous through the abortion of one of the two FRUs as suggested by Eames (1952), not via fusion of the uppermost pair of FRUs as held by Thoday and Berridge (1912). No traces of fusion of the two FRUs were found in over a thousand female cones dissected. An earlier study of the ontogeny of normally triovulate cones in *E. intermedia* found that biovulate cones in that species also originate from the early abortion of one of the three FRUs (Yang 2004). Based on those results and the present study it appears that asymmetrical development of ovulate units in a female cone may be common in *Ephedra* and may have offered the variation from which natural selection produced strictly uniovulate or biovulate cones (assuming that the ancestral condition was three or more ovules per cone). Unfortunately, other studies of *Ephedra* cone ontogeny have not focused in species with variable ovule numbers (Chen and Qian 1979, Takaso 1985, Kevorkova 1987, Sklonnaya and Ruguzov 2002, Mundry and Stützel 2004).

If aborted ovules had reached the stage of micropylar tube development, one might suspect that abortion might be due to insufficient pollination or fertilization. However, aborted ovulate units always seem to lack a micropylar tube (Fig. 2D, F), while their sister ovulate units have well developed tubes, indicating

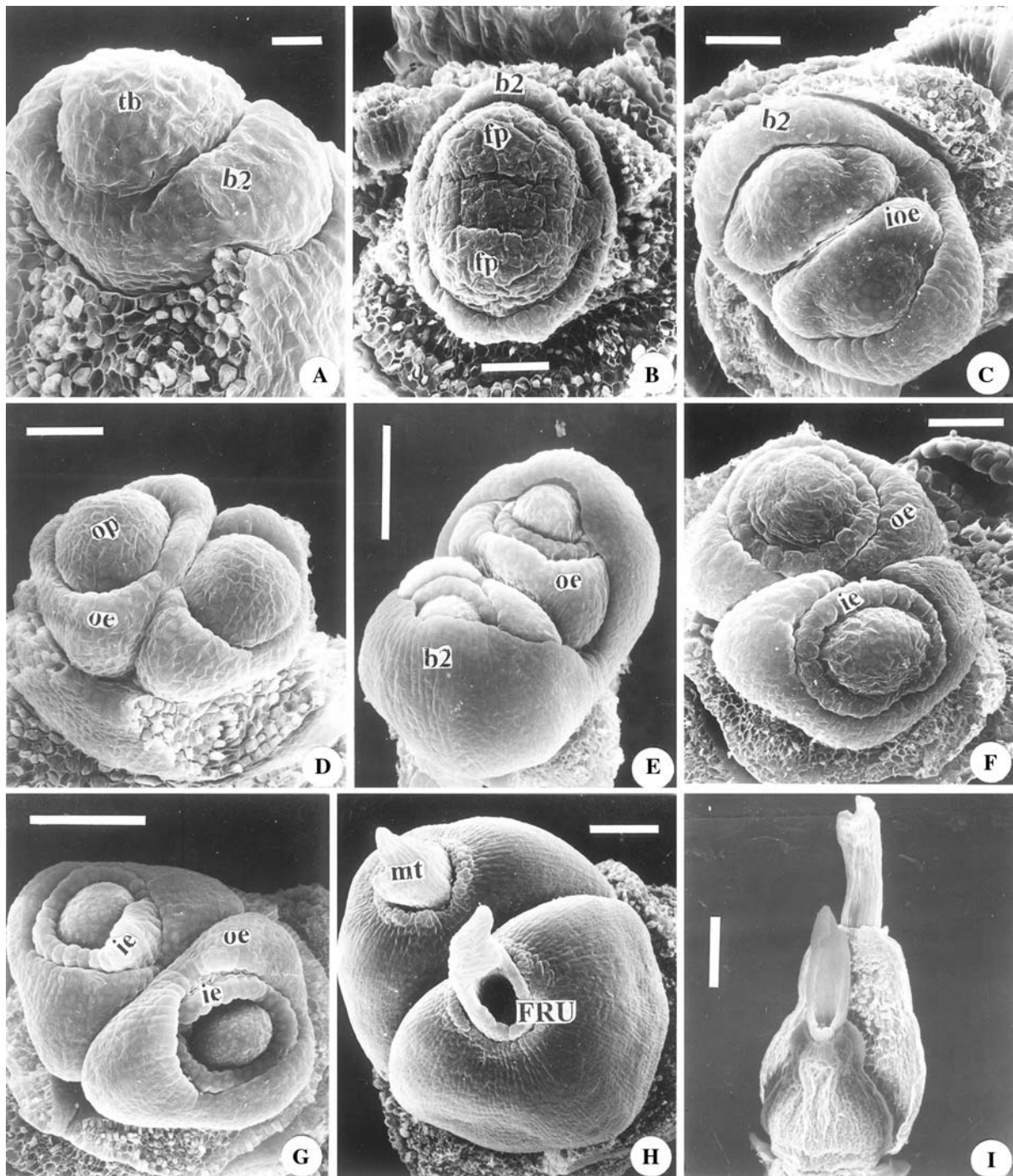


Fig. 1. Development of female reproductive units (FRUs) in *Ephedra rhytidosperma*. **A** Vegetative bud before shift to reproductive growth, bar: 20 μm ; **B** Initiation of FRU, bar: 40 μm ; **C** Initiation of the outer envelope, showing the two adaxial lateral protuberances, bar: 50 μm ; **D** Zonal growth of the outer envelope, bar: 50 μm ; **E–F** Initiation of the inner envelope, bar: 100 μm , 50 μm ; **G–H** Further development of the outer envelope and the inner envelope, bar: 100 μm , 100 μm ; **I** Asymmetrical development of FRUs and abortion of one of the two FRUs, bar: 200 μm . Abbreviations: *tb* terminal bud; *b2* the second pair of apical bracts; *fp* female reproductive unit primordium; *ioe* initiation of the outer envelope; *op* ovule primordium; *oe* the outer envelope; *ie* the inner envelope; *mt* micropylar tube; *FRU* female reproductive unit

