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Phylogenetic Relationships in *Ephedra* (Gnetales): Evidence from Nuclear and Chloroplast DNA Sequence Data

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ABSTRACT. Sequences from the nuclear ribosomal internal transcribed spacer region 1 (nrDNA ITS1) and the plastid *rps4* gene from the genus *Ephedra* (Ephedraceae, Gnetales) were obtained in order to infer phylogenetic relationships, character evolution, and historical biogeography in the genus. Within *Ephedra* the length of the nrDNA ITS1 varied from 1,081 to 1,143 basepairs (bp), in contrast to dramatically shorter lengths in the outgroups (*Gnetum*, *Welwitschia*, and *Pinus*). The *rps4* locus varied in length from 645 to 661 bp in the same set of taxa. Both parsimony and maximum likelihood analyses of these sequences resulted in a well-resolved phylogeny that supports the monophyly of *Ephedra*, but not its subdivision into the traditional sections *Ephedra*, *Asarca*, and *Alatae*. The resulting phylogeny also indicates a derivation of the New World clade from among the Old World taxa. Among the Old World species three highly-supported monophyletic groups are recognized that are highly concordant with morphological evidence. The New World clade includes two main subclades of North and South American species that are strongly supported, while the position of two, mostly Mexican species *E. peduncula*ta and *E. compacta* remains unresolved. Character reconstruction of ovulate strobilus types in *Ephedra* indicates that fleshy bracts are ancestral, with shifts to dry, winged bracts having occurred multiple times. Low levels of sequence divergence within the North American clade suggest either recent and rapid ecological radiation or highly conservative ribosomal DNA evolution within the clade.

Ephedra L., the single genus of the family Ephedraceae (Gnetales), is a well-delimited genus of ca. 60 species that are distributed in deserts and other arid regions in both the Old and the New Worlds. Its unique combination of morphological and biochemical characters includes small, decussate or whorled, ephemeral leaves on photosynthetic stems (Kubitzki 1990), vessels and dimorphic tracheary elements (Carlquist 1996), ribbed pollen (Steeves and Barghoorn 1959), ovules with a single integument subtended by a pair of fleshy or wing-like bracts, and the presence of ephedrine and other alkaloids (Stapf 1889; Martens 1971; Takaso 1984; Caveney et al. 2001). The genus has generally been considered monophyletic (Price 1996; Huang 2000; Rydin et al. 2002), although this has not been tested rigorously. Chromosome numbers indicate a base number of $x = 7$, with evidence of polyploidy and interspecific hybridization in both Old World and New World forms (Hunziker 1953; Pachomova 1971; Ickert-Bond 1999).

Ephedra is one of the few gymnosperms adapted to extreme aridity, and, as such, is highly reduced vegetatively. Both the small number of characters and the convergent evolution of species occurring in xeric habitats have limited taxonomic study. Early taxonomic work on *Ephedra* was based mostly on features of the ovule-bearing strobilus. Meyer (1846) divided the genus into two informal groups: section I. *Plagiostoma* C. A. Mey., and section II. *Discostoma* C. A. Mey., on the basis of characters of the ovule and the extension of the micropyle, called the tubillus. Later, Stapf (1889) proposed three formal subgroups based on ovule morphology. Section *Pseudobaccatae* is the most species-rich and includes those with fleshy, succulent bracts, while section *Alatae* is comprised of those with dry winged bracts from both the Old World (*E. alata* Decne., *E. przewalskii* Stapf, *E. lomatolepis* Schrenk, *E. strobilacea* Bunge) and New World (*E. trifurca* and *E. torreyana*). Section *Asarca* has two New World species, *E. californica* and *E. aspera*, which are neither winged nor fleshy and have ovulate strobili with bracts having scarious margins. Other investigators have attempted to test these relationships using additional morphological characters, including pollen (Steeves and Barghoorn 1959), cuticle micromorphology (Pant and Verma 1974), and wood anatomy (Carlquist 1989, 1992, 1996). None of these studies supported the subdivision of *Ephedra* proposed by Stapf (1889).

In a group of plants where morphologically-based taxonomy has been so difficult, the use of molecular sequence data provides an essential tool for resolving relationships. To date, however, little molecular information is available for *Ephedra*. The genus was included in a survey of *rbcL* sequences of the Gnetales (Hasebe et al. 1992), with only a few of the more than 50 species of *Ephedra* included (Price 1996), or as exemplars in higher-level analyses of land plants using *rbcL, atpB,* 26S, and 18S ribosomal DNA (Magallón and Sanderson 2001; Rydin et al. 2002; Soltis et al. 2002). Infrageneric relationships were studied by Huang (2000) using the *matK* gene and nrDNA ITS locus, but limited sampling and a taxon bias toward New World species obscured inferences about the genus as a whole, and many clades were not well supported.

The present study seeks to increase our knowledge of diversity and evolution in the genus *Ephedra* by presenting hypotheses for infrageneric relationships using DNA sequence data from both the chloroplast and nuclear genomes. This study endeavors to: 1) test the monophyly of *Ephedra*; 2) provide a preliminary test of the validity of the traditional taxonomic divisions into sections *Alatae*, *Asarca* and *Ephedra* (Stapf 1889; Mussayev 1978; Freitag and Maier-Stolte 1994); 3) enhance our understanding of infrageneric relationships among species of *Ephedra*; and 4) provide a preliminary test of the biogeographical diversification of the genus. Character evolution is discussed as it relates to the resulting phylogeny.

Materials and Methods

Taxon Sampling. A total of 71 taxa were sampled in this study (Appendix 1), including 51 species from all lineages of *Ephedra* as defined by Stapf (1889), two recently described South American species (Hunziker 1994; Matthei 1995), and two recently described Old World species (Assadi 1996; Freitag and Maier-Stolte 2003). Multiple accessions of some species were used to confirm questionable identifications. Sampling of the New World species included all known taxa, except for *Ephedra trifurcata* Zoellner. This species is only known from the type specimen, collected in 1978, and could not be relocated by the first author while visiting the type locality (Marga Marga, Chile) in 2001. Selection of Old World species was based on consultation with M. Maier-Stolte and H. Freitag, University of Kassel, Germany, who are currently revising those taxa (Freitag and Maier-Stolte 1992, 1994, 2003). Outgroup taxa include *Gnetum*, *Welwitschia* Hooker f., and the more distantly related *Pinus* L. All new sequences have been deposited in Genbank (http://www.ncbi.nlm.nih.gov/), and the final data matrices of both the nrDNA ITS1 and the plastid *rps4* gene have been deposited in TreeBASE (study accession number S1144, matrix accession numbers M1966–M1967).

DNA Extraction and PCR Amplification. Fresh or silica-dried material was available for most taxa, but herbarium material was used for a few samples. Genomic DNAs were isolated using DNA extraction kits (QIAGEN Corporation, Alameda, California). DNA sequences were amplified by polymerase chain reaction (Palumbi 1996) on a DNA engine Dyad (MJ Research, Waltham, Massachusetts) with the following conditions: initial denaturation (92°C, 2 min), followed by 40 cycles of denaturation (92°C, 45 sec), annealing (55°C, 30 sec), and extension (72°C, 30 sec), and concluding with a final extension (72°C, 7 min). The PCR amplifications were performed in 25 μl reactions containing 10–100 ng genomic DNA, 0.2 mM deoxyribonucleotide triphosphates (equimolar), 0.5 units Platinum *Taq* polymerase (Invitrogen), oligonucleotide primers at 0.5 μM, and Mg^{2+} at 1.5 mM. Glycerol was substituted with 5% DMSO to relax the secondary structure of ITS (Liston et al. 1996).

PCR amplification of the internal transcribed spacer region of the nuclear ribosomal DNA (nrDNA ITS1) used the primer ITS5* and 5.8SR (Liston et al. 1996) and a pair of internal primers designed specifically for *Ephedra* (ITS1-Ep1S, 5′-GGACGGTCTTTGACCAGTTTATA-3′; ITS1-Ep2R, 5′- GCGACGTAGGAAAGGAAATAG-3′; modified from Huang 2000). Amplification yielded a single product of ca. 1200 nucleotides for *Ephedra*, whereas sequences from outgroup taxa varied greatly in length, ranging from 400 to 900 bp. Amplification of the ca. 700 bp plastid gene *rps4* used the primers trnSR2 (5′-GCTTACCGGGGTTCGAATC-3′) and rps5F (5′-ATGTTCCCGTTATCGAGGACCT-3′) designed by R. Cranfill, University of California, Berkeley. This sequence includes ca. 30 bp of the intergenic spacer between the 3′ end of the *rps4* gene and the *trnS* gene. Most taxa were amplified according to reaction conditions given as above, but an annealing temperature of 55°C and 40 amplification cycles were used.

Sequencing. Purified PCR products (QIAquick® PCR purification kit, QIAGEN) were sequenced for complimentary strands. Sequencing reactions employed the same primers as PCR for the plastid *rps4* gene. Because the nrDNA ITS1 region in *Ephedra* spans ca. 1,200 bp, both the external PCR primers and the additional pair of internal primers (ITS1-Ep1S and ITS1-Ep2R), were used to generate sequences across the full length of nrDNA ITS1 in both directions. PCR products were cycle-sequenced using fluorescent dye-labeled chemistry (Big Dyes, Perkin-Elmer, Foster City, California). Separation and identification of cycle-sequence products was conducted on an ABI automated 377XL sequencer at the Arizona State University DNA Laboratory.

Alignment and Analysis. Sequences were assembled into contigs using Sequencher, version 4.1 (Gene Codes Corporation, Ann Arbor, Michigan). Sequence alignments were initially made in ClustalX (Jeanmougin et al. 1998) and edited manually. The plastid gene *rps4* codes for a protein product and is easily alignable by eye. The nrDNA ITS1 sequences are somewhat more difficult to align due to dramatic length variation (ranging from 400 to 1,200 bp) and high sequence divergence between *Ephedra* and all known outgroups.

