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EVOLUTIONARY RELATIONSHIPS IN *EPHEDRA* (GNETALES), WITH IMPLICATIONS FOR SEED PLANT PHYLOGENY

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Evolutionary relationships in *Ephedra* are difficult to resolve, mainly because there are few informative characters in investigated loci and long distances to outgroups. We address these problems by using a large data set that includes information from seven plastid and nuclear loci and 204 vascular plants. The deepest divergences in *Ephedra* are weakly supported and differ by analytical method, but they indicate a basal grade of species distributed in the Mediterranean area. New World species are monophyletic, with a South American clade possibly nested within a North American clade. A mainly Asian clade comprises several well-supported subgroups, of which some are endemic to restricted geographic regions in East or Central Asia; others have a broad distribution that may extend into Europe (*E. distachya*, *E. major*) and/or Africa (*E. pachyclada*–*E. somalensis*). *Ephedra laristanica* and *E. somalensis* are nested within other species, whereas the recognition of *E. milleri* as a separate species is supported. Our results provide another example of how exceptionally difficult it is to disentangle the early divergences of seed plants. Bayesian analysis strongly supports the "gnetifer" hypothesis, a result rarely found in the literature, but it conflicts with our results from only chloroplast data ("gne-cup") and with results of most maximum parsimony analyses ("Gnetales sister").

Keywords: *Ephedra*, Gnetales, gnetifer hypothesis, seed plant phylogeny.

Online enhancement: appendix table.

Introduction

Ephedra L. (Gnetales) is a morphologically distinct group of seed plants with a long and diverse evolutionary history; ephedroid fossils are known from the Early Cretaceous of Asia, Europe, and North America (Krassilov 1982; Sun et al. 2001; Yang et al. 2005; Rydin et al. 2006*a*, 2006*b*) and perhaps also Australia (Krassilov et al. 1998) and South America (Mohr et al. 2004). Extant species diversity is limited to ~40 species distributed in arid, warm-temperate to subtropical environments of the Northern Hemisphere and South America (Kubitzki 1990).

The extant species of *Ephedra* are very similar in gross morphology, and attempts to find morphological support for subgeneric divisions and/or classifications (Stapf 1889; Steeves and Barghoorn 1959; Mussayev 1978; Freitag and Maier-Stolte 1994) have proved difficult and have resulted in different hypotheses. One reason may be that many characters—from, e.g., pollen, leaf, and cone morphology—not only differ among species but also show substantial intraspecific variation (Huang et al. 2005). For example, the number of bracts and seeds per cone are characters that vary between species in an obvious way; however, often enough these characters are also variable within species (see floral work on *Ephedra*). Similarly, Ickert-Bond et al. (2003) report pronounced pollen dimorphism in three New World species, and El-Ghazaly and Rowley (1997) find all four types of *Ephedra* pollen proposed in Steeves and Barghoorn's (1959) classification within a single microsporangium of *Ephedra foliata*. These (at least seemingly) confusing variation patterns may conceivably lead to problems with species delimitation, and there are a few indications of this, in two previous studies (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004), that deserve further investigation.

In groups where homology assessments and evolutionary significance of morphological traits have been considered confusing, analyses of DNA sequence data may provide important insights and a basis for a better understanding of the morphological evolution in the group. So far, this has, however, not been the case in *Ephedra*. Previous studies have shown that the phylogenetic information in all investigated loci is surprisingly low (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004; Huang et al. 2005; Wang et al. 2005), a finding that is usually explained by hypotheses of a very recent origin of extant species diversity (Huang and Price 2003; Renner 2005). Although previous studies provided new information on the phylogeny of *Ephedra*, species representation was limited, especially among Old World species, and groups were often poorly supported.

Robust phylogenies are an important basis for further studies of biology and biogeography. A restricted knowledge of the phylogeny and morphology of living *Ephedra* is, for

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example, a major reason why fossils have been difficult to place within the ephedroid lineage (Rydin et al. 2004, 2006*a*, 2006*b*). Better phylogenetic resolution among living and fossil species of *Ephedra* would be important for further studies on divergence times, biogeography, and morphological evolution in a historical perspective and may subsequently also provide clues to phylogeny and evolution in the seed plant clade. We therefore make further attempts to resolve relationships among extant species of *Ephedra*.

We address the phylogeny of *Ephedra*, with special emphasis on Old World species, using a large data set comprising data from seven loci and 104 ingroup terminals. Specific aims are to test classification schemes in *Ephedra* based on morphology, to test the generally poorly supported phylogenetic results of previous molecular studies, to test the monophyly of species, and to resolve the systematic position of species not included in previous studies.

A distant relationship between *Ephedra* and other seed plants is supported by molecular data (Magallón and Sanderson 2005), by the pronounced differences between the extant genera of the Gnetales in morphology, ecology, and distribution (Arber and Parkin 1908), and by the diverse fossil record (see Rydin et al. 2006*a*, 2006*b* for a summary), which indicate that extant species of the Gnetales are a small remnant of former diversity. To address the problem of the long distance between *Ephedra* and outgroups, we have included a large sample of taxa from the remaining clades of vascular plants.

Material and Methods

Taxon Sampling and Laboratory Procedures

We selected 104 ingroup terminals for this study (table A1 in the online edition of the *International Journal of Plant Sciences*): 74 terminals representing Old World species of *Ephedra* and 30 from the New World. Care was taken to include, where possible, a broad sample of recognized subgeneric groups and geographical diversity, and many *Ephedra* species are represented by several individuals. We further selected 100 outgroup species from the remaining major clades of vascular plants (lycopods, ferns, cycads, *Ginkgo*, conifers, and angiosperms). The trees were rooted on *Huperzia selago*, which belongs to the sister clade of all other vascular plants, the lycopods (Qiu et al. 2007).

DNA sequence data were retrieved from seven loci: three regions from the nuclear ribosomal DNA (18S, 26S, and the nrITS region [nrITS1, 5.8S, and nrITS2]), and four chloroplast regions (the protein-coding genes *rbcL* and *rps4*, the *rpL16* intron, and the $trnS^{UGA}$-$trnfM^{CAU}$ intergenic spacer). A total of 222 sequences are new to this study. These were analyzed along with sequences retrieved from GenBank (GenBank accession numbers are given in table A1). DNA was extracted, amplified, and sequenced with standard procedures described previously (Anderberg et al. 2005). References to primers are given in table 1. Sequence fragments were assembled with the Staden (1996) package.