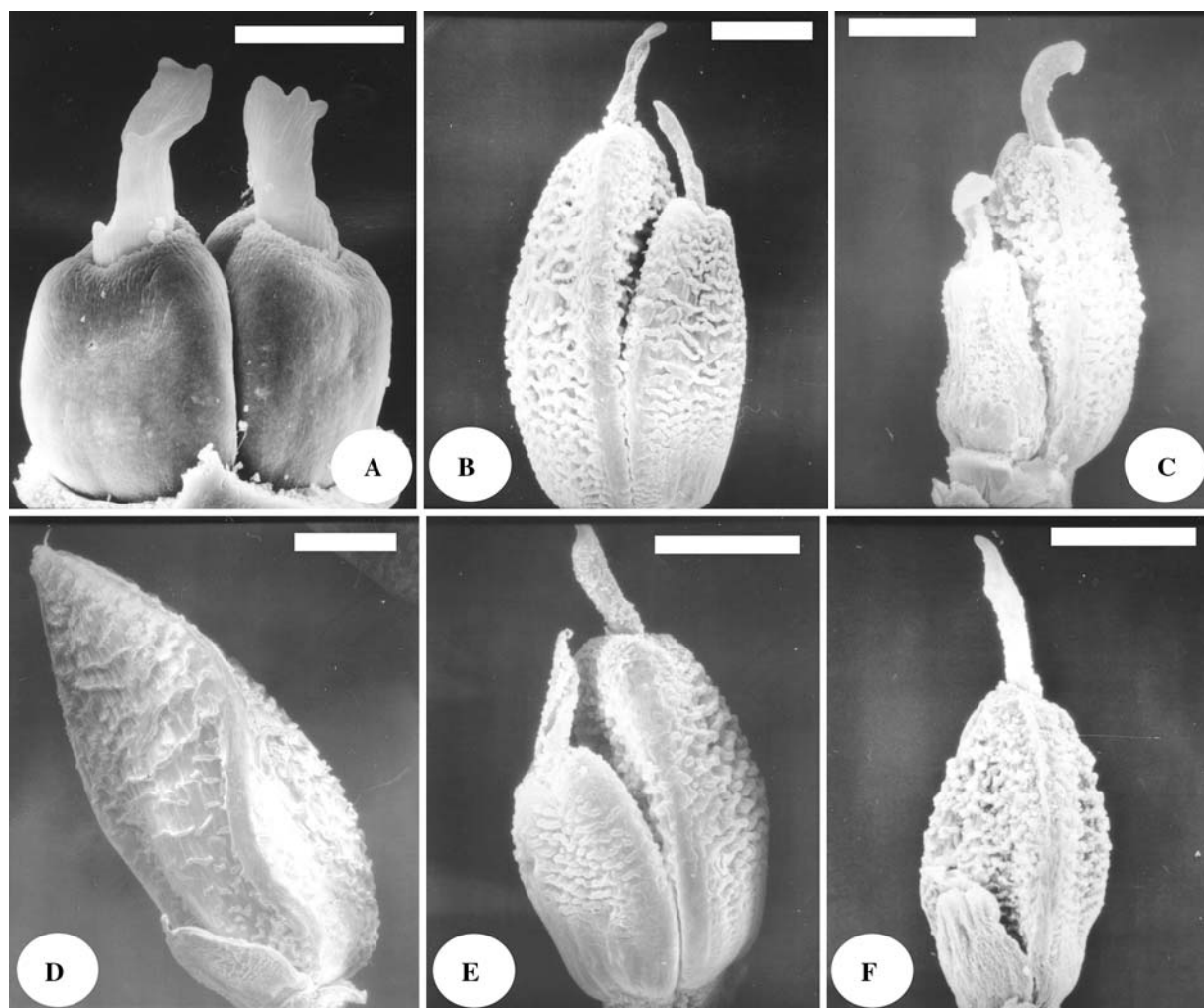
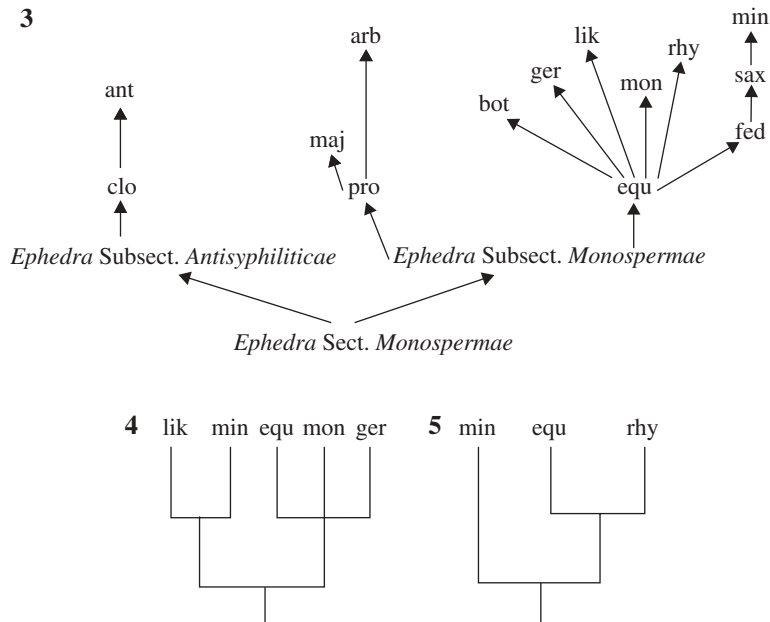


Fig. 2. Asymmetrical development of ovulate cones and formation of uniovulate cones in *Ephedra rhytidosperma*. **A** Two normal FRUs, bar: 200 μm . **B–F** Reduction of FRUs showing one normal FRU (the longer one) and one abortive FRU (the shorter one) in different developmental stages. **B** bar: 400 μm ; **C** bar: 300 μm ; **D** bar: 500 μm ; **E** bar: 400 μm ; **F** bar: 400 μm

that abortion is not due to insufficient pollination.

As described in the *Introduction*, the number of seeds per cone has been used in *Ephedra* classification. Thus, Pachomova (1971) placed five species of *Ephedra* that always or predominantly have just one seed in his section *Monospermae*, and nine more were added by Mussayev (1978) (*E. antisiphilitica* C. A. Meyer, *E. arborea* Lag. ex Bertol., *E. botschantzevii* Pachomova, *E. clokeyi* Cutler, *E. equisetina*, *E. fetschenkoaei*, *E. gerardiana*

Wall. ex C. A. Meyer, *E. likiangensis* R. Florin, *E. major* Host, *E. minuta* R. Florin, *E. monosperma*, *E. procera* C. A. Meyer, *E. rhytidosperma*, *E. saxatilis*). However, as shown here, some of these “monospermous” species, such as *E. rhytidosperma*, ontogenetically are biovulate, becoming monospermous only by abortion of the second FRU, while others are strictly uniovulate from the beginning (at least *E. equisetina* and *E. monosperma*, Yang, personal observation). As expected from this morphological heterogeneity, molecular