PAUP* 4.0 beta10 (Swofford 2003) was used to reconstruct phylogenetic relationships. All characters were unordered and weighted equally. Parsimony analyses were conducted using heuristic search methods and employed all addition sequence options (SIMPLE, CLOSEST, RANDOM addition sequences) in combination with tree-bisection reconnection (TBR) branch-swapping and the MULTREES option, which saves all minimal trees, with MAXTREES set to 5,000. Fourteen indels (insertions/deletions) in the nrDNA ITS1 data set (varying from one to 25 base positions in length for a total of 72 characters) and were routinely excluded from all analyses. However, the indels were also coded as separate binary characters and added at the end of the data set and analyzed to determine whether they were phylogenetically informative. For all data sets, support for individual tree branches was estimated using non-parametric bootstrap methods (Felsenstein 1985). Bootstrap proportions (BP) were obtained from 100 replicates of heuristic searches as described above (1000 random addition sequences, TBR branch swapping, and MULTREES selected).

The monophyly of previously proposed taxonomic groupings (sects. *Ephedra*, *Alatae*, and *Asarca*) was investigated by comparing trees consistent with topological constraints to trees obtained from unconstrained analyses. Trees with topological constraints were constructed using MacClade 4.03 (Maddison and Maddison 2000) and loaded into PAUP*. Heuristic searches were then conducted to find the shortest trees consistent with each imposed constraint.

Maximum likelihood (ML) analyses were conducted with the nrDNA ITS1 dataset using PAUP* to compare with results from parsimony analyses. A single tree derived from parsimony or neighbor joining analysis was used as the starting tree for a heuristic search (addition sequence ''ASIS'') with TBR branch swapping and MULTREES in effect. Base frequencies were empirically determined, and the transition: transversion (K) ratio and gamma shape parameter (Γ) were estimated on tree number 6 (of 198 most parsimonious trees) from parsimony analysis (3.283000 and 0.0535576, respectively) and the tree derived from neighbor joining (3.48352 and 0.073199, respectively) under the HKY85 model of sequence evolution, chosen using results from ModelTest version 3.06 (Posada and Crandall 1998). Rate heterogeneity across sites followed a gamma distribution with four categories. Pairwise distances were calculated across all sites as 'uncorrected p' values using PAUP*.

Rooting followed the outgroup method for the *rps4* data, but was problematic within the nrDNA ITS1 sequences due to dramatic length differences between sequences of the outgroup and ingroup. Multiple analyses were undertaken both with and without inclusion of outgroups. One approach to deal with large length variation and sequence divergence between the outgroup and the ingroup was proposed by Simmons and Freudenstein (2003) that utilizes ClustalX (Jeanmougin et al. 1998) and was employed here. In ClustalX, blocks of 30 invariant bases were added to the se-

TABLE 1. Comparison of rate constancy of nrDNA ITS sequence evolution in *Ephedra* based on likelihood ratio tests, with placement of the root in all possible positions. The log likelihood values with assumption of molecular clock ($-\ln L_0$) and without ($-\ln L_1$): values for molecular clock assumption are consistently 3168.24174. Molecular clock in these data is rejected based on chi-square values (df = 46, $P = 0.01$, CV = 71.201). Taxa abbreviations: EA1 = *Ephedra antisyphilitica* 900, EAM = *Ephedra americana* Argentina, EAP1 = *Ephedra aphylla* Fr 14a 01, EAP2 = *Ephedra aphylla* F30181, EAS = *Ephedra aspera,* EBO = *Ephedra boelkei,* EBR = *Ephedra breana,* EC1 = *Ephedra coryi* SIB 953, EC2 = *Ephedra coryi* SIB 952, ECA = *Ephedra californica,* ECH = *Ephedra chilensis,* ECO = *Ephedra compacta,* ECU = *Ephedra cutleri,* EDI = *Ephedra distachya,* EFA = *Ephedra fasciculata,* EFE = *Ephedra fedtschenkoae,* EFL = *Ephedra frustillata,* EFN = *Ephedra funerea,* EFR = *Ephedra fragilis,* EFU = *Ephedra funerea* SIB 964, EGR = *Ephedra gracilis,* EIN = *Ephedra intermedia,* ELA = *Ephedra laristanica,* EMO = *Ephedra monosperma,* EMU = *Ephedra multiflora,* ENE = *Ephedra nevadensis,* EOC = *Ephedra ochreata,* EPE = *Ephedra pedunculata,* ERA = *Ephedra rupestris* Arg, ERE = *Ephedra regeliana,* ERU = *Ephedra rupestris,* ESA = *Ephedra saxatilis,* ESI = *Ephedra sinica* var. *pumila,* ESO = *Ephedra somalensis,* EST = *Ephedra strobilacea,* ETA = *Ephedra triandra,* ETI = *Ephedra transitoria,* ETO = *Ephedra torreyana* Crimmins, ETP = *Ephedra torreyana powelliorum,* ETR = *Ephedra trifurca,* ETS = *Ephedra torreyana* AZ, ETW = *Ephedra tweediana,* EVI = *Ephedra viridis.*

Root placed along the branch between taxa listed and the remaining taxa	Molecular clock assumption $-\ln L_0$	Without the molecular clock assumption $-\ln L_1$	Likelihood ratio $-2nLR$
ELA	3202.69271609	3168.24174	68.901955
EPE	3214.48556536		92.4870657
EVI	3232.76675754		129.05004
ESO	3235.55968010		134.63589
EDI	3242.99145664		149.49944
EMO, ESA	3251.54758526		166.61169
ECA	3260.65509676		184.82672
EAP2, EFR	3265.36480176		194.24613
ECH	3268.73771349		200.99195
EST, ETI	3271.93190747		207.38034
ECO4, EAM, ERU	3273.17757967		209.87168
ESI, EIN	3281.40952837		226.33558
EFE, ERE, EC1, EAP1	3285.32565718		234.16784
ETW, ETR, ETA	3289.90698269		243.33049
EBR	3292.51888925		248.5543
ECO1, EC2	3296.12217738		255.76088
ETO	3311.93671356		287.38995
ETP	3314.14447345		291.80547
EGR	3320.35824580		304.23302
EMU, EBO	3324.21645634		311.94944
EA1	3329.17170745		321.85994
ERA, EOC	3336.83603110		337.18859
ETS, EFN	3339.02081343		341.55815
EAS	3342.21571065		347.94794
EFU	3355.44379305		374.40411
ENE, ECU, EFA	3358.60790784		380.73234

quences at the 5′ and 3′ ends of nrDNA ITS1. These blocks kept the 5′ and 3′ ends of the *Ephedra* sequences consistently aligned with outgroup sequences, reducing overall sequence divergence. In the program, alignment parameters for pairwise alignments were set to ''slow-accurate,'' with a ''gap opening cost'' of 10, and a ''gap extension cost'' of 5 for both pairwise and multiple alignments. In multiple alignment mode, ''delay divergent sequences'' was set to 40% and ''DNA transition weight'' was set to 0.50. These invariant sequence blocks were then excluded from all phylogenetic analyses.

Two alternative methods of rooting were explored. The first was midpoint rooting, which assigns the root to the midpoint of the longest path between two terminal taxa (i.e., between the two most divergent lineages in the ingroup, Swofford et al. 1996). Second, reconstruction of a tree under the assumption of a molecular clock was used to infer the root of the tree. Analyses were conducted to determine likelihood scores for a tree of ingroup taxa, generated by the neighbor-joining method, both with and without enforcement of a molecular clock, where the root was forced to attach to each of the possible branches on the same ingroup topology (see Schultheis and Baldwin 1999). The number of ingroup sequences was reduced to represent only one accession per taxon to minimize the number of necessary calculations (Table 1). Estimated nucleotide frequencies and the substitution model parameter values, consistent with the General Time Reversal model [GTR+I+Γ] under the Akaike Information Criterion, were determined using ModelTest version 3.06 (Posada and Crandall 1998). Rate heterogeneity across all lineages was then assessed using a likelihood ratio test (Felsenstein 1988; Huelsenbeck and Rannala 1997).

RESULTS

Nuclear Locus. Results from likelihood ratio analyses of nrDNA ITS1 data with and without enforcement of a molecular clock (Table 1) for the ingroup taxa show that clock-like evolution can be rejected for all root placements within *Ephedra* except along the lineage between the Old World species *E. laristanica* and the remaining taxa. In all other rootings examined, branch lengths were not consistent with equal rates of nucleotide change. Midpoint rooting also resulted in placement of the root between *E. laristanica* and all remaining taxa. Results from analyses of the dataset in which invariant blocks were included at both the 3′ and 5′ ends of the nrDNA ITS1 of both the ingroup