Alignment

Alignments were performed with Seaview 2.2 (Galtier et al. 1996), Se-Al v.2.0 (Rambaut 1996), and MacClade, version 4.07b13 (Maddison and Maddison 2005). For the *rpL16* and $trnS^{UGA}$-$trnfM^{CAU}$ regions, an initial ClustalW analysis was run with BioEdit (Hall 1999), and the output constituted the basis for further assessments by eye. Insertions or deletions (indels) were present in the alignments, and highly divergent regions resulting in problematic homology assessments were excluded from the analyses. Furthermore, it was not possible to trace homology between sequences from *Ephedra* and the outgroups for the nrITS, *rpL16*, and $trnS^{UGA}$-$trnfM^{CAU}$ regions, and these were scored as question marks for outgroup taxa. The potential phylogenetic information of the indels was not taken into consideration in the analyses (i.e., no "gap coding" was performed).

Phylogenetic Analyses

The single-gene data sets (18S, 26S, nrITS, *rbcL*, *rpL16*, *rps4*, $trnS^{UGA}$-$trnfM^{CAU}$), a combined data set including all seven regions, and separate data sets including the nuclear and chloroplast regions were analyzed with a Bayesian Markov chain Monte Carlo (B/MCMC) approach and equally weighted maximum parsimony (MP). The B/MCMC analyses were performed with the parallel version of MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and the MP analyses with PAUP*, version 4.0b10 (Swofford 2002). All analyses were performed on the Duke University Shared Cluster Resource (Durham, NC).

Bayesian (B/MCMC) analyses. The Perl script MrAIC, version 1.4 (Nylander 2004), in combination with PHYML, version 2.4.4 (Guindon and Gascuel 2003), was used to choose nucleotide substitution models for each of the seven regions studied and for the data sets with nuclear and chloroplast regions. The choice of model was based on the corrected Akaike Information Criterion; see table 2 for a summary of models used). Different settings for the B/MCMC runs were needed for the data sets to reach convergence (table 2).

The values sampled for different parameters were examined with Tracer v1.4 (Rambaut and Drummond 2007) and AWTY (Wilgenbusch et al. 2004; Nylander et al. 2008) to determine whether the parameters had converged. We also examined the standard deviation of the split frequencies among the independent runs, as calculated by MrBayes. For each analysis, every thousandth tree was sampled, and after analysis of the parameter values, the initial trees were discarded as "burn-in" (table 2). Trees from each of the independent analyses (except those discarded as burn-in) were pooled before calculation of a majority-rule consensus tree for each region.

MP analyses. The MP analyses for each data set included a bootstrap analysis with 5000 replicates, each with 10 random-sequence-addition replicates, and with multrees off, heuristic search, and TBR branch swapping.

Combinability of data sets. The resultant consensus topologies from each of the seven single-region analyses were examined for potential conflicts. Comparisons were made among analytical methods and among data sets. First, topologies based on the same single-region data set but analyzed with different methods were compared (e.g., the B/MCMC and MP topologies of the *rbcL* data set were compared). Second, the topologies resulting from different data sets were

Table 1

Primers

DNA locus (primer name)	Primer sequence 5′-3′	Reference
rbcL forward (rbcL 5′)	ATG TCA CCA CAA ACA GAG AC	Zurawski and Clegg 1987
rbcL reversed (rbcL 3′)	TCA AAT TCA AAC TTG ATT TCT TTC CA	Wikström and Kenrick 1997
rps4 forward (rps4Fb)	CGA TCT TCT CGA CCC TGG TGG	Rydin et al. 2004
rps4 reversed (rps4Rb)	CCG TCG AGA ATA ATA TTC TAT	Rydin et al. 2004
18S forward (18S1)	GCT TGT CTC AAA GAT TAA GCC	Rydin et al. 2004
18S reversed (18Srev)	CCT TCC TCT AAA CGA TAA GGT TC	Rydin et al. 2004
26S forward (26S1)	CGA CCC CAG GTC AGG CG	Kuzoff et al. 1998
26S reversed (1229R)	ACT TCC ATG ACC ACC GTC CT	Kuzoff et al. 1998
ITS forward (ITS-18SF)	GAA CCT TAT CGT TTA GAG GAA GG	Rydin et al. 2004
ITS reversed (ITS-26SR)	CCG CCA GAT TTT CAC GCT GGG C	Rydin et al. 2004
rpL16 forward (F71)	GCT ATG CTT AGT GTG TGA CTC GTT G	Small et al. 1998
rpL16 reversed (R1516)	CCC TTC ATT CTT CCT CTA TGT TG	Small et al. 1998
$trnS^{UGA}$ (forward)	GAG AGA GAG GGA TTC GAA CC	Demesure et al. 1995
$trnfM^{CAU}$ (reversed)	CAT AAC CTT GAG GTC ACG GG	Demesure et al. 1995

compared. For each analytical method, all topologies from the seven data sets were compared (i.e., B/MCMC topologies were compared with each other, and MP topologies were compared with MP topologies). Comparisons were also made between the nuclear and chloroplast data sets. The data sets were subsequently combined into a single data set. For some taxa, sequences were not present from all regions (table A1). In the combined data set, these sequences were treated as missing data.

Analyses of the combined data set. When B/MCMC analyses were run on the combined data set using seven partitions, one for each of the DNA regions, some of the B/MCMC parameters failed to converge. Changing settings, such as increasing the number of generations and chains or lowering the temperature parameter, improved the convergence statistics, but these were still not optimal. We therefore simplified the B/MCMC analyses by decreasing the number of partitions to two, one for the nuclear regions (18S, 26S, and nrITS) and one for the chloroplast regions (*rbcL*, *rpl16*, *rps4*, and $trnS^{UGA}$-$trnfM^{CAU}$). Each partition was assigned the same model used in the separate B/MCMC analysis (table 2).

Results

The number of taxa and characters included in the analyses and tree statistics for the MP analyses are summarized in table 2. Convergence of runs was sometimes a problem and was carefully investigated with Tracer v1.4 (Rambaut and Drummond 2007), AWTY (Wilgenbusch et al. 2004; Nylander et al. 2008), and diagnostics available in MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Independent chains that did not converge were excluded from consensus calculations (table 2). The "poorly converging" seven-partition analysis was not used; we present results from the "better-converging" two-partition analysis, but no conflicts were found between the topologies resulting from these two independent analyses. The result of the analyses of the two-partition combined data set is shown in figures 1 and 2 (fig. 1 shows relationships among *Ephedra* and the outgroups and fig. 2 relationships within the ingroup).

