On the evolutionary history of *Ephedra*: Cretaceous fossils and extant molecules

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Communicated by Peter R. Crane, Royal Botanic Gardens, Kew, Surrey, United Kingdom, October 14, 2004 (received for review June 30, 2004)

Gnetales comprise three unusual genera of seed plants, *Ephedra*, *Gnetum*, and *Welwitschia*. Their extraordinary morphological diversity suggests that they are survivors of an ancient, more diverse group. Gnetalean antiquity is also supported by fossil data. Dispersed "ephedroid" (polyplicate) pollen first appeared in the Permian >250 million years ago (Myr), and a few megafossils document the presence of gnetalean features in the early Cretaceous. The Cretaceous welwitschioid seedling *Cratonia cotyledon* dates the split between *Gnetum* and *Welwitschia* to before 110 Myr. Ages and character evolution of modern diversity are, however, controversial, and, based on molecular data, it has recently been suggested that *Ephedra* is very young, only 8–32 Myr. Here, we present data on the evolutionary history of *Ephedra*. Fossil seeds from Buarcos, Portugal, unequivocally link one type of Cretaceous polyplicate pollen to *Ephedra* and document that plants with unique characters, including the peculiar naked male gametophyte, were established already in the Early Cretaceous. Clades in our molecular phylogeny of extant species correspond to geographical regions, with African species in a basal grade/clade. The study demonstrates extremely low divergence in both molecular and morphological characters in *Ephedra*. Features observed in the fossils are present in all major extant clades, showing that modern species have retained unique reproductive characters for >110 million years. A recent origin of modern species of *Ephedra* would imply that the Cretaceous *Ephedra* fossils discussed here were members of widespread, now extinct sister lineage(s), and that no morphological innovations characterized the second diversification.

molecular phylogeny | fossil record | Gnetales | molecular dating

The extraordinary form and habit of the three extant genera of Gnetales have fascinated and puzzled botanists ever since the discovery of *Welwitschia* (1) in the Namib desert in 1860, and the group continues to be central in ongoing discussions about seed plant phylogeny (2–8). However, we do not yet have a clear understanding of the phylogenetic position of the Gnetales, and its fossil history is poorly known. *Drewria potomacensis* (9) and *Eoantha zherikhinii* (10) can unambiguously be assigned to the Gnetales, and a welwitschioid fossil seedling, *Cratonia cotyledon* (11), from the Early Cretaceous of Brazil, clearly belongs to crown group Gnetales, based on the presence of an embryo feeder and a unique venation pattern, shared by the fossil and *Welwitschia* (11–13). These fossils show that Gnetales were more diverse in the past than they are today, and *Cratonia* provides a minimum age of ≈110 million years (Myr) for the split between *Gnetum* and *Welwitschia*. Morphological and molecular analyses of relationships within the Gnetales have been congruent in placing *Ephedra* as sister to *Gnetum* and *Welwitschia* (3, 5, 8), and the split between *Ephedra* and the *Gnetum-Welwitschia* lineage must therefore be older. This early divergence of major gnetalean lineages does not necessarily imply that modern diversity was established in the Early Cretaceous. The age of extant *Ephedra* was recently estimated to 8–32 Myr (14) by using molecular sequence data (*rbcL*) and assuming a constant rate of evolution calculated by landmark event calibration (14). The result reflects the low amount of divergence between *rbcL* sequences within the genus and the assumption of clock-like substitution rates.

The 35–45 modern species of *Ephedra* (15) are similar also in gross morphology, with decussate branching, reduced leaves, and ovulate cones, often with fleshy bracts and seeds with an inner integument and an outer envelope. The apical part of the integument is extended into a micropylar tube that serves for pollen reception. The pollen cones consist of stalked microsporangia surrounded by paired bracts. Pollen grains are distinct, with longitudinal ridges and valleys (polyplicate), due to alternation of thicker and thinner exine regions. During germination, the exine is shed, leaving the male gametophyte naked (16), and the shed exine curls up in a characteristic way, resulting in transverse striations (16). Dispersed polyplicate pollen, similar to that of *Ephedra* and *Welwitschia*, occurs in sediments ranging back to the Permian, >250 Myr (17), and is particularly common in the Early Cretaceous (18), but their presumed affinity to the Gnetales has rarely been confirmed. *Eoantha zherikhinii* (10), an Early Cretaceous gnetalean ovule with an extending micropylar tube, contains polyplicate pollen of *Ephedripites*-type with intact exines. Further, *Welwitschia*-type pollen was found in association (not *in situ*) with *Drewria potomacensis* (9), but in general, little has been known about the plants that produced the so called "ephedroids."

The discovery presented here, of exceptionally well-preserved fossil seeds with unique *Ephedra* characters and *in situ* polyplicate pollen, documents that some Cretaceous polyplicate pollen was produced by *Ephedra* plants. The fossils and a molecular phylogeny contribute data on the evolutionary history of *Ephedra*.