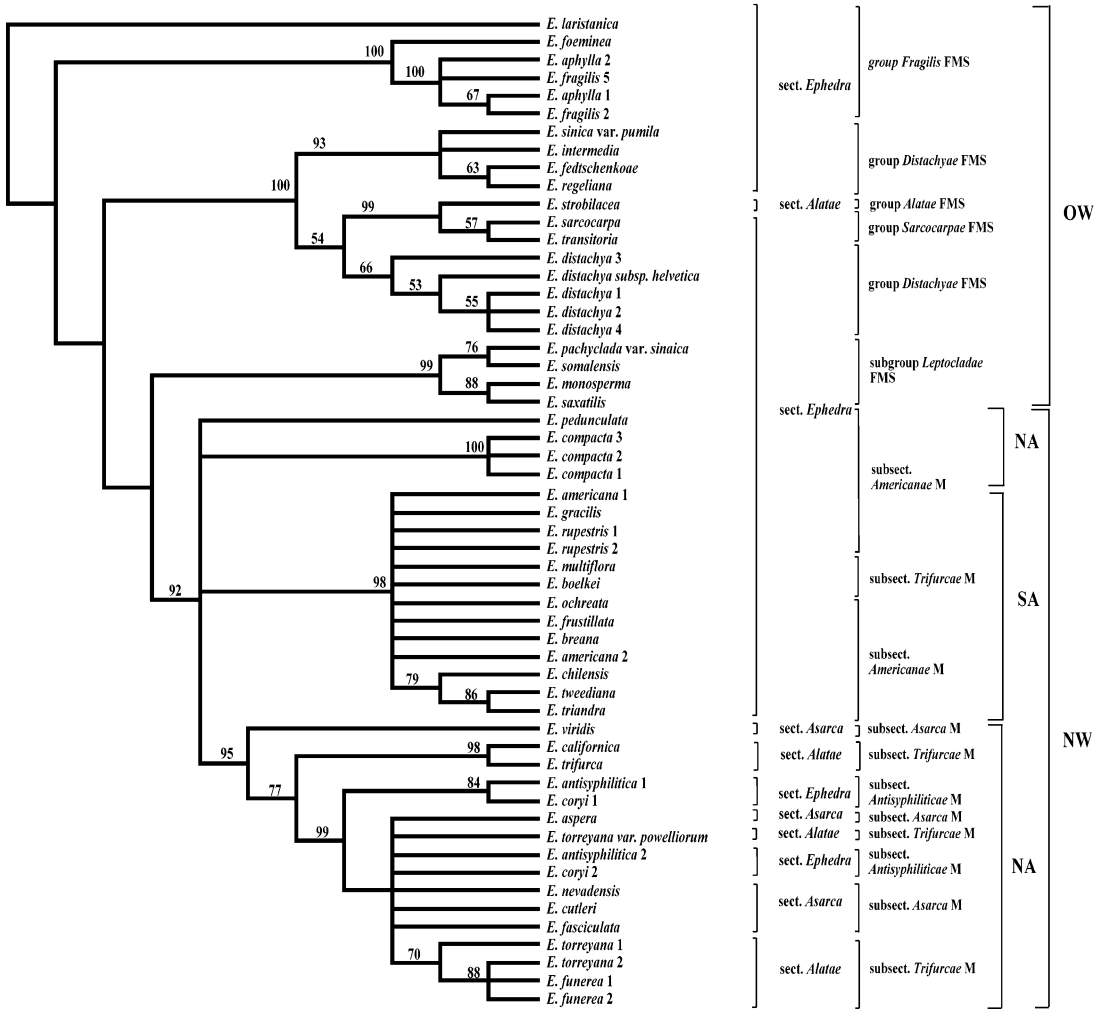

FIG. 1. Phylogenetic relationships in *Ephedra* based on parsimony analysis of nrDNA ITS1 sequence data. Tree is strict consensus of 198 equally parsimonious trees of length 234 steps (CI = 0.7179; RI = 0.9282) derived from heuristic analyses (random addition sequence, TBR branch swapping, MULTREES in effect). Bootstrap values are given above branches of clades that were supported in both the strict consensus and the bootstrap consensus trees. Named groups following classifications by Stapf (1889), except as noted: M = Mussayev (1978); FMS = Freitag and Maier-Stolte (1994); and their geographic distribution (NW, New World; NA, North America; SA, South America; OW, Old World) appear in margin at right.

and the outgroup are compatible with results from both midpoint rooting of the ingroup taxa alone and from root placement based on a molecular clock assumption (i.e., between *E. laristanica* and the remaining taxa). For subsequent analyses and presentation of results, trees were rooted with *E. laristanica*.

The aligned length of the nrDNA ITS1 region for 55 ingroup taxa was 1,158 bp. There were 14 indels that were also added to the data set as binary characters, but the inclusion of these extra characters had no effect on the overall tree topology. The nrDNA ITS1 data set included 112 parsimony-informative characters and yielded 198 most parsimonious trees (234 steps). Pairwise distances ranged from 0–5.5% within *Ephedra*.

The strict consensus of these trees (Fig. 1), when rooted with *Ephedra laristanica*, shows the New World species of *Ephedra* comprising a well-supported clade, while the Old World taxa form a paraphyletic grade of three well-supported clades at the base of the tree. Within the New World clade there is strong support (BP=95) for a clade comprising all species from North America except for two primarily Mexican species, *E. compacta* and *E. pedunculata*. Within the main North American clade, *E. viridis* is sister to the remaining species. Species of section *Asarca* do not form a monophyletic group within the main North American clade, whereas two species of section *Alatae*, subsection *Trifurcae*, *E. torreyana* and *E. funerea*, form a moderately-supported clade (BP=70) within this main clade. Although all South American species belong to one high-

ly-supported clade (BP=98), relationships among these species are not well resolved, except the clade including *E. tweediana*, *E. triandra*, and *E. chilensis*.

Among the Old World taxa, there is strong support for several clades. *Ephedra monosperma*, *E. pachyclada* var. *sinaica*, *E. saxatilis*, and the newly described *E. somalensis* comprise a very well-supported clade (BP=99) that is congruent with *Ephedra* section *Ephedra*, group *Distachyae*, subgroup *Leptocladae*. In this clade, *E. monosperma* and *E. saxatilis* are sister taxa, and this clade is sister to a clade of *E. somalensis* and *E. pachyclada* var. *sinaica*. Further, *Ephedra* section *Ephedra*, group *Distachyae*, subgroup *Distachyae*, which includes *E. sinica* var. *pumila*, *E. intermedia*, *E. fedtschenkoae*, and *E. regeliana*, is strongly supported (BP=93) as monophyletic. Within this clade, *E. fedtschenkoae* and *E. regeliana* are weakly (BP=63) supported as sister species. *Ephedra strobilacea* of section *Alatae* comprises a clade with *E. transitoria* and *E. sarcocarpa*, with *E. strobilacea* sister to *E. transitoria* and *E. sarcocarpa* of group *Sarcocarpae*. Finally, the species representing *Ephedra* section *Ephedra*, group *Fragilis* are well resolved as monophyletic.

Comparison of results from parsimony and maximum likelihood analyses (Figs. 2, 5) are generally congruent with respect to overall topology and estimated branch lengths. For example, a highly-supported clade of New World taxa is nested within Old World groups, and both reconstruction methods are also consistent in showing taxa with the largest number of inferred nucleotide substitutions to be *E. laristanica*, *E. aphylla*, *E. fragilis*, and *E. foeminea*.

Chloroplast Locus. The aligned length of the *rps4* gene and the partial sequence of the intergenic spacer region between *rps4* and *trnS* ranged from 673 to 685 bp. Pairwise distance ranged from 0–1.9% within *Ephedra*. Results of parsimony analyses using *Pinus*, *Welwitschia*, and *Gnetum* as outgroups, strongly supports the monophyly of the genus *Ephedra* (Fig. 3). Although these data provide very little resolution within *Ephedra* (13 parsimony informative characters within the ingroup, not including indels), they do suggest the New World *Ephedra* are nested within Old World lineages (Fig. 3). *Ephedra somalensis* is sister to the rest of *Ephedra*, with relationships of the remaining taxa unresolved except for a few weakly-supported clades. Consistent with results from the nrDNA ITS1 data (Fig. 1), the New World species are nested within Old World groups, although bootstrap support is weak. Branch lengths estimated by parsimony (Fig. 4) show very few character changes in *Ephedra* compared to *Gnetum* and *Welwitschia*, and long branches separate the genera.

Discussion

Molecular Evolution. Based on results of comparison of uncorrected pairwise distances the nrDNA ITS1 region in *Ephedra* evolves approximately three times faster than the protein-coding region of plastid *rps4* and the adjacent intergenic spacer region. Although this region is only twice as long as the *rps4* region, it has ten times as many the number of informative characters. It is therefore evident that the nrDNA ITS1 region is more phylogenetically informative for deducing relationships in *Ephedra*. The ITS1 region has been used for phylogeny reconstruction in several gymnosperm studies (Liston et al. 1996; Gernandt et al. 2001; Li et al. 2001; Sinclair et al. 2002) and extensively in angiosperms (Baldwin et al. 1995; Álvarez and Wendel 2003). However, it should be noted that the *rps4* gene has shown phylogenetic utility in studies of pteridophytes (Pryer et al. 2001; Schneider et al. 2004) and monocots (Nadot et al. 1994).

Low levels of sequence divergence in nrDNA ITS1 (as in *rps4*) in the New World clade (Fig. 2), as compared to that observed within Old World groups, suggests that either *Ephedra* underwent a more recent or rapid radiation in the New World or that evolution of the nrDNA region has been more conservative in New World *Ephedra*. A similar pattern has recently been shown in the New World thistle *Cirsium* Mill., where the western North American lineage exhibits a lower sequence divergence compared to other New World groups (Kelch and Baldwin 2003). Molecular phylogenetic analysis of the conifer genus *Torreya* Arn. also shows well-supported Old and New World clades but low levels of sequence divergence within each of them, pointing to a recent origin of extant taxa (Li et al. 2001), a pattern similar to the results for *Ephedra*. Other examples of a long stem lineage and low divergence within resulting crown groups have been noted recently in Myristicaceae (Sauquet et al. 2003) and Chloranthaceae (Zhang and Renner 2003). For example, in the Chloranthaceae the fossil record dates back to the Early Cretaceous (Doyle et al. 2003), but age estimates based on molecular data (Zhang and Renner 2003) date the divergence time of the crown group as considerably more recent, in the Tertiary (14–45 MYA).

Monophyly of Ephedra. Previous studies have provided morphological and molecular evidence consistent with the monophyly of *Ephedra*, within the broader context of seed plant relationships (Price 1996; Huang 2000; Rydin et al. 2002; Huang and Price 2003; Won and Renner 2003). In this study we used a more comprehensive sampling of *Ephedra* as well as outgroup taxa to address this issue. Molecular sequence data from the plastid *rps4* gene strongly support the monophyly of *Ephedra* (Figs. 3, 4), as well as the monophyly of those species sampled from *Gnetum*.