The Combined Data Set: Ingroup Relationships and Conflicts between Analytical Methods

Ephedra is strongly supported as monophyletic (B/MCMC posterior probability [PP] 1.00/MP bootstrap support [BS] 99%). In the B/MCMC analysis, *Ephedra foeminea* is separated from the remaining species of *Ephedra* (0.69/-), which constitute a trichotomy comprising *E. alata*; a clade consisting of *E. fragilis*, *E. altissima*, *E. major* (specimens 88, 98), and *E. aphylla* (0.66/-); and the remaining species of *Ephedra* (0.73/-; fig. 2). The four specimens of *E. foeminea* are collapsed into two clades in the B/MCMC analysis but are monophyletic in the MP analysis (-/97; not shown). *Ephedra fragilis* is polyphyletic: two species from Morocco group with *E. altissima* (1.00/72), whereas *E. fragilis* from Jordan and Europe group with *E. aphylla* and *E. major* (specimens 88, 98; 0.99/92). The next divergence shows *E. milleri* as sister to the remaining species (the core *Ephedra*, 0.85/-), followed by *E. foliata*, with *E. laristanica* and *E. ciliata* nested within it (1.00/75).

These results partly differ from those of the MP analysis, in which *E. foemina*, *E. alata*, *E. fragilis*, *E. altissima*, *E. major* (specimens 88, 98), *E. aphylla*, and *E. milleri* form a clade (-/76). Relationships within this clade are largely collapsed, but resolved results (e.g., polyphyly of *E. fragilis*) do not differ from those of the B/MCMC analysis. "Core *Ephedra*" is defined as all other species of *Ephedra* (*E. foliata* to *E. distachya* in fig. 2).

The remaining species of *Ephedra* comprise two large sister clades (0.86/-), the New World clade (0.97/81) and the mainly Asian clade (0.91/-). No conflicts between B/MCMC and MP analyses are found among these species, but the MP analysis is less well resolved. Within the New World clade, *E. pedunculata* is sister to the remaining species (0.62/-), which constitute a South American clade (0.98/96) and a North American clade (0.63/-). The Mexican species *E. compacta* is sister to remaining North American taxa (0.99/80).

Ephedra minuta and *E. likiangensis* (0.98/91) are sister to the remaining species in the mainly Asian clade (0.98/-), which comprises two sister clades. One consists of *E. pachyclada* to *E. equisetina* (fig. 2; 0.99/83) and the other of *E.*

Table 2

Description of Data and Analyses

	Combined data set	Nuclear data set	Plastid data set	18S	26S	nrITS	*rbcL*	*rps4*	*rpL16*	*trnS*^{UGA}-*trnfM*^{CAU}
Total taxa	204	199	201	144	136	103	167	160	62	59
Ingroup taxa	104	104	101	64	61	103	67	91	62	59
New sequences	222	60	162	19	19	22	15	15	67[a]	65[a]
Total characters	8077	4589	3488	1715	1257	1617	1344	607	700	837
Excluded characters[b]	306	102	204	5	88	9	0	167	27	10
Number of variable characters	2716	1631	1085	672	613	346	715	321	20	29
Number (%) of parsimony-informative characters including outgroup	1897 (24.4)	1037 (23.1)	860 (26.2)	408 (23.9)	402 (34.4)	NA	563 (41.9)	270 (61.4)	NA	NA
Number (%) parsimony-informative characters within *Ephedra*	322 (4.1)	268 (6.0)	54 (1.6)	11 (.6)	31 (2.7)	226 (14.1)	22 (1.6)	6 (1.4)	8 (1.2)	19 (2.3)
Best-fitting evolutionary model AICc weights	GTRIG + GTRIG[c]	GTRIG	GTRIG	GTRIG	GTRIG	GTRG	GTRIG	K2PG	F81	GTRG
Supported conflicts between Bayesian and parsimony results	Yes[d]	No	Yes[e]	No	No	No	Yes[e]	No	No	No
Bayesian analyses:										
Generations (10^6)	8	8	8	8	8	12	8	5	5	5
Runs	4	4	4	4	4[f]	4[f]	4	2	2	4[f]
Chains	8	8	8	8	8	8	8	8	8	8
Temp[g]	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
Burn-in (10^6)	6	6	6	6	6	6	6	3	3	3

Note. AICc = corrected Akaike Information Criterion; NA = not applicable.

[a] Sequences from the outgroup (*Gnetum* and *Welwitschia*) were not used in the final analyses but have been submitted to GenBank.

[b] Characters excluded because of problematic homology assessments.

[c] Analysis based on two partitions, one including the nuclear data and one the plastid data.

[d] Conflicts concern seed plant relationships and relationships within *Ephedra*.

[e] Conflicts concern seed plant relationships.

[f] One of the runs did not converge; trees from the other three runs were pooled.

[g] Temperature setting in MrBayes (Huelsenbeck and Ronquist 2001).