Methods

Morphological Studies. The fossils were collected from the Early Cretaceous Buarcos locality, situated north of Figueira da Foz, Portugal, locality details in Friis *et al.* (19). The plant-bearing sediments were previously assigned to the "Arenitos de Carrascal" and thought to be of preAlbian age (Barremian or Aptian) (19). The sequence is now included in the lowermost member (Calvaria Member) of the Figueira da Foz Formation established recently by Dinis (20). The age of this part of the sequence is late Aptian to early Albian, or perhaps early Albian (21). The coalified fossils were extracted from the sediment by sieving in water and cleaned with hydrochloric and hydrofluoric acid, and water. They were initially investigated by using a stereomicroscope. For light microscope studies, seeds were macerated by using nitric acid followed by ammonia or by using sodium

Abbreviations: Myr, million years (ago); *rbcL*, ribulose-bisphosphate carboxylase large subunit; *rps4*, small ribosomal protein 4 gene; ITS, internal transcribed spacer.

Data deposition: The DNA sequences reported in this paper have been deposited in the GenBank database (accession nos. AY755660–AY755857).

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Table 1. Presence of papillae on the apical, inner surface of the outer envelope

No.	Taxa	Voucher	Papillae on seed envelope	Stapf 1889 Section/"Tribe"
83	*E. alata* Decne.	C-303 (S)	Present	Alatae/Tropidolepides
81	*E. altissima* Desf.	C-620 (S)	Present	Ephedra/Scandentes
	E. altissima Desf.	C-628–629 (S)	Present	Ephedra/Scandentes
	E. antisyphilitica Berl. & C. A. Mey.	C-612 (S)	Present	Ephedra/Antisyphiliticae
80	*E. aphylla* Forssk.	C-7791 (S)	Present	Ephedra/Scandentes
	E. californica S. Watson	C-602 (S)	Present	Asarca/Ascara
	E. campylopoda C. A. Mey.	C-7619, C-593 (S)	Present	Ephedra/Scandentes
60	*E. chilensis* C. Presl.	E00130260 (E)	Present	Ephedra/
75	*E. chilensis* C. Presl.	49.0542 (UC)	Present	Ephedra/
	E. clokeyi Cutler	C-589-590 (S)	Present	Ascara/
	E. cutleri Cutler	C-583 (S)	Present	Ascara/
	E. distachya L.	C-493 (S)	Present	Ephedra/Leptocladae
	E. equisetina Bunge	C-460 (S)	Present	Ephedra/Leptocladae
	E. fasciculata A. Nelson	C-442 (S)	Present	Ascara/
	E. fedtschenkoae Paulsen	– (S)	Present	Ephedra/Leptocladae
84	*E. foliata* Boiss. & C. A. Mey.	C-7816 (S)	Present	Ephedra/Scandentes
	E. funera Cov.	C-360 (S)	Present	Ascara/
	E. gerardiana Wall. & Florin	C-353 (S)	Present	Ephedra/Leptocladae
66	*E. intermedia* Schrenk & C. A. Mey.	C-325 (S)	Present	Ephedra/Pachycladae
02	*E. likiangensis* Florin	03–926 (S)	Present	Ephedra/Leptocladae
	E. lomatolepis Schrenk	C-287 (S)	Present	Ephedra/
07	*E. minuta* Florin	03–930 (S)	Present	Ephedra/Leptocladae
	E. pachyclada Boiss.	C-219 (S)	Present	Ephedra/Pachycladae
71	*E. procera* C. A. Mey.	04903487 (Mo)	Present	Ephedra/Leptocladae
73	*E. rupestris* Benth.	87.1368 (UC)	Present	Ephedra/Antisyphiliticae
33	*E. sinica* Stapf	EtOH (S)	Present	Ephedra/Leptocladae
76	*E. tweediana* Fisch. & C. A. Mey.	66.0742 (UC)	Present	Ephedra/Antisyphiliticae

hypochlorate. For scanning electron microscopy (SEM) studies, specimens were mounted on aluminum stubs, coated with gold in a sputter coater, and examined with a Hitachi 4300 field emission scanning electron microscope at 5 kV. Twenty-five species of extant *Ephedra* (Table 1) were investigated for the presence of papillae on the apical, inner surface of the outer envelope, by using a stereomicroscope.

Taxon Sampling and Gene Sequencing. Taxon sampling was designed to investigate the phylogeny of *Ephedra*. Representatives from all major continents were selected (Table 2). We have sequenced two chloroplast genes [the ribulose-bisphosphate carboxylase large subunit (*rbcL*) and the small ribosomal protein 4 gene (*rps4*)], and three nuclear ribosomal regions [1,230 bp from the large subunit (26S), the small subunit (18S), and the internal transcribed spacer 1+2 (ITS)]. The intron and spacer of the chloroplast *trnL-F* region were also sequenced, but they contained no variation. Primer sequences and references are given in Table 3. The ITS primers 18SF and 26SR were designed for seed plants and have proven useful on a range of land plants. DNA was extracted, amplified, and sequenced by using standard methods. Fragments were assembled and edited by using the STADEN package (22).