Figs. 3–5. Phylogenetic hypotheses for *E. rhytidosperma* and its relatives. **3** Redrawn from Mussayev (1978); **4** Redrawn from Long et al. (2004) and Rydin et al. (2004); **5** Redrawn from Wang et al. (2005). Abbreviation: *ant* *E. antisyphilitica*; *arb* *E. arborea*; *bot* *E. botschantzevii*; *clo* *E. clokeyi*; *equ* *E. equisetina*; *fed* *E. fedtschenkoae*; *ger* *E. gerardiana*; *lik* *E. likiangensis*; *maj* *E. major*; *min* *E. minuta*; *mon* *E. monosperma*; *pro* *E. procera*; *rhy* *E. rhytidosperma*; *sax* *E. saxatilis*

studies have not supported the monophyly of *Ephedra* section *Monospermae* (Huang and Price 2003, Rydin et al. 2004, Ickert-Bond and Wojciechowski 2004, Huang et al. 2005), indicating instead that species with monospermous cones evolved repeatedly in clades with dispermous or trispermous cones. Notably, however, Asian species of section *Monospermae* so far sequenced, namely *E. equisetina*, *E. gerardiana*, *E. likiangensis*, *E. minuta*, *E. rhytidosperma*, and *E. saxatilis*, turned out to be monophyletic (Rydin et al. 2004, Long et al. 2004, Wang et al. 2005).

The only molecular study to include *E. rhytidosperma* (Wang et al. 2005) found it as sister species of the uniovulate *E. equisetina*, with both then sister to *E. minuta*, although this last relationship had low statistical support from chloroplast genes and none from nuclear loci (Wang et al. 2005). Wang et al. (2005) did not sample all 15 Chinese species of *Ephedra*, but based on morphology and another molecular study (Long et al. 2004),

which sampled *E. gerardiana*, *E. equisetina*, and six other species (but not *E. rhytidosperma*), the clade that includes *E. equisetina*, *E. minuta*, and *E. rhytidosperma*, also includes *E. gerardiana* and *E. monosperma*. Since *E. equisetina* and *E. monosperma ab initio* have uniovulate cones, while *E. gerardiana*, *E. minuta*, and *E. rhytidosperma* have biovulate cones (this study, and personal observation for *E. gerardiana* and *E. minuta*), the uniovulate condition of female cones may be the derived condition, fitting with the molecular tree of Wang et al. (2005) in which the biovulate species *E. minuta* forms the outgroup to the biovulate *E. rhytidosperma* and the uniovulate *E. equisetina*. Reduction of the number of ovules, and therefore seeds, is an important evolutionary trend in conifers (to which *Ephedra* and the remaining Gnetales may belong [Bowe et al. 2000, Chaw et al. 2000, Burleigh and Mathews 2004, although seed plant relationships are not yet securely resolved]), and reductions from three- or

bi-ovulate cones to fewer-ovulate cones in *Ephedra* may be a further instance of this.

The Early Cretaceous fossil seeds described as *Ephedra archaeorhytidosperma* Y. Yang et al. (2005) have uniovulate or biovulate cones, showing that asymmetric development of ovules in *Ephedra* dates back to the Early Cretaceous. Even though molecular clocks consistently indicate that extant species of *Ephedra* diverged from each other relatively recently, namely in the Oligocene and Miocene (Huang and Price 2003; Huang et al. 2005, Wang et al. 2005, Won and Renner 2006), the genus appears to exhibit stasis in certain morphological traits, including the seed coat in fossil *E. archaeorhytidosperma* and living *E. rhytidosperma* (Yang et al. 2005) and perhaps the asymmetrical development and abortion of FRUs.

Based on habit, bark texture of the twigs, cone shape, and geographic range, Mussayev (1978) suggested that *E. rhytidosperma* evolved from *E. equisetina*. Mussayev's hypothesis implies that the initially always biovulate cones of *E. rhytidosperma* evolved from the *ab initio* uniovulate cones of *E. equisetina*. Results of the present study do not fit with Mussayev's hypothesis and instead suggest that the two species probably shared a common biovulate ancestor.

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