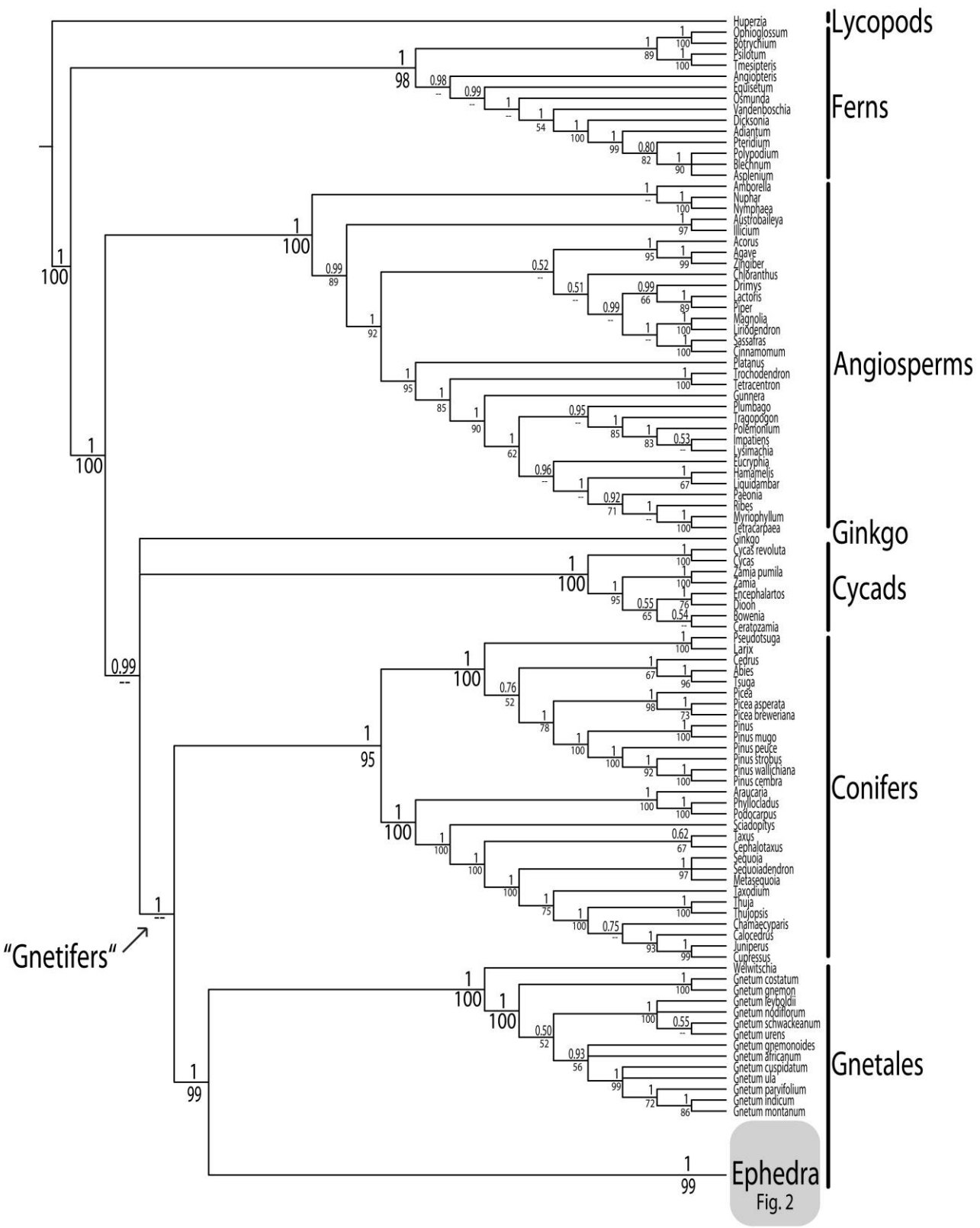

Fig. 1 Relationships among vascular plants: 50% majority-rule consensus tree from Bayesian analysis of data from seven loci (nuclear ribosomal 18S, 26S, and the nrITS region [nrITS1, 5.8S, and nrITS2]; chloroplast *rbcL* and *rps4* genes; *rpL16* intron; and $trnS^{UGA}$-$trnfM^{CAU}$ intergenic spacer). Posterior probabilities of clades are indicated above branches; bootstrap indices (under maximum parsimony) are mapped below branches.

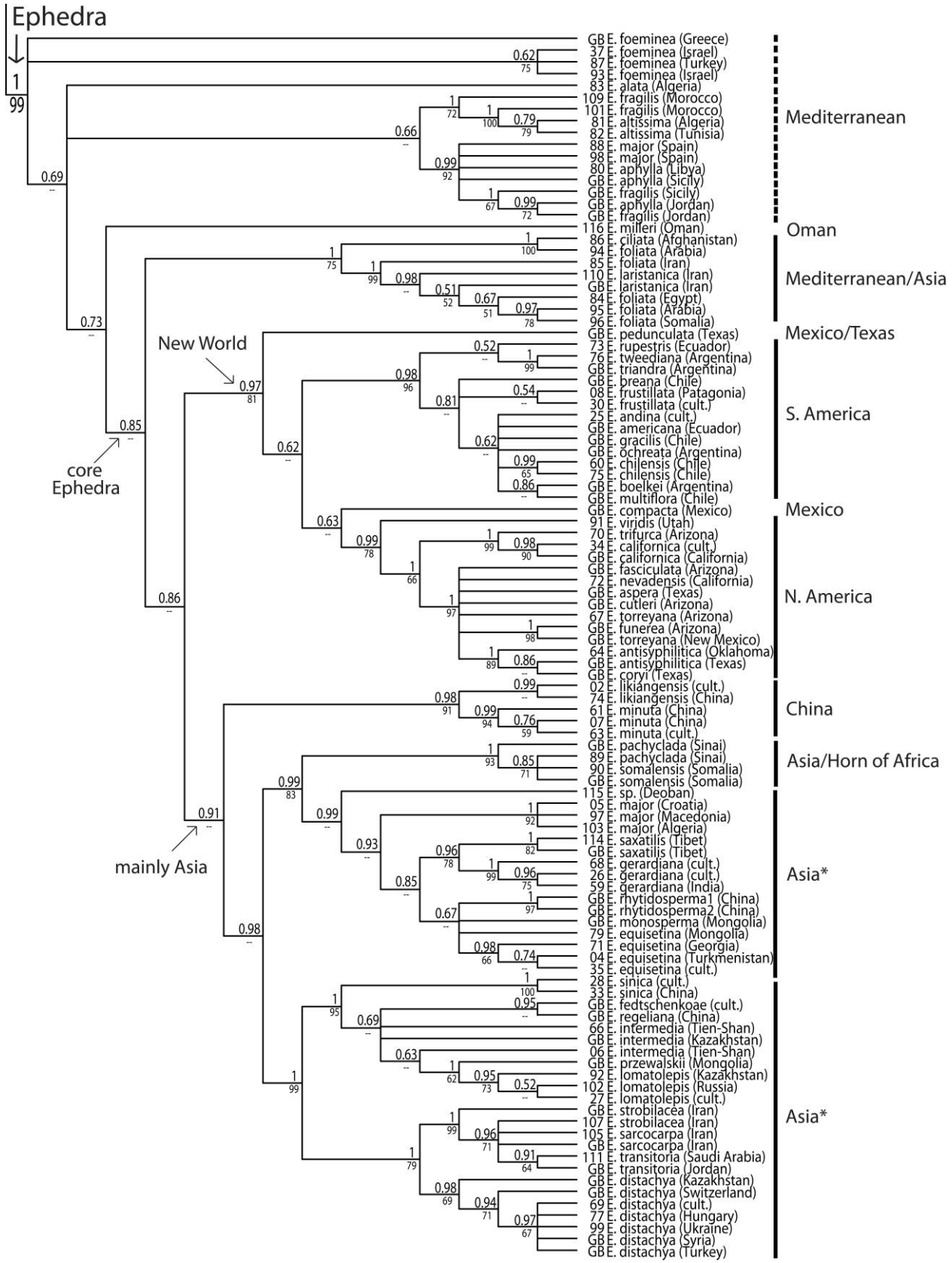

Fig. 2 Relationships within *Ephedra*: 50% majority-rule consensus tree from Bayesian analysis of data from seven loci (nuclear ribosomal 18S, 26S, and the nrITS region [nrITS1, 5.8S, and nrITS2]; chloroplast *rbcL* and *rps4* genes; *rpL16* intron; and $trnS^{UGA}$-$trnfM^{CAU}$ intergenic

sinica to *E. distachya* (fig. 2; 1.00/99). Relationships within the Asian subclades are generally well resolved, well to moderately supported (fig. 2), and consistent between analytical methods. Results in the New World clade are less well resolved (fig. 2) but are consistent between methods.