Alignment and Analyses. All sequences were aligned by using computer software BIOEDIT (23). No insertion/deletion events were found in *rbcL*. A 6-bp deletion was inferred in all *rps4* sequences of *Gnetum* and *Welwitschia*, and an additional 3-bp deletion for *Welwitschia*. Insertion/deletion events in the nuclear 18S, ITS, and 26S rDNA sequences were inferred by eye. Gaps were treated as missing data in the alignment and added as binomial characters (absent or present) at the end of the matrix. All trees were rooted on the sister group of *Ephedra*, the *Gnetum-Welwitschia* clade (8).

Bayesian analyses were performed with MRBAYES 3.0 (24) by using the general time reversible (GTR) model with γ distributed rates and a proportion of invariable sites. The data set was partitioned into six data partitions (18S+26S; ITS; *rbcL+rps4* 1st codon positions; *rbcL+rps4* 2nd pos; *rbcL+rps4* 3rd pos; and indels). Each partition was allowed to have its own unique GTR plus gamma plus proportion of invariable sites model. The indels were treated as a morphological data partition. We ran 600,000 generations, with a sample frequency of 100, four parallel chains, and all other options at their default values. The majority rule consensus of trees from the last 100,000 generations was calculated in PAUP* 4.0B10 (25). Most parsimonious trees were calculated by using the heuristic search option in PAUP*, 500 random sequence additions, tree bisection reconnection branch swapping, and multrees off. Support values were obtained by using bootstrap in PAUP*, performing 1,000 replicates with 10 random sequence additions.

Results and Discussion

The seeds, discovered from the Early Cretaceous Buarcos locality in Portugal, are ovoid, 0.85–1.2 mm long, rounded at the base, and provided with an acuminate micropyle (Fig. 1*A*). The seeds have two tissues: an inner, membranous integument and an outer envelope of sclerenchymatic cells. The outer envelope often splits apically into four valves, exposing distinct papillae (Fig. 1*B*) on their inner surface. The papillae are identical in shape and position to those described for extant *Ephedra distachya* and *Ephedra altissima* (26), where they are thought to provide support for the micropylar tube, and to close the gap between the micropylar tube and the outer envelope (26). Polyplicate pollen occurs *in situ* in the micropylar tube (Fig. 1*C*). The pollen grains are ribbed with 10–15 visible ribs. The surface of the pollen wall is indistinctly rugulate (Fig. 1*D*), a feature also observed for *Ephedra foliata* (27). Maceration of the seeds revealed shed, upcurled exines inside the integument (Fig. 1*E*), matching completely the unusual shed exines of extant *Ephedra* (16). The shed exines indicate that the pollen grains had germinated in the ovule, leaving the male gametophyte naked in the same peculiar way described for extant *Ephedra* (16).