Phylogenetic Relationships Within Ephedra. Results from both data sets are consistent in showing that *Ephedra* comprises a basal paraphyletic grade of Old World lineages, within which a highly-supported New World clade is nested (Figs. 1–5). Results presented

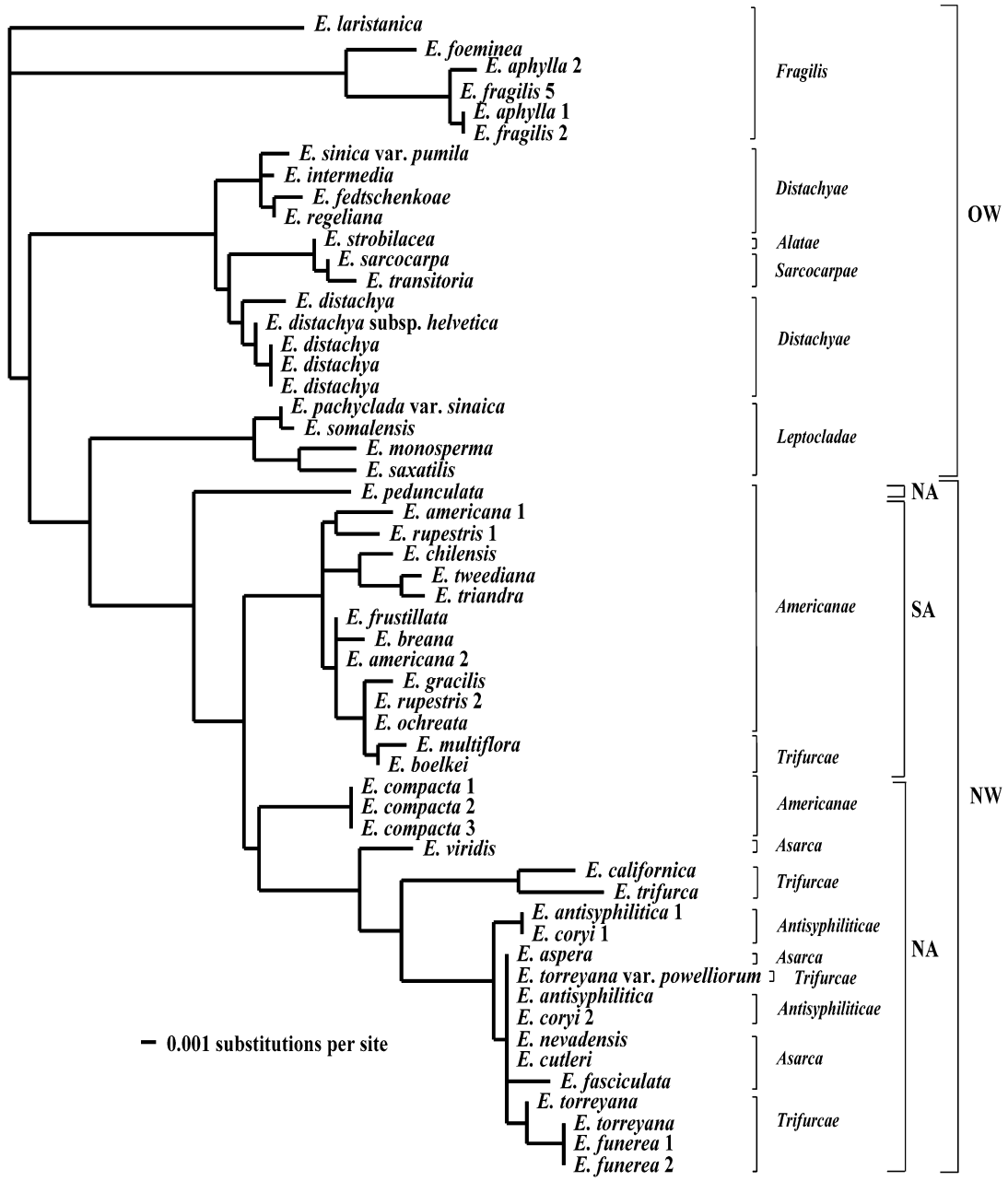

FIG. 2. Maximum likelihood tree of nrDNA ITS1 data showing estimated branch lengths. Relationships among major clades of *Ephedra* are similar to those inferred from parsimony analyses of the same data, except that *Ephedra compacta* is now sister to main North American clade. Phylogram shown is one of four trees ($-\ln L = 3048.49402$) derived from maximum likelihood analyses under a HKY85+Γ model of nucleotide substitution. Geographic distribution of taxa (NW, New World; NA, North America; SA, South America; OW, Old World) appear in margin at right.

here suggest that neither the subdivision of *Ephedra* into the three traditional sections, *Alatae*, *Asarca*, and *Pseudobaccatae* (= *Ephedra* L.) as circumscribed by Stapf (1889), nor the slightly modified system by Mussayev (1978), who recognized five sections (*Alatae*, *Asarca*, *Ephedra*, *Scandentes* (Stapf) Mussayev, *Monospermae* Pachom.), is supported. All the sections based on morphology by Stapf (1889) and Mussayev (1978) are distributed worldwide, except for the strictly North American section *Asarca*. Within the New World clade four subclades are highly-supported, one composed of most of the North American taxa and one of strictly

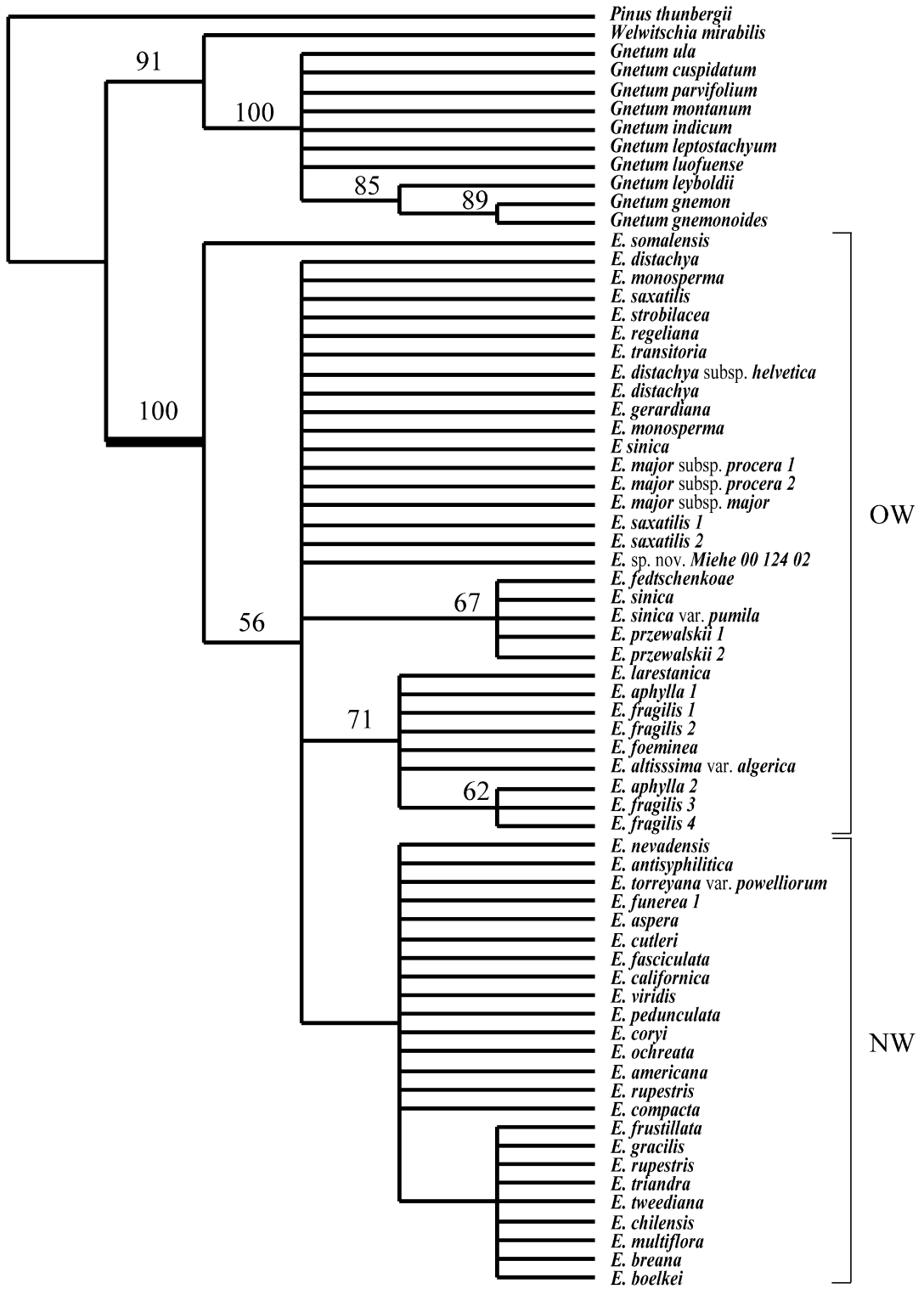

FIG. 3. Phylogeny of *Ephedra* based on parsimony analysis of plastid *rps4* (including *rps4* gene and *rps4- trn*S intergenic spacer) sequence data. Tree is strict consensus of 1000 (MAXTREES) equally parsimonious trees of length 465 steps (CI = 0.892; RI = 0.962) derived from heuristic analyses (random addition sequence, TBR branch swapping, MULTREES in effect). Bootstrap values are given above branches for clades resolved in strict consensus. Geographic distribution of taxa indicated in margin at right (NW, New World; OW, Old World).

