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Phylogenetic position of *Ephedra rhytidosperma*, a species endemic to China: Evidence from chloroplast and ribosomal DNA sequences

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Abstract **The chloroplast genes *mat*K and *rbc*L, ribosomal gene 18S and ITS regions of nuclear ribosomal DNA from *Ephedra rhytidosperma*, a species endemic to China, were sequenced and its phylogenetic position was investigated. Independent and combined phylogenetic analyses for the DNA sequences from 16 taxa representing 15 species of the genus *Ephedra* were performed using the maximum parsimony (MP), neighbor-joining (NJ), minimum evolution (ME) and maximum likelihood (ML) methods. The results indicate that *E. rhytidosperma* is closely related to *E. equisetina*. The divergence time between them is estimated to be 10.85±2.44 Ma based on the results of the relative-rate tests and the evolutionary rate of *rbc*L gene.**

Keywords: ***Ephedra*, *Ephedra rhytidosperma*, cpDNA *mat*K gene, Chloroplast *rbc*L, Nuclear ribosomal 18S, nrDNA ITS regions, phylogenetic analysis.**

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The gymnosperm class *Gnetopsida*, which share some important characteristics with angiosperms such as double fertilization and vessel[1,2], have a special significance in plant phylogenetic studies. Recent morphological and molecular studies have suggested that the orders Gnetales, Welwitschiales, and Ephedrales form a monophyletic group, in which the monotypic order *Ephedrales* occupied a basal position[3,4]. However, systematic relationships of extant *Ephedra* have still not been well resolved, which is ascribed to two aspects: difficulties in sampling and complex interspecific relationship. Searching for the primitive group has also been a key issue for a better understanding the evolution of *Ephedra*[5−8].

Fossil records are essential to the study of the origin and evolution of plants. Although many ephedroid fossils have been found, few of them are megafossils and their structural details are lacking compared with the extant *Ephedra*[9,10]. Recently, some ephedroid megafossils were recorded in Lower Cretaceous (125 Ma ago) from western Liaoning Province, China[11−13], among which *E. archaeorhytidosperma*[13] is the only one bearing morphological characters similar to those of extant *E. rhytidosperma* Pachomova. Although this finding has an important implication for the origin and evolution of *Ephedra*, the authors did not deem that *E. rhytidosperma* is the most primitive species in living *Ephedra*, and this species has not been included in recent molecular phylogenetic studies of the genus[7,8,14−16]. We therefore sequenced the chloroplast genes *mat*K and *rbc*L and ribosomal gene 18S as well as nrDNA ITS regions of *E. rhytidosperma* for the first time. Phylogenetic position of *E. rhytidosperma* was then detected using sequence data and the origin time of this species was also estimated.

Fresh leaves of *E. rhytidosperma* were collected from two populations in the Mt. Helan, Ningxia Hui Autonomous Region, China. Voucher specimens are deposited in the herbarium of the Institute of Botany, the Chinese Academy of Sciences (PE). Total genomic DNA was extracted from silica-dried leaves using the CTAB procedure[17]. The PCR-amplification primers for the *mat*K gene were *trn*K-2R and *trn*K-3914F, and the primers for *rbc*L and 18S followed a previous work[8]. The ITS regions were amplified using the primers ITS4 and ITS5[18]. The amplified products were purified with the Waston PCR purification kit. The purified PCR fragments were sequenced using the BigDye cycle sequencing kit and run in an Automated Sequencer 377 (Applied Biosystems, CA). The sequencing was done in the State Key Laboratory for Biocontrol at Zhongshan University and Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering at Fudan University. All sequences have been deposited in GenBank (for accession numbers, see Table 1). DQ028779—DQ028782 and DQ212957—DQ212960 represent the sequences from the two populations of *E. rhytidosperma*, respectively, and the sequences of *Gnetum* are from *G. hainanense*, *G. urens*, *G. leyboldii*, and *G. gnemon*. The sequences were assembled and aligned using Clustal-X program[19] and corrected manually.

Phylogenetic analyses of *Ephedra* based on the DNA sequences of chloroplast genes *mat*K and *rbc*L and ribosomal gene 18S as well as nrDNA ITS regions were performed using the maximum parsimony (MP)[20], neighbor-joining (NJ)[21], minimum evolution (ME)[22] and maximum likelihood (ML)[23] methods. *Welwitschia* and

* These authors contributed equally to this work.