The Combined Data Set: Outgroup Relationships and Conflicts between Analytical Methods

Ephedra is consistently and with high support (1.00/99) retrieved as sister to a clade including the two other gnetalean genera, *Gnetum* and *Welwitschia* (1.00/100; fig. 1). The monophyly of the Gnetales is also well supported (1.00/99), but their relationship to other seed plants is unclear and differs by analytical method and the data set analyzed. In the combined analyses, Gnetales are either sister to conifers (B/MCMC analysis: 1.00 PP; fig. 1) or sister to all other seed plants (MP analysis: 89% BS; not shown). The monophyly of conifers is well supported in the combined analyses (1.00/95). Angiosperms (1.00/100) are sister to gymnosperms (0.99/-) in the B/MCMC analysis (fig. 1) and sister (-/0.88) to a *Ginkgo*-cycads-conifer clade (-/93) in the MP analysis (not shown). The position of *Amborella* differs: *Amborella* is sister to the Nymphaeales in the B/MCMC analysis (1.00/-) but sister to all other angiosperms (-/89) in the MP analysis. The position of *Ginkgo* is unresolved within gymnosperms (0.99/-) in the B/MCMC analysis and unresolved within a *Ginkgo*-cycads-conifer clade (-/93) in the MP analysis. Monilophyta sensu Cantino et al. (2007; Ophioglossales, Psilotaceae, leptosporangiate ferns, *Angiopteris*, and *Equisetum*) constitute a monophyletic group (1.00/98) sister to seed plants (1.00/100; fig. 1).

The Single-Genome Data Sets: Relationships within Ephedra *and Topological Conflicts*

We report incongruences between the combined topologies and those obtained from single-genome analyses here (chloroplast data and nuclear data). Single-gene matrices contained very little information on ingroup relationships, and trees resulting from analyses of the separate gene regions were consequently largely unresolved within *Ephedra*. Single-gene results are therefore not further reported or discussed.

Chloroplast data. There are no deviations between results from the combined Bayesian analysis and those obtained from the Bayesian analysis of the subset including only chloroplast data. In the MP analysis of chloroplast data, many nodes are collapsed, but two incongruences are found among the Asian taxa: *E. minuta*, *E. gerardiana*, and *E. saxatilis* (specimen 114) form a clade (-/75), and *E. likiangensis* groups with *E. equisetina* and *E. rhytidosperma* (-/54).

Nuclear data. In the Bayesian analyses of the subset including only nuclear data, *E. minuta* and *E. likiangensis* form a clade with *E. foliata* (including *E. laristanica*; 0.82/-), and this clade is sister to the clade comprising *E. sinica* to *E. distachya* (fig. 2). *Ephedra ciliata* is unresolved within a clade that also comprises the clade *E. pachyclada* to *E. equisetina* (fig. 2) and New World species (0.84/-). *Ephedra compacta* and *E. pedunculata* group with South American species (0.56/-). There are no deviations between results from the combined MP analysis and those obtained from the MP analysis of the subset including only nuclear data.

The Single-Genome Data Sets: Outgroup Relationships and Topological Conflicts

Generally, single-gene and single-genome analyses (not shown) produce the same topologies as those described above, but partly collapsed. There is one exception, however; there are pronounced conflicts regarding seed plant phylogeny, and we discuss below incongruences between results from the combined analyses and those obtained from the chloroplast and nuclear data sets. Results from single-gene analyses also differed, showing Gnetales as sister to other seed plants (*rbcL* MP; *rps4* B/MCMC and MP), Gnetales as sister to Pinaceae (*rbcL* B/MCMC), Gnetales as sister to conifers (18S B/MCMC and MP), or unresolved relationships among the major groups (26S B/MCMC and MP). Results from single-gene analyses have generally been discussed in previous studies and are not further commented on here.

Chloroplast data. The B/MCMC analysis of the subset including only chloroplast data results in a sister relationship between Gnetales and Cupressophyta sensu Cantino et al. (2007; i.e., Cupressaceae, "Taxodiaceae," Cephalotaxaceae, Taxaceae, Sciadopityaceae, Araucariaceae, and Podocarpaceae; 0.98/-). In the MP analysis of chloroplast data, Gnetales are sister to all other seed plants (-/96), and conifers are monophyletic (-/92).

Nuclear data. The subset including only nuclear data results in a Gnetales-conifer ("gnetifer") clade in B/MCMC as well as MP analyses (1.00/61).

Discussion

This study provides new information on phylogenetic relationships in *Ephedra* and the monophyly of species, both in terms of number of species/specimens investigated and in statistical support for the topology. There are still unresolved questions, however, mainly concerning the deepest divergences in *Ephedra* and among New World species.

Deep Divergences in Ephedra

In the Bayesian analysis, *Ephedra foeminea* is found in an unresolved trichotomy at the base of *Ephedra*. However, the result is weakly supported and incongruent with our parsimony analysis and with Bayesian results in Rydin et al. (2004), where *E. foeminea* is part of a moderately supported

spacer). Posterior probabilities of clades are indicated above branches; bootstrap indices (under maximum parsimony) are mapped below branches. Collection sites of specimens are indicated in parentheses. The asterisk after "Asia" indicates that a few species in the Asian clade have a broad distribution area that extends outside of Asia.

clade of Mediterranean species. In Rydin et al. (2004), this clade was sister to all other *Ephedra* species. Further, both these results conflict with those obtained by Ickert-Bond and Wojciechowski (2004). Because homology assessments between *Ephedra* and the *Gnetum-Welwitschia* clade are impossible for nrITS data, Ickert-Bond and Wojciechowski (2004) did not include outgroup taxa in their study, but likelihood ratio analyses of nrDNA ITS1, as well as midpoint rooting, resulted in a placement of the root between *E. laristanica* and all remaining taxa (Ickert-Bond and Wojciechowski 2004). In our study, *E. laristanica* is nested within *E. foliata*, with high support in Bayesian as well as MP analyses. These differences are, however, caused only by the different rooting approaches employed. To address the problem of the long distance between *Ephedra* and outgroups (Magallón and Sanderson 2005), we included a large sampling of outgroup taxa, and our results support the position of *E. foeminea* in the most basal divergence within the genus. When we reroot the tree obtained by Ickert-Bond and Wojciechowski (2004) on *E. foeminea*, our results are largely congruent.