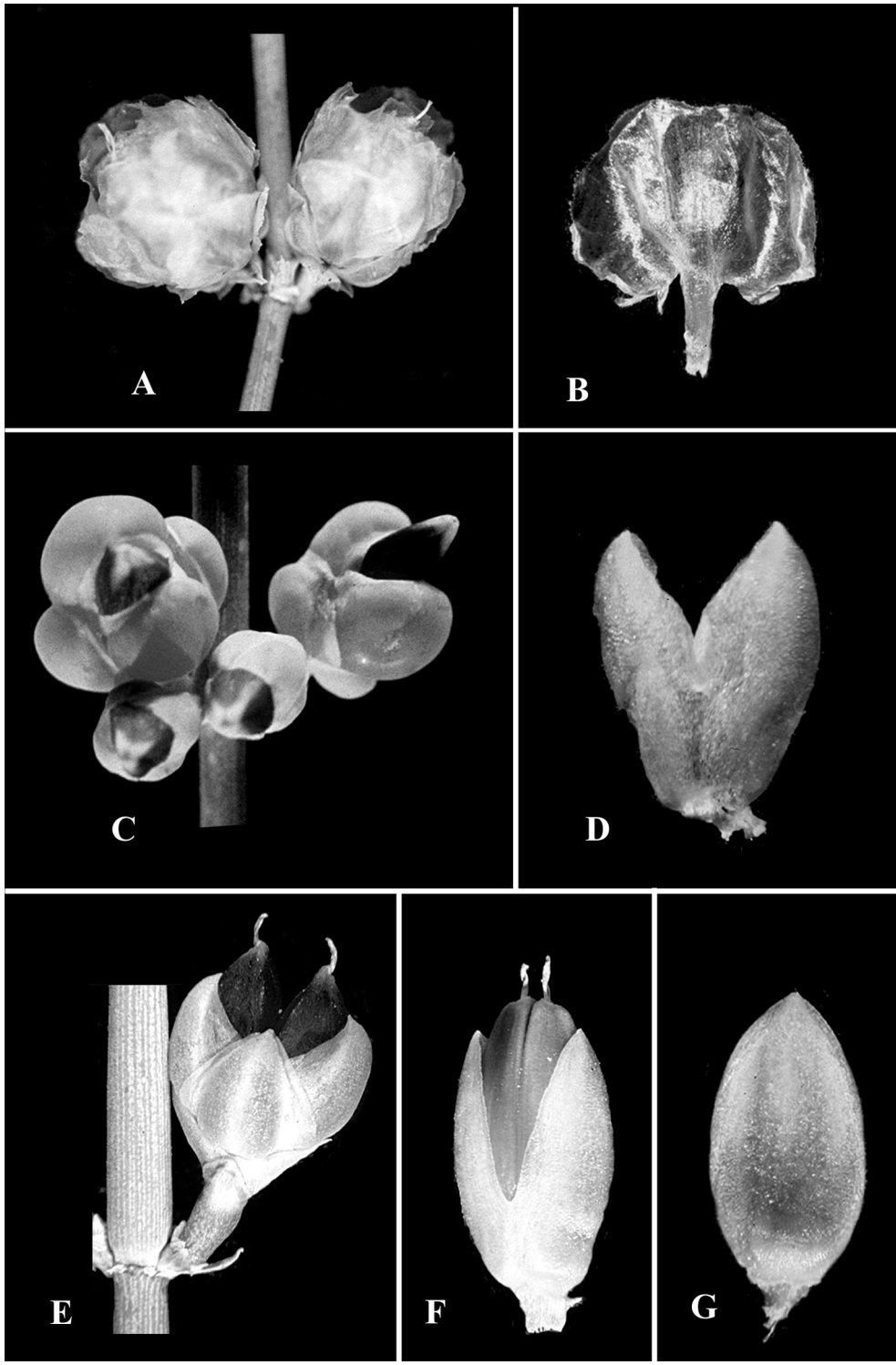

FIG. 4. General morphology of the ovulate bract in *Ephedra*. A. *Ephedra torreyana*, of section *Alatae*, subsection *Trifurcae* showing dry, wing-bracted ovulate strobili (× 3.5). B. Detail of individual bract, note stalk at base and margin elaborated into wing (× 6). C. *Ephedra antisyphilitica* of section *Ephedra*, subsection *Antisyphiliticae* showing fleshy bracts of the ovulate strobili (× 3.9). D. Detail of bracts showing fusion of more than 50% of the entire lengths of the bract, note absence of stalk at base (× 7.6). E. *Ephedra nevadensis* of section *Asarca* showing bracts that are dry, not papery or fleshy (× 3.5). F. Detail of bracts fused basally, but well below midpoint (× 6.4). G. Detail of single bract with margin entire, not elaborated into wing (× 6.5).

South American species. The wing-bracted section *Alatae* as circumscribed by Stapf (1889) and Mussayev (1978) is not monophyletic in this analysis (Fig. 1). To achieve monophyly, trees derived from the MP analysis would have to be 55 steps (ca. 22%) longer. *Ephedra strobilacea* is the only species with dry, winged bracts from the Old World section *Alatae*, *Tropidolepides* Stapf (= subsection *Alatae* (Stapf) Mussayev) included in this analysis. It is strongly supported as sister to a clade of two species (*E. sarcocarpa* and *E. transitoria*) with fleshy bracts (BP=99). In Stapf's classification, *E. sarcocarpa* was placed in section *Pseudobaccatae* Stapf, subsection *Pachycladae* Stapf, among other fleshy-bracted ephedras of the Old World, while *E. transitoria* was not described until after Stapf's treatment (Riedl 1961). Freitag and Maier-Stolte (1994) provide an alternative classification, including *E. sarcocarpa*, *E. transitoria*, and a third species, *E. lomatolepis*, in their group *Sarcocarpae* Freitag and Maier-Stolte, which is monophyletic in this analysis (Figs. 1, 2; *Sarcocarpae* clade), although with weak support (BP=57). Our molecular results are consistent with the distribution of similar morphological characters, such as wide hyaline margins of the otherwise fleshy bracts (Fig. 5A, B), distinctly stalked microsporangia (Fig. 5C), and pollen morphology, as first noted by Freitag and Maier-Stolte (1994).

The New World subsection of section *Alatae*, *Habrolepides* Stapf (= subsection *Trifurcae* Mussayev), is not monophyletic in this analysis, although two species, *E. funerea* and *E. torreyana*, are closest relatives (BP=70) in the main North American clade. It appears that dry-winged bracts originated multiple times in the New World. Other members of Stapf's original subsection *Haprolepides*, which includes the North American *E. trifurca* and the South American *E. multiflora* and *E. boelkei*, do not cluster with the other wing-bracted taxa from the New World, although *E. boelkei*, a species with winged bracts recently described from Argentina (Roig 1984), forms a clade with *E. multiflora* (Figs. 2, 5) in the South American clade. These two species are very closely related and at least one specialist (J. Hunziker, pers. comm., Instituto Darwinion, San Isidro, Argentina, 2000) considers *E. boelkei* to be a polyploid derivative of *E. multiflora* that has subsequently become morphologically distinct. This hypothesis needs to be investigated using both molecular and cytological methods.

The second traditionally-described section, *Asarca* Stapf, which includes *E. californica*, *E. fasciculata*, and *E. aspera* (Mussayev 1978), is not supported as monophyletic in our analysis. For these taxa to form a clade, trees derived from parsimony analysis would require 17 more steps than the globally most parsimonious trees (~ 7% longer). It is interesting to note that *E. californica*, a species with dry bracts that are marginally winged that has commonly been placed in section *Asarca* (Stapf 1889; Mussayev 1978; Price 1996), is highly supported as the closest relative of *E. trifurca* of section *Alatae*, subsection *Trifurcae* (BP=98), which is delineated by winged bracts of the ovule-bearing strobili (Fig. 5A, B).

Results from analyses presented here also suggest that the third section, *Pseudobaccatae* (= *Ephedra*), the fleshy-bracted ephedras, is polyphyletic. Monophyly of these taxa would require trees with 40 additional steps (~ 16% longer). However, within section *Pseudobaccatae*, several relationships are highly supported. Most species of subsection *Scandentes* Stapf (= section *Scandentes* (Stapf) Mussayev, subsection *Scandentes*) (= group *Fragilis* Freitag & Maier-Stolte) form a highly-supported monophyletic group (Figs. 1, 2; *Fragilis* clade). This grouping is consistent with putative shared development of ''large'' leaves (up to four cm in *E. foliata* Boiss.), sessile microsporangia (Fig. 5C), *Fragilis*-type pollen, and a general climbing habit (Fig. 5D; Freitag & Maier-Stolte 1994). The newly described *E. laristanica* from Iran (Assadi 1996) was used as the outgroup to the rest of *Ephedra* in this analysis but fits well within subsection *Scandentes* Mussayev (=group *Fragilis*) morphologically (Freitag and Maier-Stolte, pers. comm., University of Kassel, Germany, 2003).

Another well-supported clade within the Old World section *Ephedra* corresponds to Freitag and Maier-Stolte's subgroup *Leptocladae*, which includes *E. monosperma* and *E. saxatilis* (from the Himalayas and Mongolia) and *E. somalensis* and *E. pachyclada* var. *sinaica* (from Somalia and Israel) (BP = 99). These taxa share the morphological characters of typically 1-seeded ovulate strobili, sessile microsporangia (Fig. 5C) and *Distachya*-type pollen (Freitag and Maier-Stolte 1994, 2003). The disjunct distribution of these four species between east-central Asia and east Africa may reflect a combination of vicariance and extinction events, an explanation suggested for a similar distribution of taxa in the family Nyctaginaceae (Thulin 1994). *Ephedra somalensis* grows in mesophytic habitats, which may indicate that it is a relictual species of a possible, once more widespread Tertiary laurophyllous vegetation in northeast Africa (Axelrod 1979; Freitag and Maier-Stolte 2003). Species of subgroup *Leptocladae* were traditionally placed in subsection *Leptocladae* Stapf, or by Mussayev (1978) in section *Monospermae* Pachomova, subsection *Monospermae*. To render subsection *Monospermae* monophyletic would require 15 additional steps relative to the most parsimonious trees (~ 6% longer).