Table 2. Species included in molecular analyses

	Taxa	Voucher	rbcL	rps4	26S	18S	ITS	Comments	Sec./''Tribe''*	Distrib.
83	*E. alata* Decne.	C-303 (S)	AY755805	AY755851	AY755732	AY755698	AY755774	Algeria 1980	Alatae/Tropidolepides	Africa-Asia
82	*E. altissima* Desf.	C-7688 (S)	AY755804	AY755850	AY755731	AY755697	AY755773	Tunisia 1972	Eph./Scandentes	Africa
81	*E. altissima* Desf.	C-628 (S)	AY755803	AY755849	AY755730	AY755696	AY755772	Algeria-Morocco 1936	Eph./Scandentes	Africa
25	*E. andina* Poepp. ex C.A. Mey.	10140 (K)	AY755782	AY755821	AY755707	AY755670	AY755744	Kew 1967–25610 (cult)	Eph./Antisyphiliticae	S. America
64	*E. antisyphilitica* Berl. & C.A. Mey.	04–488 (S)	AY755789	AY755834	AY755715	AY755682	AY755757	Oklahoma 2001	Eph./Antisyphiliticae	N. America
80	*E. aphylla* Forssk.	C-7791 (S)	AY755802	AY755848	AY755729	AY755695	AY755771	Libya 1982	Eph./Scandentes	Africa/Asia
34	*E. californica* S. Watson	68–154 (O)	AY056569	AY755827	AY755708	AY755676	AY755750	Oslo Univ Bot Gard (cult)	Asarca/Ascara	N. America
87	*E. campylopoda* C.A. Mey.	C-7605 (S)	AY755808	AY755855	AY755736	AY755701	AY755777	Turkey 1971	Eph./Scandentes	Eur-Afr-Asia
60	*E. chilensis* C. Presl.	E00130260 (E)	AY755786	AY755831	AY755712	AY755679	AY755754	Talca, Chile 1980 (cult)	Eph./	S. America
75	*E. chilensis* C. Presl.	49.0542 (UC)	AY755799	AY755844	AY755725	AY755691	AY755767	Chile 1949 (cult)	Eph./	S. America
86	*E. ciliata* Fisch. & C.A. Mey.	C-591 (S)	AY755807	AY755854	AY755735	AY755700	AY755776	Afganistan 1963	Eph./Scandentes	Africa-Asia
69	*E. distachya* L	S04–481 (S)	AY755793	AY755838	AY755719	AY755686	AY755761	68–226 (cult)	Eph./Leptocladae	Europe/Asia
77	*E. distachya* L	03–684 (S)	—	AY755846	AY755727	AY755693	AY755769	Hungary 1993	Eph./Leptocladae	Europe/Asia
04	*E. equisetina* Bunge	03–928 (S)	AY755781	AY755817	AY755705	AY755666	AY755740	Ashkhabad 1938 (cult)	Eph./Leptocladae	Asia
35	*E. equisetina* Bunge	90–536 (O)	AY755783	AY755828	AY755709	—	AY755751	Oslo Univ Bot Gard (cult)	Eph./Leptocladae	Asia
79	*E. equisetina* Bunge	C-465 (2) (S)	AY755801	AY755847	AY755728	AY755694	AY755770	Mangolia 1972	Eph./Leptocladae	Asia
84	*E. foliata* Boiss. & C.A. Mey.	C-7816 (S)	—	AY755852	AY755733	—	—	Egypt 1929	Eph./Scandentes	Africa-Asia
85	*E. foliata* Boiss. & C.A. Mey.	C-7808 (S)	AY755806	AY755853	AY755734	AY755699	AY755775	Iran 1960	Eph./Scandentes	Africa-Asia
37	*E. fragilis* Desf.	E00130258 (E)	AY755784	AY755829	AY755710	AY755677	AY755752	Jerusalem, Israel 1992 (cult)	Eph./Scandentes	Europe/Africa
08	*E. frustillata* Miers	04–482 (S)	AY056670	AY755820	AY755706	AY755669	AY755743	Patagonia, Argentina 1994	Eph./	S. America
30	*E. frustillata* Miers	10218 (K)	AY056564	AY755825	AY056490	AY755674	AY755748	Kew 1988–8057 (cult)	Eph./	S. America
26	*E. gerardiana* Wall. & Florin	10141 (K)	AY056560	AY755822	AY056486	AY755671	AY755745	Kew 1989–8369 (cult)	Eph./Leptocladae	Asia
59	*E. gerardiana* Wall. & Florin	E00130259 (E)	AY755785	AY755830	AY755711	AY755678	AY755753	Sikkim, India 1983 (cult)	Eph./Leptocladae	Asia
68	*E. gerardiana* Wall. & Florin	74–460 (O)	AY755792	AY755837	AY755718	AY755685	AY755760	Oslo Univ Bot Gard (cult)	Eph./Leptocladae	Asia
06	*E. intermedia* Schrenk & C.A. Mey.	03–925 (S)	AY056566	AY755818	AY056492	AY755667	AY755741	Tien-Shan 1971 (cult)	Eph./Pachycladae	Asia
66	*E. intermedia* Schrenk & C.A. Mey.	04–483 (S)	AY755790	AY755835	AY755716	AY755683	AY755758	Tien-Shan 1971 (cult)	Eph./Pachycladae	Asia
02	*E. likiangensis* Florin	03–926 (S)	AY755780	AY755816	AY056485	AY755665	AY755739	Denver Bot Gard 1988 (cult)	Eph./Leptocladae	Asia
74	*E. likiangensis* Florin	94.0389 (UC)	AY755798	AY755843	AY755724	AY755690	AY755766	Yunnan, China 1994 (cult)	Eph./Leptocladae	Asia
88	*E. major* Host	03–164 (S)	AY755809	AY755856	AY755737	AY755702	AY755778	Andalusia, Spain 1995	Eph./Leptocladae	Eur-Asia-Afr
07	*E. minuta* Florin	03–930 (S)	AY056567	AY755819	AY056493	AY755668	AY755742	China, Sikang 1934 (cult)	Eph./Leptocladae	Asia
61	*E. minuta* Florin	04–485 (S)	AY755787	AY755832	AY755713	AY755680	AY755755	China, Sikang 1934 (cult)	Eph./Leptocladae	Asia
63	*E. minuta* Florin	04–486 (S)	AY755788	AY755833	AY755714	AY755681	AY755756	Univ of Stockholm (cult)	Eph./Leptocladae	Asia
27	*E. monosperma* C.A. Mey.	10142 (K)	AY056561	AY755823	AY056487	AY755672	AY755746	Kew 1998–499 (cult)	Eph./Leptocladae	Asia
72	*E. nevadensis* S. Watson	66.1033 (UC)	AY755796	AY755841	AY755722	AY755688	AY755764	California 1966 (cult)	Eph./Antisyphiliticae	N. America
89	*E. pachyclada* Boiss	C-7844 (S)	AY755810	AY755857	AY755738	AY755703	AY755779	Sinai 1974	Eph./Pachycladae	Asia
71	*E. procera* C.A. Mey.	04903487 (Mo)	AY755795	AY755840	AY755721	—	AY755763	Tiblisi Georgia 1999 (cult)	Eph./Leptocladae	Europe-Asia
73	*E. rupestris* Benth.	87.1368 (UC)	AY755797	AY755842	AY755723	AY755689	AY755765	Ecuador 1987 (cult)	Eph./Antisyphiliticae	S. America
28	*E. sinica* Stapf	10143 (K)	AY056562	AY755824	AY056488	AY755673	AY755747	Kew 1991–154 (cult)	Eph./Leptocladae	Asia
33	*E. sinica* Stapf	EtOH (S)	AY056565	AY755826	AY056491	AY755675	AY755749	Hebei, China 2000	Eph./Leptocladae	Asia
67	*E. torreyana* S. Watson	04–487 (S)	AY755791	AY755836	AY755717	AY755684	AY755759	New Mexico 2001	Alatae/Habrolepides	N. America
70	*E. trifurca* Torr.	04630447 (Mo)	AY755794	AY755839	AY755720	AY755687	AY755762	Arizona 1994 (cult)	Alatae/Habrolepides	N. America
76	*E. tweediana* Fisch. C.A. Mey.	66.0742 (UC)	AY755800	AY755845	AY755726	AY755692	AY755768	Buenos Aires, Argentina	Eph./Antisyphiliticae	S. America
01	*G. gnemon* L	03–926 (S)	L12680	AY755811	AF036488	AY755660	—	Fidji 2000	—	
31	*G. castatum* K. Sch.	10219 (K)	AY056576	AY755812	AY056497	AY755661	—	Kew 1964–47701 (cult)	—	
32	*G. parvifolium* (Warb.) W.C. Cheng	EtOH (S)	AY056577	AY755813	AY755704	AY755662	—	Namping, Xiyc. gorje 2000	—	
38	*G. indicum* Merr.	E00130257 (E)	AY056574	AY755814	AY056495	AY755663	—	Hong Kong 1955 (cult)	—	
39	*G. montanum* Markgr.	E00130261 (E)	AY056575	AY755815	AY056496	AY755664	—	Hong Kong 1979 (cult)	—	
36	*W. mirabilis* Hook. f	67–1177 (O)	AJ235814	AY188246	AY056484	AF207059	—	Oslo Univ Bot Gard (cult)	—	