Ephedra alata is adapted to extremely arid conditions (Freitag and Maier-Stolte 1994) and is one of few Old World species with dry cone bracts at seed maturity. The three Old World species with this morphology (*E. alata*, *E. strobilacea*, and *E. przewalskii*) are here shown to be only distantly related, and the feature is, in addition, present in the New World clade. Dry cone bracts have thus evolved separately in several lineages, likely as an ecological adaptation to desert conditions, in which animal dispersal of seeds might be less productive. Preliminary results (C. Rydin and P. K. Endress, unpublished observations) indicate that reevaluation of pioneer work, e.g., that by Stapf (1889), in the light of our current phylogenetic hypothesis of the group might be worthwhile.

Ephedra fragilis, *E. altissima*, *E. aphylla*, and *E. major* (specimens 88, 98) form a poorly supported monophyletic group, within which species delimitations are unclear (further discussed below). These species have their distribution in the Mediterranean area—in southern Europe and/or the Near East—and (like *E. foeminea* and *E. alata*) they are all present in northern Africa. An origin of crown group *Ephedra* in the Mediterranean area has previously been hypothesized by Mussayev (1978), and the B/MCMC topology presented here lends weak support to that theory. However, further studies are needed to elucidate relationships and species delimitations in the basal assemblage of species.

Ephedra milleri, a recently described species (Freitag and Maier-Stolte 1992), has to our knowledge not been included in any previous cladistic study. *Ephedra milleri* is sister to core *Ephedra* (fig. 2) in the Bayesian analysis. In the MP analysis, *E. milleri* is poorly supported as sister to the remaining species in the Mediterranean clade. According to the original description, *E. milleri* has an overall habit similar to that of *E. pachyclada*, but because of its papillose to ciliate margin and its pollen characters, Freitag and Maier-Stolte (1992) considered it a new species more closely related to *E. fragilis* and *E. aphylla*. Our results support the distinction between *E. pachyclada* and *E. milleri* and indicate that the similarities between the latter and *E. fragilis* and *E. aphylla* may be plesiomorphic.

Relationships in Core Ephedra

Within core *Ephedra* (fig. 2), relationships are better supported and are consistent between analytical methods (although partly collapsed in the MP analysis). There are a few incongruences between results from the combined analyses (fig. 2) and those obtained from subsets of data (chloroplast data vs. nuclear data; see "Results"), but they are supported by a PP of <0.85. A clade comprising *E. minuta*, *E. gerardiana*, and *E. saxatilis* (specimen 114) was moderately supported in the MP analysis of chloroplast data. This information, which mainly comes from the $trnS^{UGA}$-$trnfM^{CAU}$ intergenic spacer, is not congruent with results from other molecular markers.

Ephedra foliata (including *E. laristanica* and *E. ciliata*; see below) is sister to the remaining species in core *Ephedra*. *Ephedra foliata* and *E. milleri* sometimes have leaves, ovules, and cone bracts in whorls of three (Freitag and Maier-Stolte 1992), whereas the species of the basal assemblage have one or two ovules in each cone and decussate arrangement of leaves and cone bracts. While *E. milleri* has a very restricted distribution (it is known only from Oman in the Near East), *E. foliata* has a broad distribution ranging from Morocco to India but is not present north of the Mediterranean Sea.

The sister group of *E. foliata* comprises two major subclades: New World species and the mainly Asian clade. In the former, *E. pedunculata*, a species restricted to Texas and northern Mexico (Stevenson 1993), is sister to the remaining New World species. The result is poorly supported but was also indicated (nonsupported) in Ickert-Bond and Wojciechowski (2004). The utilized sequences of *E. pedunculata* were produced from a plant collected in Texas (Ickert-Bond and Wojciechowski 2004), but an unpublished nrITS sequence from a Mexican specimen supports the monophyly of *E. pedunculata* and its sister relationship to the remaining New World species. North American species are thus not monophyletic but comprise two clades. Another Mexican species, *E. compacta*, is sister to the remaining species in the larger North American clade (fig. 2). Relationships among New World taxa, outlined in Ickert-Bond and Wojciechowski (2004), are confirmed here. However, many nodes are unresolved or poorly supported in our study, as well as in Ickert-Bond and Wojciechowski (2004), and future studies should add more data in order to further test the monophyly of species and their interrelationships.

In previous studies, Asian species were resolved as several separate clades (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004; Huang et al. 2005). Here, with an expanded taxon sampling, the species present in Central and eastern Asia (except *E. foliata*) form a clade that is well supported in the Bayesian analysis. Two species with restricted distribution in central China (*E. minuta* and *E. likiangensis*; Fu et al. 1999) are sister to the remaining species, which in turn are divided into two large clades (fig. 2). The latter two clades are well resolved, often also at the species level. Some species in the Asian clade have a restricted distribution (e.g., *E. minuta*, *E. likiangensis*, *E. rhytidosperma*, and *E. sinica*); other species have a broader distribution in Asia (e.g., *E. equisetina* and *E. intermedia*), and a few species have a very large distribution area, ranging from the central to eastern parts of Asia to Western Europe (e.g., *E. distachya* and *E. major*). Al-

though weakly supported, our results indicate that the mainly Asian clade has its origin in Asia and that dispersals westward into Africa and Europe are more recent events.

Ephedra rhytidosperma is a Chinese species with a restricted distribution; it is endemic to Helan Mountain between Nei Mongol and Ningxia (Fu et al. 1999; Yang 2007). Its seeds have tiny but dense transverse laminar protuberances on the surface of the seed envelope (Yang 2007), and the structure has been compared to that preserved in an Early Cretaceous fossil, *E. archaeorhytidosperma* (Yang et al. 2005). To get a first indication of the phylogenetic position of *E. rhytidosperma*, Wang et al. (2005) included it in a phylogenetic study based on molecular data but using a restricted species representation of *Ephedra* consisting of 16 terminals. Their study provided moderate support for a sister relationship between *E. rhytidosperma* and *E. equisetina* (Wang et al. 2005). A close relationship is confirmed (but poorly supported) under the much denser taxon sampling employed here. However, *E. monosperma* is also a member of the clade (fig. 2), and the relationships among these three species are unresolved.

The Monophyly and Distribution of Species

Morphological variation in *Ephedra* has often appeared confusing from an evolutionary perspective, with intraspecific variation (Huang et al. 2005) and parallelism (Ickert-Bond and Wojciechowski 2004). Species determination requires extensive knowledge and experience (Freitag and Maier-Stolte 1994). Generally, recognized species investigated here appear distinct, and their monophyly is in most cases supported by molecular data. This is, for example, true for one of the best-known and most commonly cultivated *Ephedra* species in Europe, *E. distachya*. Despite its wide distribution in the wild, ranging from Spain to Kazakhstan (and perhaps to China), the seven specimens investigated here constitute a well-supported clade, with the easternmost (collected from Kazakhstan) as sister to remaining specimens. Chinese specimens have sometimes been treated as a (delicate) variety of *E. distachya* (Freitag and Maier-Stolte 1994; Fu et al. 1999) and sometimes as a separate species, *E. pseudodistachya* (Pachomova 1968). Most recent studies indicate that the latter classification may be the more accurate (Freitag and Maier-Stolte 2009).