Freitag and Maier-Stolte's group *Distachyae* is not supported as monophyletic in this analysis, but *E. distachya* and *E. distachya* subsp. *helvetica* form a weakly-supported clade (BP=66) that itself is weakly-supported (BP=54) as the sister group to a highly-supported clade (BP=99) of *E. strobilacea* from section *Alatae* and group *Sarcocarpae* (Figs. 1, 2, 5). The other

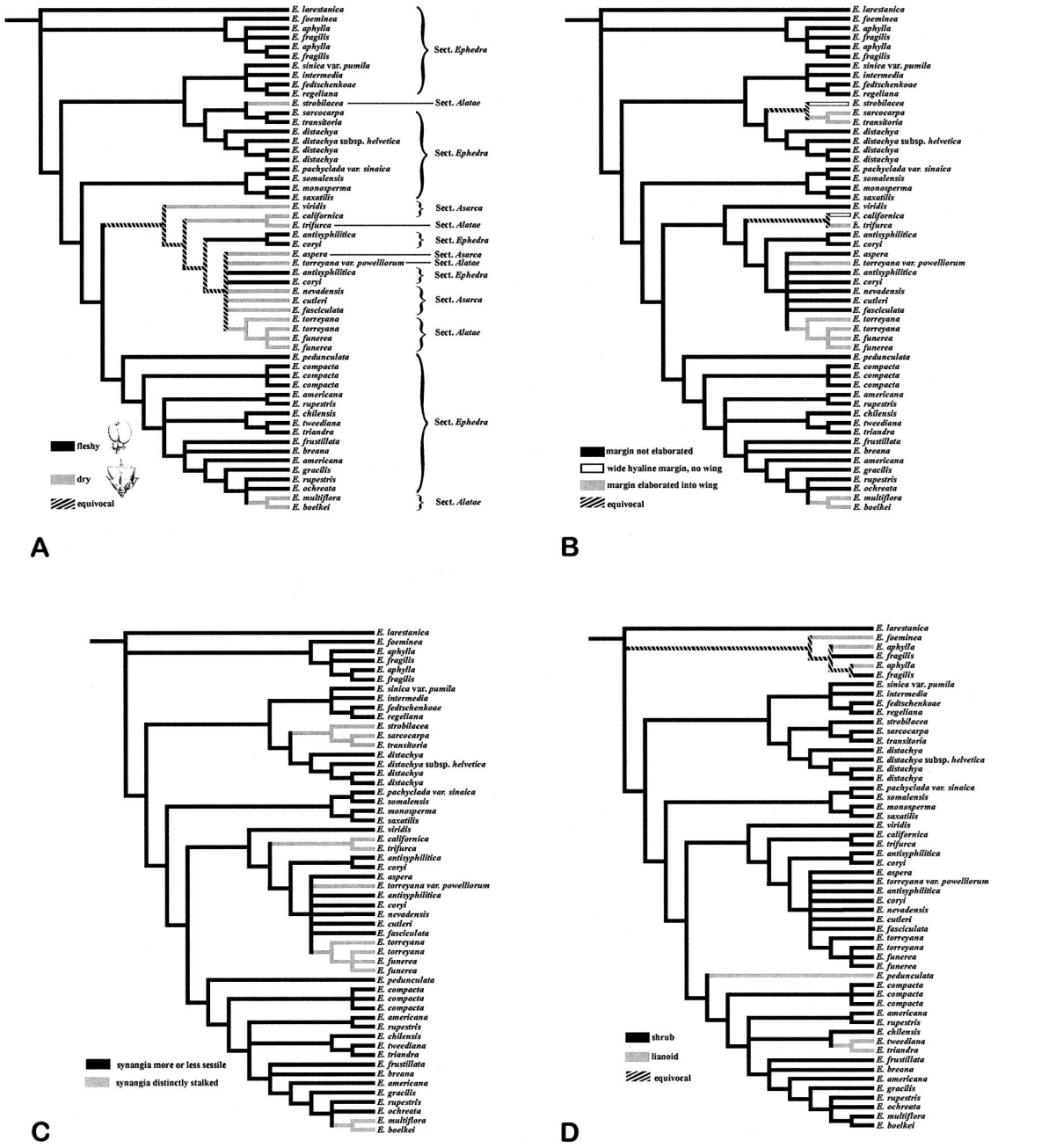

FIG. 5. Parsimony reconstruction of character state evolution of several unordered morphological key characters optimized on one of 198 equally parsimonious trees of length 234 steps (shown in Fig. 1) using MacClade. A. Ovulate bract characters. Named sections follow classifications by Stapf (1889). B. Wing margin of ovulate bracts. FIG. 5. Continued. C. Synangia characters of staminate strobili. D. Plant habit.

members of the group *Distachyae* form a highly-supported clade (BP=93), but relationships within this clade are not well resolved.

New World species of section *Ephedra* (=*Pseudobaccatae* Stapf) are not resolved as monophyletic but are scattered within the New World among North American and South American groups. Monophyly of this clade in the New World would require the addition of 30 steps (~ 12% longer than the most parsimonious trees). It is interesting to note that all of the South American species of section *Pseudobaccatae*, subsection *Americanae* Mussayev, form a highly-supported clade

(BP=98), while the two remaining members of subsection *Americanae*, *E. pedunculata* and *E. compacta* remain unresolved within the New World clade.

Character Evolution. Traditional delimitations of infrageneric relationships in *Ephedra* as proposed by Stapf (1889) and Mussayev (1978) based on a few characters of the ovulate bracts are not supported by molecular data (Fig. 5). None of the three sections, the fleshy-bracted ephedras of section *Ephedra* (= *Pseudobaccatae* Stapf), the intermediate ephedras of section *Asarca* Stapf, or the wing-bracted ephedras of section *Alatae* Stapf, are supported herein as clades. Based on our phylogenetic results, it appears that fleshy bracts are the ancestral condition and that dry, winged bracts originated multiple times (Fig. 5). The question of origin(s) of the intermediate condition (section *Asarca*) cannot be fully addressed here because relationships within the main North American clade are not well-resolved. It is noteworthy that bracts characterizing section *Asarca* are partially connate as in section *Ephedra* but distinct in section *Alatae*. Furthermore, the bracts of members of section *Asarca*, particularly in *E. nevadensis*, swell considerably during the growing season (S. Ickert-Bond, pers. obs.), but subsequent development into fleshy bracts does not occur.

While the results from analysis of molecular data do not confirm classifications based on ovulate bract characters, the larger number of species possessing fleshy-bracted ovules is intriguing and might be correlated with greater dispersal ability or different rates of speciation. The occurrence of red, fleshy bracts in section *Ephedra* is generally considered indicative of endozoochory (Stapf 1889; Freitag and Maier-Stolte 1994; Danin 1996; Hódar et al. 1996). In contrast, the dry, wing-bracted diaspores of section *Alatae* are a good example of anemochory (Stapf 1889; Danin 1996). The seeds of section *Asarca* often accumulate at the stem base, and are carried off by packrats, other small rodents, and possibly lizards (S. Ickert-Bond, pers. obs.).

While differing dispersal mechansims have been inferred to explain the high species diversity of angiosperms compared to non-fruiting plants (Tiffney and Mazer 1995; Smith 2001), recent studies have shown that similar effects may occur in the pollination and dispersal mechanisms of some gymnosperms as well (Norstog 1990; Schneider et al. 2002). Animal dispersal of angiosperm diaspores has been thought to be responsible for greater specialization and thus increased speciation, but the situation may be more complex in gymnosperms than previously recognized. It is not the mere fact of biotic versus abiotic dispersal but a combination of traits, including dispersal type, ecological conditions, and growth form, that may explain the greater species diversity in certain plant groups (Herrera 1989; Eriksson and Bremer 1991; Tiffney and Mazer 1995; Smith 2001). The coincidence of higher species number with fleshy diaspores in *Ephedra* seems to suggest that fleshiness may have affected the rate of speciation or of extinction. In contrast, anemochorous taxa in *Ephedra* inhabit highly specialized niches, such as hyperarid deserts, sometimes in dry salt lakes often devoid of animal life (Danin 1996). Thus it may be habitat availability and not dispersal ability that has prevented increased diversity within this group, a phenomenon that has been suggested to account for the diversity of herbaceous members of Rubiaceae (Eriksson and Bremer 1991).

Habit diversity in *Ephedra* is another interesting morphological character. Most species grow as much-branched shrubs, but a few climbing or small tree-like species are known as well (Stapf 1889; Pearson 1929; Carlquist 1996; Price 1996). A lianoid habit (Fig. 5D) is reconstructed as having been derived in three different clades, in the Old World *Fragilis* group, in the North American *E. pedunculata* and in the South American clade (*E. tweediana*, *E. triandra*).

Characters of the staminate strobilus in *Ephedra* show another interesting pattern (Fig. 5C). Distinctly stalked synangia (fused sporangia) *sensu* Hufford (1996) are reconstructed as derived from sessile ones. This character had been used to imply the distinctiveness of species in section *Alatae*, both in the Old as well as the New World; however, this section is not monophyletic in our analyses (Figs. 1, 5A). Results do suggest a close relationships of the wing-bracted taxa of section *Alatae*, subsection *Alatae* with Freitag and Maier-Stolte's group *Sarcocarpae* in the Old World, which is distinguished by fleshy bracts with a wide hyaline margin.

Other morphological characters that have not been used for species identification may indeed have some utility at broader infrageneric levels. For example, Steeves and Barghoorn (1959) and El-Ghazaly and Rowley (1997) considered pollen morphology to be too variable for species identification or inferring infrageneric relationships in *Ephedra*, yet general pollen-types were used in combination with other characters to delineate clades by Freitag and Maier-Stolte (1994). With further inspection of the results of phylogenetic analyses, additional morphological characters of value may be discovered.

Polyploidy in *Ephedra* is present but generally only at the tetraploid level (Choudry 1984; Hunziker 1953, 1955), with the exception of a single hexaploid count in *Ephedra funerea* (Ickert-Bond 2003). No obvious geographic or taxonomic relationship between the distribution of diploid and polyploid taxa exists, since polyploidy occurs worldwide and in all sections of the genus (Choudry 1984).