Sec., section; Distrib., distribution; Eph., Ephedra.
*According to Stapf (1889) (29).

Table 3. Primer sequences

Primer description (name)	Sequence	Reference
rbcL forward (rbcL 5′)	ATG TCA CCA CAA ACA GAG AC	34
rbcL reversed (rbcL 3′)	TCA AAT TCA AAC TTG ATT TCT TTC CA	35
rps4 forward (rps4Fb)	CGA TCT TCT CGA CCC TGG TGG	C.R., 2003*
rps4 reversed (rps4Rb)	CCG TCG AGA ATA ATA TTC TAT	C.R, 2003*
18S forward (18S1)	GCT TGT CTC AAA GAT TAA GCC	C.R., 2003*
18S reversed (18Srev)	CCT TCC TCT AAA CGA TAA GGT TC	C.R., 2003*
26S forward (26S1)	CGA CCC CAG GTC AGG CG	36
26S reversed (1229R)	ACT TCC ATG ACC ACC GTC CT	36
ITS forward (ITS-18SF)	GAA CCT TAT CGT TTA GAG GAA GG	C.R., 2000*
ITS reversed (ITS-26SR)	CCG CCA GAT TTT CAC GCT GGG C	C.R., 2000*

*Previously unpublished.

To investigate whether the fossils could be assigned to any particular group within *Ephedra*, we studied the seed characters in a phylogenetic context. Attempts have been made previously to resolve the phylogeny of extant *Ephedra* (28), but the paucity of information in most gene regions makes it difficult to get resolved results. The phylogenetic framework used here was established by using Bayesian (Fig. 2) and parsimony analyses of five gene regions from the chloroplast and nuclear genomes (the chloroplast genes *rbcL* and *rps4*, and 18S, 26S, and ITS from the nuclear ribosomal DNA). Based on the resulting phylogeny, we conclude that Stapf's widely used classification system from 1889 (29), founded on the morphology of ovulate cone bracts, is artificial. Major well supported groups correspond to geographical regions, and dry membranous bracts are present in several clades. African species constitute a basal grade or clade within *Ephedra*. Some of the basal species are restricted to Africa (e.g., *E. altissima*); others have a broader distribution in the Old World, extending from Africa into Asia or southern Europe. *Ephedra alata*, *E. altissima*, *Ephedra aphylla*, *Ephedra campylopoda*, *Ephedra fragilis*, and *Ephedra major* share a 26-bp deletion in ITS (pos. 860–886 from the end of the primer 18SF), supporting their status as a clade. *Ephedra ciliata* and *E. foliata* do not have this deletion, and their systematic position is unclear. In Bayesian analyses, the basal (African) species form a grade (Fig. 2); in the most parsimonious trees they are monophyletic. Neither of these alternatives is well supported, and further studies are needed.