There are some potentially problematic species for which species determination is uncertain, and a few names are better treated as synonyms of other names. There are also two species for which monophyly cannot be confirmed in this study.

Ephedra fragilis is here represented by data from four individuals. Two of them, collected in Morocco, group with *E. altissima* (collected in Libya and Morocco). The other two specimens of *E. fragilis* were collected in Jordan and Europe, and they are nested within a well-supported clade that also comprises *E. aphylla* and *E. major* (specimens 88 and 98). These results indicate that morphological variation in *E. fragilis*, and probably also *E. altissima* and *E. aphylla*, must be further investigated and that species delimitations should be reconsidered and perhaps revised.

The monophyly of *E. major*, present from the Himalayas to Spain and in Africa and the Canary Islands, is also uncertain. Our data indicate that *E. major* constitutes two separate clades that are distantly related and perhaps correspond to the two subspecies *E. major* ssp. *major* and *E. major* ssp. *procera*. Two specimens of *E. major* (specimens 88, 98; ssp. *major*?), both collected in Spain, fall within the basal clade of African-Mediterranean species, whereas the remaining specimens of *E. major* (05, 97, and 103; ssp. *procera*?), collected in Eastern Europe and Algeria, are well supported within the mainly Asian clade (sister to *E. saxatilis*, *E. gerardiana*, *E. monosperma*, *E. rhytidosperma*, and *E. equisetina*). Ideally, identification of *Ephedra* should be made on fertile specimens, and the vouchers of the two specimens of *E. major* from Spain, which form a clade with *E. aphylla* and *E. fragilis*, are vegetative. Nevertheless, we consider the identification plausible. Their features (erect, sturdy branches with membranous leaves, old leaf sheets dark brown) fit the description of *E. major*. *Ephedra aphylla* is not present in Europe and has ciliate leaf sheets, and the sympatric *E. fragilis* is scrambling and has green leaves. The result is further consistent with ongoing, more extensive molecular and morphological work (C. Rydin and P. K. Endress, unpublished manuscript); a taxonomic revision of *E. major* might be needed.

Ephedra fedtschenkoae has been considered to be very similar in gross morphology to *E. regeliana* (Bobrov 1968; Freitag and Maier-Stolte 1994) and *E. monosperma* (Freitag and Maier-Stolte 2009), and the distinction between *E. fedtschenkoae* and *E. regeliana* has been questioned (Bobrov 1968). Here, the latter two species are sisters with strong support, whereas *E. monosperma* belongs in another subclade of the mainly Asian clade.

Ephedra laristanica was recently described as an Iranian endemic (Assadi 1996), but our results clearly show that this species is nested within *E. foliata*. According to the original description, *E. laristanica* differs from *E. foliata* in having an erect habit (not climbing), scalelike leaves (not foliaceous), sessile cones (not pedunculate), and female cone bracts connate only to about one-third of their length (not fully connate; Assadi 1996). This reflects the plasticity of many gross morphological characters in *Ephedra*. *Ephedra ciliata* is sister to the *E. foliata–E. laristanica* clade, and the treatment of *E. ciliata* as a synonym of *E. foliata* (Freitag and Maier-Stolte 2003) is thus congruent with results from our study.

Freitag and Maier-Stolte (1989, 1996) included *E. campylopoda* C. Meyer in *E. foeminea*. Mohr and Meeuse (1991) doubted this treatment, but our results on the position of specimen 87, "*E. campylopoda*" (*E. foeminea* in figures and tables), support the interpretations made by Freitag and Maier-Stolte (1989, 1996).

Among the species of the mainly Asian clade, only the *E. somalensis* clade (but see above on uncertainties about *E. major*) occurs in Africa, and it is restricted to the Horn of Africa. However, our results do not support the existence of a distinct *E. pachyclada* clade and a *E. somalensis* clade, and the latter is perhaps best considered the westernmost outpost of *E. pachyclada*, dispersed into the Horn of Africa, e.g., from Yemen. Freitag and Maier-Stolte (2003) considered the possibility of an affinity between *E. somalensis* and *E. pachyclada* but suggested that the species were distantly related because of differences in vegetative habit. These authors have extensive knowledge and field experience of *Ephedra*, and

this illustrates again the extreme difficulty of species determinations and delimitations in the genus. *Ephedra* is probably an example of a plant group where DNA bar-coding would be a recommended and very helpful alpha-taxonomic tool, especially if used integratively, in combination with morphological information.

Classification

Classification schemes, originally outlined by Stapf (1889) on the basis of the morphology (texture) of cone bracts (see also Soskov 1968; Mussayev 1978), have been shown to be artificial (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004). A more recent discussion of Old World species, including a subdivision into informal groups (Freitag and Maier-Stolte 1994), was based on a wider range of gross morphological characters. Ickert-Bond and Wojciechowski (2004) suggested congruency between their results (based on molecular data) and the subgroups outlined by Freitag and Maier-Stolte (1994).

Our results, however, indicate that all subgroups of *Ephedra* suggested by Freitag and Maier-Stolte (1994) are polyphyletic, except perhaps group *Leptocladae*. The group *Alatae* comprises species with cone bracts that turn dry on seed maturity (*E. alata*, *E. strobilacea*, and *E. przewalskii*), but the species belong to three different clades and are each most closely related to species with fleshy cone bracts (fig. 2). The species of group *Sarcocarpae* (*E. transitoria*, *E. sarcocarpa*, and *E. lomatolepis*) belong to two subclades in the mainly Asian clade, as do the species of group *Distachyae* (*E. distachya*, *E. regeliana*, *E. intermedia*, and *E. fedtschenkoae*; fig. 2). The group *Fragilis* (*E. fragilis*, *E. foeminea*, *E. aphylla*, and *E. foliata*) largely corresponds to a (probably) paraphyletic assemblage of Mediterranean species (fig. 2). The group *Leptocladae* (*E. pachyclada*, *E. major*, *E. monosperma*, and *E. saxatilis*) is monophyletic, according to our results, if *E. gerardiana*, *E. equisetina*, and *E. rhytidosperma* are included (but see above on *E. major*). Our findings highlight the need for a new classification of *Ephedra*, and a formal classification that takes into account the phylogenetic relationships as revealed by DNA sequence data, as well as new morphological information, will be presented in a forthcoming article.