Biogeographical Implications. Three hypotheses concerning the center of origin for *Ephedra* have been put forth: 1) the genus originated in Central Asia [hy-

pothesis 1 of Pachomova (1969)]; 2) it originated in South America [hypothesis 2 of Pachomova (1969)]; 3) it originated in the mountainous littorals of the Mediterranean [hypothesis 3 of Soskov (1968) and Mussayev (1978)]. To investigate biogeographical patterns in *Ephedra*, evidence derived from plate tectonics, fossils, and phylogenetic analysis is brought to bear on these competing hypotheses.

Unequivocal gnetalean megafossils known from Early Cretaceous strata include *Drewria potomacensis* Crane and Upchurch of Aptian age, from the Potomac Group of Virginia, USA, and *Cratonia cotyledon* Rydin, Mohr and Friis from the late Aptian-early Albian Crato Formation of Brazil (Crane and Upchurch 1987; Rydin et al. 2003). Both of these genera show a mosaic of morphological characters seen among extant genera of the Gnetales, suggesting that they represent examples of a group that was considerably more diverse during the Cretaceous, when they were also more widely distributed geographically. Characters currently unique to one or another extant genus, such as the unusual cotyledon morphology of the narrowly-endemic genus *Welwitschia*, were more broadly distributed in these two genera, suggesting later Cretaceous or early Tertiary extinction of genera with intermediate morphologies.

There are additional megafossils with strong gnetalean affinities (Crane 1996) that await more detailed study, including samples from Early Cretaceous localities in China (Guo and Wu 2000), Russia (Krassilov 1986), and Portugal (Crane et al. 1995; Rydin et al. 2004). The habit of these fossils ranges from large-leaved taxa, as in the *Gnetum-Welwitschia* lineage (Krassilov 1986; Crane and Upchurch 1987; Rydin et al. 2003) to small-leaved forms, as in the *Ephedra* lineage (Crane and Maisey 1991; Mohr et al. 2003, 2004; Rydin et al. 2004). The age and geographic distribution of these gnetalean megafossils thus suggests that the crown group of Gnetales once ranged from Eurasia across the North Atlantic land bridge to North America, and was present in eastern South America (at least the *Welwitschia* and *Ephedra* lineages). Given the present day western African distribution of *Welwitschia*, it can be hypothesized that this group had a broader northern Gondwanan distribution (Rydin et al. 2003) during the Cretaceous.

Dispersed fossil pollen grains of questionable gnetalean affinity are abundant from the Triassic to the Recent. The form genus *Equisetosporites* Daugherty (*Ephedripites* Bolkhovitina) comprises grains that are similar to those of modern *Ephedra* but also include other forms, which exceed the modern pollen diversity of the genus including grains having psilate end plates, twisted muri, etc. (Osborn et al. 1993; Hesse et al. 2000). Fossil grains assigned to *Ephedra* are known from a variety of localities since the Paleocene, including sites in southeastern and western North America and Australia (Cookson 1956; Leopold and Clay-Poole 2001). *Ephedra* pollen has been reported from the middle Eocene Green River Formation of Wyoming, where it was thought to occur on dry, well-drained slopes (Wodehouse 1933); the middle Eocene Claiborne Formation in western Kentucky, where it presumably grew in sandy xeric coastal habitats (Graham 1999); and in the late Eocene Florissant, Colorado flora, where it may have occurred on dry ridges supporting a semiarid vegetation (Leopold and Clay-Poole 2001). *Ephedra* is also present in the Tertiary of Australia, where it is now extinct (Cookson 1956). The Paleocene-Eocene period (ca. 60 MYA) might thus indicate the time when crown group *Ephedra* originated.

Proposed divergence times of the crown group Gnetales based on estimates derived from analyses of DNA sequence data range widely from about 80 to 218 MYA (Sanderson and Doyle 2001; Ickert-Bond and Wojciechowski 2002; Sanderson 2002; Soltis et al. 2002). This variation in estimated divergence times is due in part to the analysis of different genes, different partitions of the same gene, or different assumptions on seed plant topology. Using a molecular clock-based analysis of *rbcL* data, Huang and Price (2003) estimated an age for crown group *Ephedra* ranging from 8 to 32 MYA. This date is considerably younger than the well-established pollen record of *Ephedra* that begins in the Paleocene (Graham 1999).

Results from the analyses presented here appear to support the idea of an origin and early diversification of *Ephedra* in the Old World with a subsequent diversification to the New World (Figs. 1–5). This clearly refutes the second hypothesis of Pachomova (1969) for a South American origin of *Ephedra*. Our results also suggest diversification of the New World clade with derivation of the South American taxa from those of North America, but the relationships are not adequately resolved to answer this definitively.

The close relationship among the four New World clades is supported by high bootstrap values (Fig. 1). Similar close relationships of taxa occurring in deserts of North America and South America can be found in the genera *Larrea* Cav. (Cortes and Hunziker 1997; Lia et al. 2001), *Prosopis* L. (Thorne 1986), and *Fagonia* Tourn. ex L. (Porter 1974). One explanation for this recurring pattern is that these plant taxa may be of recent origin and their distributions are the result of long-distance dispersal (Axelrod 1979; Graham 1999). After North and South America separated in the Cretaceous, a large marine barrier extended across much of Central America until ca. 3.6 MYA, and it was not until ca. 2.4 MYA that land connections (the Panama land bridge) existed that would allow exchange of large terrestrial animals and plants lacking means for long-distance dispersal (Graham 1992; Burnham and

Graham 1999). It has generally been hypothesized that herbaceous genera evolved in North America and were dispersed south to temperate South America relatively recently (Raven 1963; Porter 1974; Thorne 1986, Wen et al. 2002), while woody species are considered to have a South American ancestry (Thorne 1973, 1986). Results from analyses of molecular sequence data presented here (Figs. 1–5) argue that the migration in New World *Ephedra* was not from South America to North America (Figs. 1–5), but instead proceeded from North America, including Mexico, to South America. Relationships of the four lines in the New World clade are unresolved in the strict consensus, however *Ephedra peduncula*ta, a species mainly Mexican in distribution, groups with the North American clade in some of our most parsimonious trees, this topology either shows *E. compacta* (strictly Mexican in distribution) as sister to the core North American clade, while in other most parsimonious trees these two taxa remain unresolved. This is evidence against the hypothesis that the first New World species of *Ephedra* split into a North American and a South American clade by an initial vicariance event.

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Appendix 1

List of taxa, and their voucher information for nrDNA ITS1 and plastid *rps4* sequences. Letters in parentheses refer to different accessions used in the molecular analysis. All vouchers are deposited in the ASU herbarium unless otherwise noted in parentheses, acronyms follow Index Herbariorum (Holmgren et al. 1990). c = cultivated. Infrageneric groups follow Stapf (1889), Mussayev (1978) and Freitag and Maier-Stolte (1994).

Sect. ***Alatae***, subsect. ***Alatae*** (Stapf) Mussayev ***E. przewalskii*** (Stapf) Andrz. Mongolia, Gobi Altai, *Hurka & Neuffer 12228* (KAS); *rps4* AY591484, no ITS1. Pakistan, *Eberhardt 98–743* (KAS); *rps4* AY591483, no ITS1. ***E. strobilacea*** Bunge Iran, *Rechinger 27161* (US); *rps4* AY591448, ITS1 AY599162.

Sect. ***Alatae***, subsect. ***Trifurcae*** Mussayev ***E. boelkei*** F. A. Roig Argentina, Mendoza, *Ickert-Bond 1252*; *rps4* AY591473, ITS1 AY599175. ***E. funerea*** Coville and C. V. Morton (1) U.S.A., Arizona,

Ickert-Bond 473; *rps4* AY591454, ITS1 AY599168. (2) U.S.A., California, *Ickert-Bond 964*; no *rps4,* ITS1 AY599170. ***E. multiflora*** Phil. ex Stapf Chile, Atacama Desert, *Ickert-Bond 1211*; *rps4* AY591471, ITS1 AY599173. ***E. torreyana*** S. Watson (1) U.S.A., Arizona, *Ickert-Bond 941*; no *rps4,* ITS1 AY599155. (2) U.S.A., Arizona, *Crimmins s.n.*; no *rps4,* ITS1 AY599166. ***E. torreyana*** S. Watson var. ***powelliorum*** T. Wendt U.S.A., Texas, Big Bend, *Ickert-Bond 1126*; *rps4* AY591453, ITS1 AY599147. ***E. trifurca*** Torr. ex S. Watson U.S.A., Arizona, *Ickert-Bond 753*; no *rps4,* ITS1 AY599164.