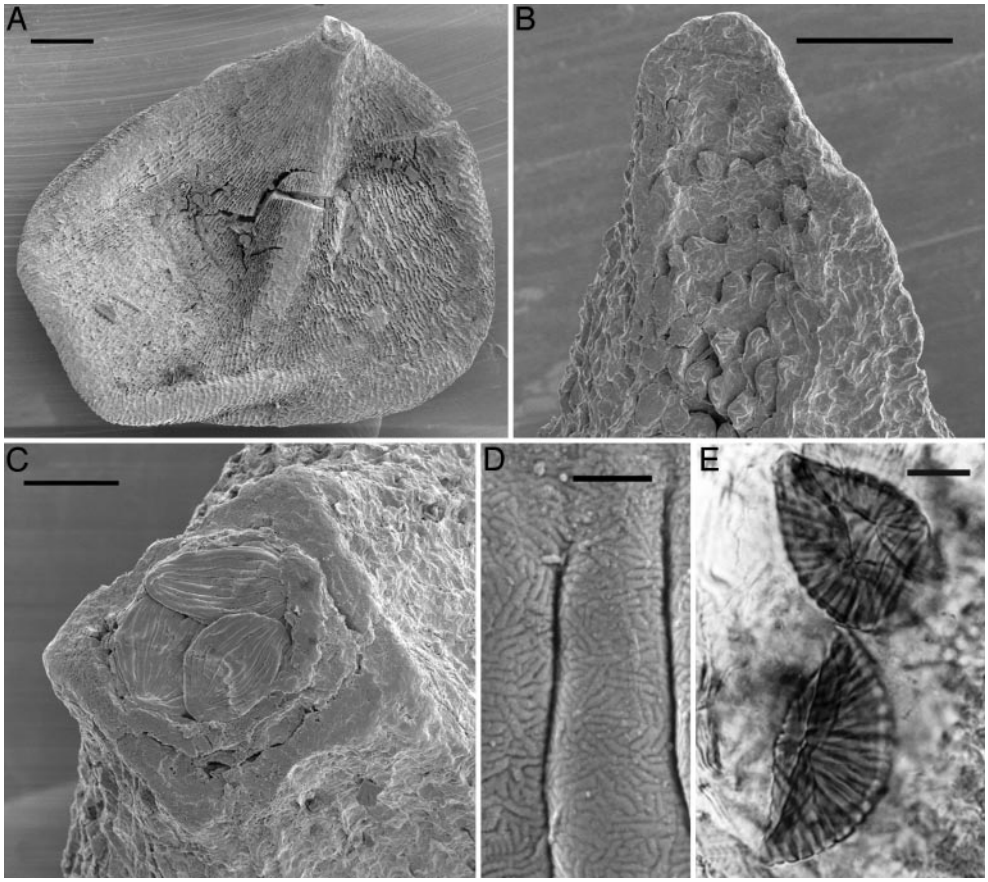

Fig. 1. Early Cretaceous *Ephedra* seeds from Buarcos, Portugal. (*A*) Overview of one of the fossil seeds (S-107680). (*B*) Details of papillae on the inner surface of the outer envelope (S-107685). (*C*) Pollen grains in the micropylar region. Note the inner circular integument and the outer squared envelope (S-107680). (*D*) Rugulate surface of the pollen grains (S-107680). (*E*) Macerated seed, exposing two shed pollen exines inside the micropyle (S-136808). [Scale bars: 100 μm (*A*); 50 μm (*B*); 25 μm (*C*); 1 μm (*D*); and 10 μm (*E*).] (*A–D*) Scanning electron micrographs. (*E*) Transmitted light micrograph.

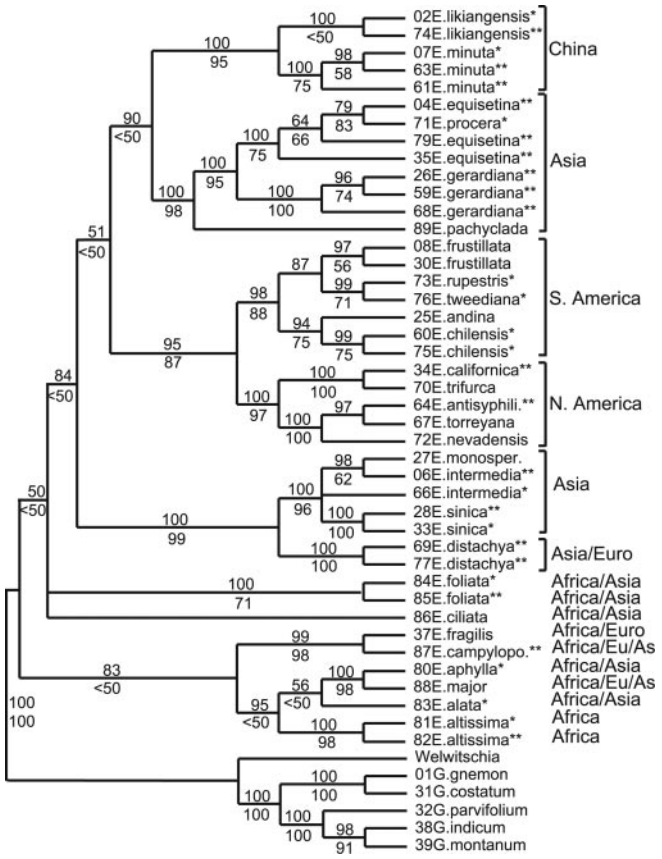

Fig. 2. Cladogram of recent *Ephedra* based on Bayesian analysis of five regions from the nuclear and chloroplast genomes (18S, 26S, ITS, *rbcL*, and *rps4*). Bayesian posterior probabilities are given above branches, parsimony bootstrap values below branches. Species marked * and ** were examined for papillae on the inner surface of apical part of the outer envelope. For species marked *, the DNA voucher material was investigated. For species marked **, other material has been used.