General Patterns, Problems, and Perspectives

Species of *Ephedra* are similar not only in gross morphology but also in terms of molecular characters. A striking and obvious fact is the limited amount of information, as demonstrated in this and in previous studies (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004; Huang et al. 2005); there are very few variable characters in all investigated loci (with the possible exception of the nrITS region; see table 2 and fig. 3). We have, in addition, tested other gene regions, i.e., *trnL-F* (Rydin et al. 2004) and several regions that have been found highly informative in other groups ($trnH^{GUG}$-*psbA*, $trnS^{GCU}$-$trnG^{UUC}$, *rpoB*-$trnC^{GCA}$, and $trnD^{GUC}$-$trnT^{GGU}$; see Shaw et al. 2005 for a review), but with unsatisfactory results in terms of estimated information value. In total, 13 loci have now been used or tested for their phylogenetic information (Ickert-Bond and Wojciechowski 2004; Rydin et al. 2004; Huang et al. 2005; this study), and still, despite these efforts, many questions on phylogenetic relationships within *Ephedra* remain to be resolved.

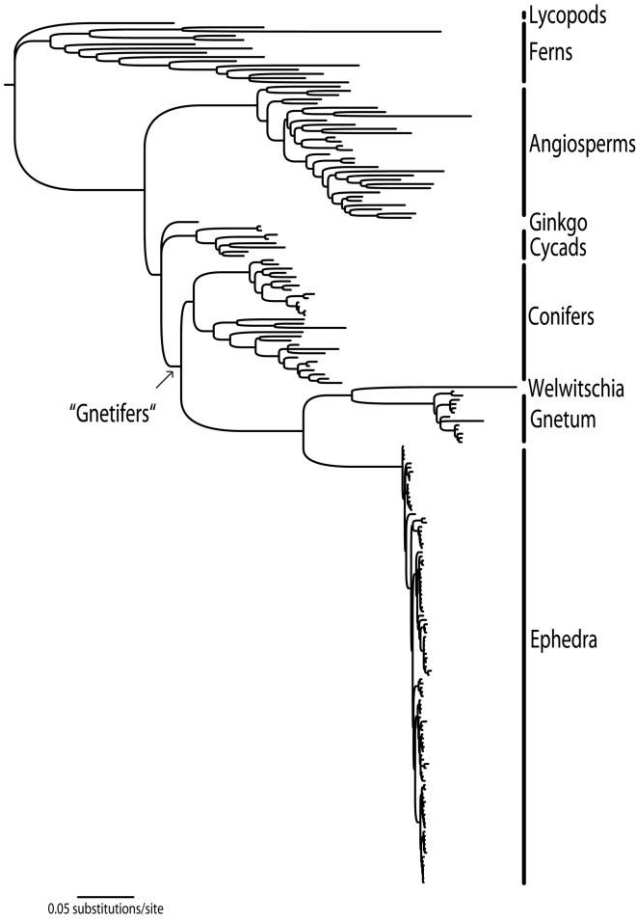

Fig. 3 Phylogram with branch lengths proportional to the number of substitutions per site, resulting from the Bayesian analysis of the combined data set (two data partitions).

Another general problem for attempts to resolve the phylogeny of *Ephedra* is the distance to the outgroup (see Rydin et al. 2002; Magallón and Sanderson 2005). More slowly evolving gene regions contain very little information within *Ephedra*, and more quickly evolving regions are difficult or impossible to align with the outgroup. The $trnS^{UGA}$-$trnfM^{CAU}$ intergenic spacer and the *rpL16* intron were selected as potential regions of "intermediate" amounts of variation, and it was possible to align the *Ephedra* sequences of these regions with those from *Gnetum* and *Welwitschia*, but because of repeated losses of longer regions in the *Ephedra* sequences (see also Wu et al. 2009), we considered the homology assessments too uncertain.

In contrast to what has been found in phylogenetic investigations of seed plants (Sanderson et al. 2000; Rydin et al. 2002; Burleigh and Mathews 2004, 2007*a*, 2007*b*), there are, however, no strong conflicts between information from different loci; the incongruences found are not well supported, and poor resolution and support appear to be more

or less entirely due to the fewness of informative characters. Since we cannot detect any strong conflicts between or within data sets, adding molecular data is likely a good strategy to accomplish further resolution. Furthermore, it is not necessarily problematical to use morphology for subdivision of *Ephedra*. Ongoing studies indicate that morphological, anatomical, and histological features can be highly diagnostic for identifying subclades of *Ephedra* (C. Rydin and P. K. Endress, unpublished observations) and that the information appears largely congruent with the present estimates of phylogeny based on molecular data.

Seed Plant Phylogeny: Another Difficult Evolutionary Problem

Like the phylogeny of *Ephedra*, relationships among the major seed plant clades have proved difficult to resolve. The ambiguous position of the Gnetales is known from many previous large-scale phylogenetic studies that show contradicting topologies for the basal divergences among seed plants, depending on taxon choice, data analyzed, and analytical methods (Magallón and Sanderson 2002; Rydin and Källersjö 2002; Rydin et al. 2002; Burleigh and Mathews 2004, 2007*a*, 2007*b*).

Here, the Bayesian analysis resulted in a topology in which conifers are monophyletic and sister to the Gnetales (fig. 1). This "gnetifer" topology has previously been presented only in a few studies based on molecular data (18S data: Bowe et al. 2000; Chaw et al. 2000; multigene study: Rydin et al. 2002), but it may be more compatible with other kinds of information (e.g., morphology, anatomy, and embryology) than the hypothesis in which Gnetales are nested within conifers (see Mathews 2009 for a recent discussion).

However, the gnetifer topology presented here conflicts with results in most studies in the literature and with those obtained from other analyses in this study. The combined MP analysis resolved Gnetales as sister to all other seed plants, a common result from equally weighted parsimony analyses (Källersjö et al. 1998; Sanderson et al. 2000; Rydin et al. 2002). Further, our B/MCMC analysis of the subset including only chloroplast data resulted in a well-supported "gne-cup" topology (Gnetales sister to Cupressophyta).

Diagnostic for the seed plant phylogeny problem is that several, often mutually incompatible, hypotheses have been put forward. Further, the different results are generally very well supported statistically, and the problem has not been resolved despite extensive efforts using large amounts of data. Over and over again, well-supported yet conflicting results are demonstrated. As a consequence of the inclusion of a large outgroup sampling, this study provides another striking example that seed plant relationships present an unresolved, exceptionally difficult phylogenetic problem. Additional data from different sources and integrative approaches are needed to progress in this field (Rydin 2005; Mathews 2009).

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