Sect. ***Ephedra*** subsect. ***Americanae*** Mussayev ***E. americana*** Humb. & Bonpl. ex Willd. (1) Ecuador, Bolivar, *Ickert-Bond 1105*; *rps4* AY591464, ITS1 AY599143. (2) Argentina, Salta, *Ickert-Bond 1219*; no *rps4,* ITS1 AY599178. ***E. breana*** Phil. Chile, El Loa, *Ickert-Bond 1234*; *rps4* AY591472. ***E. chilensis*** Miers Chile, Valparaiso, *Ickert-Bond 1240*; *rps4* AY591470, ITS1 AY599142. ***E. compacta*** Rose (1) Mexico, Nuevo León, *Puente 2238*; no *rps4,* ITS1 AY599163. (2) Mexico, Puebla-Veracruz, *Puente 2243B*; no *rps4,* ITS1 AY599161. (3) Mexico, San Luis Potosi, *Puente 1901*; *rps4* AY591474, ITS1 AY599157. ***E. coryi*** E. L. Reed (1) U.S.A., Texas, *Ickert-Bond 952*; *rps4* AY591461, ITS1 AY599153. (2) U.S.A., Texas, *Ickert-Bond 953*; no *rps4,* ITS1 AY599151. ***E. frustillata*** Miers Chile, Magallanes, *Ickert-Bond 1247*; *rps4* AY591462, ITS1 AY599174. ***E. gracilis*** Phil. Chile, Valparaiso, *Ickert-Bond 1201*; *rps4* AY591465, ITS1 AY599150. ***E. ochreata*** Miers Argentina, Mendoza, *B 380819* (B); *rps4* AY591463, ITS1 AY599176. ***E. pedunculata*** Engelm. ex S. Watson U.S.A., Texas, *Ickert-Bond 920*; *rps4* AY591460, ITS1 AY599144. ***E. rupestris*** Benth. (1) Ecuador, Cotopaxi, *Ickert-Bond 1100*; *rps4* AY591467, ITS1 AY599167. (2) Argentina, Salta, *Ickert- Bond1220*; *rps4* AY591466, ITS1 AY599171. ***E. triandra*** Tul. emend. J. H. Hunz. Argentina, Salta, *Ickert-Bond 1227*; *rps4* AY591468, ITS1 AY599165. ***E. tweediana*** Fisch and C.A. Mey. emend J. H. Hunz. Argentina, Salta, *Ickert-Bond 1225*; *rps4* AY591469, ITS1 AY599159

Sect. ***Ephedra*** subsect. ***Antisyphilticae*** Mussayev ***E. antisyphilitica*** S. Watson U.S.A., Texas, *Puente 2314*; no *rps4,* ITS1 AY599152. U.S.A., Texas, *Ickert-Bond 900*; *rps4* AY591452, ITS1 AY599148.

Group ***Distachyae,*** subgroup ***Distachyae*** s. str. Freitag and Maier-Stolte ***E. distachya*** L. (1) Syria, NE of Damascus, *Freitag 30144* (KAS); *rps4* AY591441, ITS1 AY599133. (2) Rumania, Bucarest, *Diaconescu 30205* (B); *rps4* AY591481, no ITS1. (3) S Kazakhstan, coast of Aral lake, *Wucherer 453* (KAS); no *rps4,* ITS1 AY599135. (4) Turkey, Ronya, *Freitag 28772* (KAS); no *rps4,* ITS1 AY599134. ***E. distachya*** L. subsp. ***helvetica*** (C.A. Mey.) Ascherson & Graebner (1) Switzerland, *Freitag 20332* (KAS); no *rps4,* ITS1 AY599136. (2) Switzerland, *B37921* (B); *rps4* AY591480, no ITS1. ***E. fedtschenkoae*** Pauls. Arrowhead Alpines Nursery (c), *Ickert-Bond s.n.*; *rps4* AY591442, ITS1 AY59915. ***E. intermedia*** C. A. Mey. Kazakhstan, Mosqua, *B 37886* (B); no *rps4,* ITS1 AY599179. ***E. regeliana*** Florin China, SW Xinjiang, *U. Wundisch 956* (KAS); *rps4* AY591449, ITS1 AY599160.

Group ***Distachyae,*** subgroup ***Leptocladae*** Freitag and Maier-Stolte ***E. equisetina*** Bunge Kazakhstan, *Freitag s.n.* (KAS); no *rps4,* no ITS1. ***E. gerardiana*** Wall. Pakistan, Kaghan Valley, *Miehe 4717* (KAS); *rps4* AY591482, no ITS1. ***E. major*** Host subsp. ***major*** Turkey, WSW Eregli, *Freitag & Adiguzel 28780* (KAS); *rps4* AY591489, ITS1. ***E. major*** Host subsp. ***procera*** (Fischer & C. A. Mey.) Bornm. (1) Turkey, Van-Gölö, *Freitag 2/02* (KAS); *rps4* AY591487, no ITS1. (2) Iran, Baghrot Valley, *G.& S. Miehe 4526* (KAS); *rps4* AY591488, no ITS1. ***E. monosperma*** C. A. Mey. (2) Mongolia, Gobi Altai, *Hurka & Neuffer 12182* (KAS); *rps4* AY591443, ITS1 AY599139. ***E. saxatilis*** (Stapf) Florin China, SE Tibet, *Miehe 95–18–11* (KAS); *rps4* AY591445, ITS1 AY599140. China, Xizang S Tibet, *Miehe 98–00505* (KAS); *rps4* AY591491, no ITS1. ***E. saxatilis*** (Stapf) Florin var. ***sikkimensis*** (Stapf) Florin China, Xizang, S Tibet, *Dichore 6535* (KAS); *rps4* AY591490, no ITS1. ***E. somalensis*** Freitag and Maier-Stolte Somalia, Sanaag Region, *Thulin 10944* (KAS); *rps4* AY591444, ITS1 AY599141.

Group ***Sarcocarpae*** Freitag and Maier-Stolte ***E. transitoria*** Assadi Jordan, Shaumari Wildlife Res., *Freitag 30123* (KAS); *rps4* AY591450, ITS1 AY599172. ***E. sarcocarpa*** Aitch. and Hemsl. Iran, Kavir desert near Mobarakiyeh, *Freitag 13966* (KAS); no *rps4,* ITS1 AY599137.

Group ***Fragilis*** Freitag and Maier-Stolte ***E. altissima*** Desf. var. ***algerica*** Stapf Montréal Botanical Garden (c), *JBM2830–41*; *rps4* AY591479, no ITS1. ***E. aphylla*** Forssk. (1) Jordan, Wadi Musa, *Freitag 30181* (KAS); *rps4* AY591438, ITS1 AY599128. (2) Italy, Sicily, near Manfredia, *Freitag F14a/ 01* (KAS); *rps4* AY591439, ITS1 AY599127. ***E. foeminea*** Forssk. Greece, Delphi, *Freitag 190801.* (KAS); *rps4* AY591478, ITS1 AY599131. ***E. fragilis*** Desf. (1) Italy, Sicily, *Freitag 180/01* (KAS); *rps4* AY591477, no ITS1. (2) Jordan, Wadi Musa, *Freitag 30180* (KAS); *rps4* AY591440, ITS1 AY599129. (3) Italy, Sicily, near Manfredia, *Freitag F14/ 01* (KAS); *rps4* AY591476, no ITS1. (4) Morocco, *Walters 675* (KAS); *rps4* AY591475, no ITS1. (5) SE Spain, *Freitag 27237* (KAS); no *rps4,* ITS1 AY599130. ***E. laristanica*** Assadi Iran, Fars, *Assadi & Sardabi 41781* (KAS); *rps4* AY591437, ITS1 AY599126.

Subsect. ***Pachycladae E. pachyclada*** Boiss. var. **sinaica** (Riedl) H. Freitag and M. Maier-Stolte Sinai, S Musa, *Freitag 19960* (KAS); no *rps4,* ITS1 AY599138. ***E. sinica*** Florin Mongolia, Gobi Altai, *Hurka & Neuffer 11937* (KAS); *rps4* AY591446, no ITS1. Companion plants (c), *Ickert-Bond s.n.* (ASU); *rps4* AY591486, no ITS1. ***E. sinica*** Florin var. ***pumila*** Mongolia, Hovd Aymag, *Hurka & Neuffer 10242* (KAS); *rps4* AY591447, ITS1 AY599169

Sect. ***Asarca E. aspera*** Engelm. ex S. Watson (2) U.S.A., Texas, *Ickert-Bond 1136a*; *rps4* AY591455, ITS1 AY599146. ***E. californica*** S. Watson U.S.A., California, *Ickert-Bond 968; rps4* AY591458, ITS1 AY599145. ***E. cutleri*** Peebles U.S.A., Arizona, *Ickert-Bond 1006*; *rps4* AY591456, ITS1 AY599156. ***E. fasciculata*** A. Nelson U.S.A., Arizona, *Ickert-Bond 513*; *rps4* AY591457, ITS1 AY599180. ***E. nevadensis*** S. Watson U.S.A., Nevada, *Ickert-Bond 959*; *rps4* AY591451, ITS1 AY599154. ***E. viridis*** Coville U.S.A., Arizona, *Ickert-Bond 941*; *rps4* AY591459, ITS1 AY599149.

No sectional affiliation ***E.*** sp. nov ''Bhutanica'' Bhutan, Tsochen Chen, *Miehe 00–124–02* (KAS); *rps4* AY591492, no ITS1

Gnetum L. ***G. cuspidatum*** Bl. Malaysia, *Won 551* (MO); *rps4* AY5914, no ITS1. ***G. gnemon*** L. Montréal Botanical Garden (c), *JBM2240–50*; *rps4* AY591428, no ITS1. ***G. gnemonoides*** Brongn. Malaysia, *Won 553* (MO); *rps4* AY591429, ITS1 AY624586. ***G. indicum*** Merrill Royal Botanical Garden Edinburgh (c), *E19550226* (E); *rps4* AY591434, ITS1 AY624585. ***G. leptostachyum*** Bl. Malaysia, *San 157112* (MO); *rps4* AY591435, no ITS1. ***G. leyboldii*** Tul. Costa Rica, La Selva, *Landrum s.n.*; *rps4* AY591432, no ITS1. ***G. lufoense*** C.Y.Cheng Singapore, *Won 600* (MO); *rps4* AY591436, ITS1 AY624584. ***G. montanum*** Mgf. Royal Botanical Garden Edinburgh (c), *E19791010* (E); *rps4* AY591433, no ITS1. ***G. parvifolium*** (Warb.) Cheng University of Tokyo Botanical Garden (c), *Kato s.n.*; *rps4* AY591431, no ITS1. ***G. ula*** Brogn. University of California Botanical Garden (c), *Ickert-Bond s.n.*; *rps4* AY591427, no ITS1.

Pinus thunbergii Parl. No locality or voucher; *rps4* D17510, ITS1 AF037025

Welwitschia mirabilis Hooker f. Hunt Botanical Garden (c), *Cranfill s.n.*; *rps4* AY188246, no ITS1. California State University Fullerton (c), no voucher; no *rps4,* ITS1 U50740.