All non-African species belong to a clade that probably originated in Asia. Here, Bayesian and parsimony analyses produced congruent topologies. Of the four investigated European species, three are present also in Africa (*E. major*, *E. fragilis*, and *E. campylopoda*) and they belong to the basal grade/clade of African species. The European species *Ephedra distachya* has a broad distribution extending from Spain to Russia and China, but is absent in Africa. The two representatives of *E. distachya* included in this study are highly supported within one of the Asian clades. According to our results, *Ephedra procera*, which is generally considered a subspecies of *E. major*, is instead related to *Ephedra equisetina*. This finding should be further investigated with a more extensive species sampling. New World species are monophyletic and may have originated from within the Asian clade. They comprise two well-supported groups: a South American and a North American clade. Dry membranous bracts occur in the North American clade but also in one of the basal African species. The character differs in detailed morphology between species and has probably evolved several times. The Cretaceous flora probably contained species with dry bracts, as well as species with fleshy bracts, but this result has not been thoroughly studied yet.

From the correspondence between clades and geographical regions, it is clear that major groups originated after the final rifting of the Gondwana continent. A possible origin of *Ephedra* in Africa is interesting because the diversity of ephedroid pollen grains is particularly high in Early Cretaceous palaeoequatorial regions of Africa-South America (30). However, this connection is only relevant if the molecular dating of extant *Ephedra* to 8–32 Myr (14) is incorrect. The Cretaceous seeds presented here share a number of unique characters with extant *Ephedra*. Polyplicate pollen lacking a colpus and seeds with an outer envelope with apical papillae constitute obvious derived characters shared by the fossils and extant *Ephedra*. Shedding and upcurling of the pollen exine during germination represent another synapomorphy unique to *Ephedra*. The polyplicate pollen grains of *Welwitschia* have a distinct colpus through which germination occurs and the exine is not shed (C.R. and E.M.F., unpublished results). All characters of the fossils are present in all extant groups, and it was not possible to include the seeds in any particular subgroup. Papillae were found on the apical, inner surface of the outer envelope in all investigated specimens (representatives from all major extant clades, species marked * in Fig. 2, and other species; see Table 1). Our analyses indicate that there is very little variation in key reproductive structures in fossil and extant *Ephedra*. The same is true for molecular data where we have investigated three nuclear and three chloroplast sequences. They contained few informative characters, and one sequence, the *trnL-F* region, was nearly invariable.

Concluding Remarks. The co-occurrence of dispersed ephedroid (polyplicate) pollen and megafossils with distinct gnetalean vegetative morphology, for example in the Araripe Group (11, 31, 32) and in the Potomac Group (9), has strongly suggested that these Early Cretaceous ephedroid grains were produced by gnetalean plants. The new discovery of *Ephedra* seeds with polyplicate pollen *in situ* provides direct proof for the association. The fossils document that plants with unique *Ephedra* characters were already present in the Early Cretaceous, characters such as the apical papillae on the seed envelope and the peculiar shedding of the pollen exine, which leaves the male gametophyte naked. *Ephedra* may even have been diverse and widespread at that time. Dispersed polyplicate pollen occurs frequently in Cretaceous sediments from low palaeolatitudes (18, 30). Our results support the idea that *Ephedra*-plants produced at least some of these grains. Further, Early Cretaceous fossils from the Crato Formation in Brazil (B. Mohr, personal communication) and the Potomac Group, zone 1, USA (P. Crane, personal communication) have a morphology very similar to extant *Ephedra*. The same has also been reported from China (33) and now from Portugal. Together, these findings indicate that both reproductive and vegetative features characterizing extant *Ephedra* were fully established and widespread in the Early Cretaceous flora, and suggest that crown group *Ephedra* might be of Mesozoic origin. The alternative hypothesis inferred from molecular dating (14), that the crown group is young (8–32 Myr), needs more research. We have tested the proposed age for *Ephedra* with commonly used methods for molecular age estimates, but found that without a calibration point within *Ephedra*, which may be difficult (impossible?) to attain due to the conserved morphology of the genus, the results of penalized likelihood analyses are dubious. A late differentiation of crown group *Ephedra* would imply that the lineage experienced two major radiation events, one in the Early Cretaceous resulting in widespread, but now extinct stem group(s), and a second radiation in the late Cenozoic resulting in the modern diversity. The implication of this hypothesis is that all characters of modern *Ephedra* have remained unchanged for >110 million years, through the second major diversification. This scenario seems incompatible with the hypothesis of constant substitution rates within the lineage (14), but clearly further testing and development of methods for molecular dating is needed to clarify conflicts between molecular signals and the fossil record.

We thank David Cantrill, Petra Korall, Mari Källersjö, Hervé Sauquet, and Niklas Wikström for valuable comments on the manuscript, and Ron Hoggard (University of Oklahoma, Norman) for sending plant material. This study was supported by grants from the Swedish Research Council (to E.M.F.) and from the Carlsberg Foundation (to K.R.P.).

1. Welwitsch, F. (1861) *J. Proc. Linn. Soc. Bot.* **5,** 182–187.
2. Doyle, J. A. (1996) *Int. J. Plant Sci.* **157,** Suppl., S3–S39.
3. Crane, P. R. (1985) *Ann. Mo. Bot. Gard.* **72,** 716–793.
4. Bowe, L. M., Coat, G. & de Pamphilis, C. W. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4092–4097.
5. Chaw, S.-M., Parkinson, C. L., Cheng, Y., Vincent, T. M. & Palmer, J. D. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4086–4091.
6. Donoghue, M. J. & Doyle, J. A. (2000) *Curr. Biol.* **10,** R106–R109.
7. Magallón, S. & Sanderson, M. J. (2002) *Am. J. Bot.* **89,** 1991–2006.
8. Rydin, C., Källersjö, M. & Friis, E. M. (2002) *Int. J. Plant Sci.* **163,** 197–214.
9. Crane, P. R. & Upchurch, G. R. (1987) *Am. J. Bot.* **74,** 1722–1736.
10. Krassilov, V. A. (1986) *Rev. Palaeobot. Palynol.* **47,** 9–16.
11. Rydin, C., Mohr, B. & Friis, E. M. (2003) *Biol. Lett. R. Soc. London* **270,** 29–32.
12. Rodin, R. J. (1953) *Am. J. Bot.* **40,** 371–378.
13. Rodin, R. J. (1958) *Am. J. Bot.* **45,** 90–95.
14. Huang, J. & Price, R. A. (2003) *Mol. Biol. Evol.* **20,** 435–440.
15. Kubitzki, K. (1998) *The Families and Genera of Vascular Plants* (Springer, Berlin), Vol. 1.
16. El-Ghazaly, G., Rowley, J. R. & Hesse, H. (1998) *Plant Syst. Evol.* **213,** 217–231.
17. Wilson, L. R. (1962) *Okla. Geol. Surv. Bull.* **49,** 5–50.
18. Crane, P. R. & Lidgard, S. (1989) *Science* **246,** 675–678.
19. Friis, E. M., Crane, P. R. & Pedersen, K. R. (1997) *Grana* **36,** 225–244.
20. Dinis, J. L. (2001) *Comun. Inst. Geol. e Mineiro* **88,** 127–160.
21. Heimhofer, U., Hochuli, P. A. & Weissert, H. (2004) *Polen* **14,** 178–179.
22. Staden, R. (1996) *Mol. Biotechnol.* **5,** 233–241.
23. Hall, T. (1997) *Bioedit* (North Carolina State University, Raleigh).
24. Huelsenbeck, J. P. & Ronquist, F. R. (2001) *Bioinformatics* **17,** 754–755.
25. Swofford, D. L. (1998) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)* (Sinauer, Sunderland, MA).
26. Thoday, M. G. & Berridge, E. M. (1912) *Ann. Bot.* **26,** 953–985.
27. El-Ghazaly, G. & Rowley, J. R. (1997) *Palynology* **21,** 7–18.
28. Huang, J. (2000) Ph.D. dissertation (University of Georgia, Athens).
29. Stapf, O. (1889) *Denkschr. Math-Nat. wiss. Cla. Kaiserl. Akad. Wiss. Wien* **56,** 1–112.
30. Crane, P. R. (1996) *Int. J. Plant Sci.* **157,** Suppl., S50–S57.
31. Martill, D. M., Brito, P. M., Wenz, S. & Wilby, P. R. (1993) in *Palaeontological Association Field Guides to Fossils Series 5*, ed. Jarzembowski, E. A. (Palaeontolgical Association, London), pp. 1–159.
32. Osborn, J. M., Taylor, T. N. & de Lima, M. R. (1993) *Rev. Palaeobot. Palynol.* **77,** 171–184.
33. Guo, S.-X. & Wu, X.-W. (2000) *Acta Palaeont. Sin.* **39,** 81–91.
34. Zurawski, G. & Clegg, M. T. (1987) *Annu. Rev. Plant Physiol.* **38,** 391–418.
35. Wikström, N. & Kenrick, P. (1997) *Int. J. Plant Sci.* **158,** 862–871.
36. Kuzoff, R. K., Sweere, J. A., Soltis, D. E., Soltis, P. S. & Zimmer, E. A. (1998) *Mol. Biol. Evol.* **15,** 251–263.