Table 1 Species and GenBank accession numbers used in this study

Species	GenBank Accession No.				Sample location	Section[a)]
	*rbc*L	*mat*K	18S	ITS		
E. antisyphilitica	AY492031	AY492008	AY755682	AY755757	North America	*Alatae*
E. aphylla	AY755802	AY492009	AY755695	AY755771	Africa	*Ephedra*
E. californica	AY492033	AY492011	AY755676	AY755750	North America	*Asarca*
E. chilensis	AY492036	AY492012	AY755679	AY755754	South America	*Alatae*
E. distachya	AY492035	AY492013	AY755686	AY755769	Asia, Europe	*Ephedra*
E. equisetina	AY755783	AY492014	AY755666	AY755770	Asia	*Ephedra*
E. fragilis	AY755784	AY492015	AY755677	AY755752	Africa, Europe	*Ephedra*
E. intermedia	AY056566	AY492016	AY755683	AY755758	Asia	*Ephedra*
E. minuta	AY492041	AY492019	AY755681	AY755756	Asia	*Ephedra*
E. nevadensis	AY492042	AY492020	AY755688	AY755764	North America	*Ephedra*
E. rhytidosperma	DQ028779[b)]	DQ028780[b)]	DQ028781[b)]	DQ028782[b)]	Asia	*Ephedra*
	DQ212957[b)]	DQ212960[b)]	DQ212959[b)]	DQ212958[b)]		
E. sinica	AY492046	AY492024	AY755675	AY755749	Asia	*Ephedra*
E. torreyana	AY492047	AY492025	AY755684	AY755759	North America	*Alatae*
E. trifurca	AY492048	AY492026	AY755687	AY755762	North America	*Alatae*
E. tweediana	AY492049	AY492027	AY755692	AY755768	South America	*Alatae*
Gnetum[c)]	AY296546	AY449629	L24045	AY449561		
Welwitschia[c)]	AJ235814	AF280996	AF207059	U50740		

a) Classification based on Stapf (1889); b) represent the samples sequenced in this study; c) outgroups.

Gnetum were selected as complex outgroups[8]. To improve the accuracy of the phylogenetic analyses, two combined datasets of the chloroplast and ribosomal sequences respectively from 16 taxa, including Sect. *Alatae,* Sect. *Asarca*, Sect. *Ephedra*, and the common species of the four genes, were analyzed using ML method. The reliability of the combined dataset was evaluated with partition homogeneity test[24]. The HKY model[25] and gamma distribution were used for constructing the ML trees. Bootstrap analysis with 1000 replicates was used to assess the relative support for each branch of the ML trees. The relative-rate test was used to detect the significantly different rates among branches[26,27]. Under the hypothesis of molecular clock, the origin time of *E. rhytidosperma* was estimated with Jukes-Cantor model, according to the divergence time (290 Ma) of seed plants[7,28]. All of the analyses were implemented in PAUP 4.0, MEGA 3.0, and RRT 1.1[24,28,29].

The results of phylogenetic analyses showed that: (1) evidence from the molecular data does not support the traditional taxonomy of *Ephedra* based on morphological characters. For example, Sect. *Alatae* and Sect. *Ephedra* did not form a monophyletic group (Fig. 1), which is consistent with the conclusion of Ickert-Bond and Wojciechowski[14] and Huang et al.[15]. There are three groups (South America, North America, and the Old World) in the ML trees based on the 15 species, corresponding with their geographical distribution. Thus, it could be deduced that the radiation of *Ephedra* happened after Gondwana division; (2) all of the phylogenetic trees (MP, NJ, ME, and ML) are in support of the conclusion that *E. rhytidosperma* and *E. equisetina* form a monophyletic group, with relatively high bootstrap support values (74%—100%), validating the viewpoints of Pachomova[30] and Mussayev[31]; (3) the partition homogeneity tests of the chloroplast and ribosomal datasets (p= 0.433 and p= 0.763, respectively) suggest that the combined analysis might improve phylogenetic accuracy. The tree based on the combined data of the four genes is similar in topological structure to those based on a single gene, but each independent analysis has a relatively low bootstrap support (<50%), which could be attributed to the interspecific hybridization in *Ephedra*[32]; and (4) the molecular clock was detected in *rbc*L and 18S with the relative-rate test, but the phylogenetic tree of 18S is too rough to divide the species into the New World and Old World; thus the time *E. rhytidosperma* and *E. equisetina* parted was estimated to be (10.85±2.44) Ma based on the *rbc*L data only, and Huang and Price ($(2.353\pm0.205)\times10^{-10}$ substitution/site)[7]. This suggests that the relationship between *E. rhytidosperma* and the *E. archaeorhytidosperma* found in western Liaoning Provice might not be rather close, and more information for determining the relationships between fossil and the living species with morphological data is still needed. In general, the phylogenetic analyses in our study show that *E. rhytidosperma* is very close to *E. equisetina*, but whether it is the most primitive species in *Ephedra* remains unclear. An accumulation of samples and molecular data might help resolve the problem. Our future work will focus on the origin and distribution of ephedrine compounds in the order *Ephedrales* and gymnosperms[1)].

1) Wang, Q. et al., Molecular phylogenetic evidence of the origin of ephedrine compounds in gymnosperms (unpublished data).

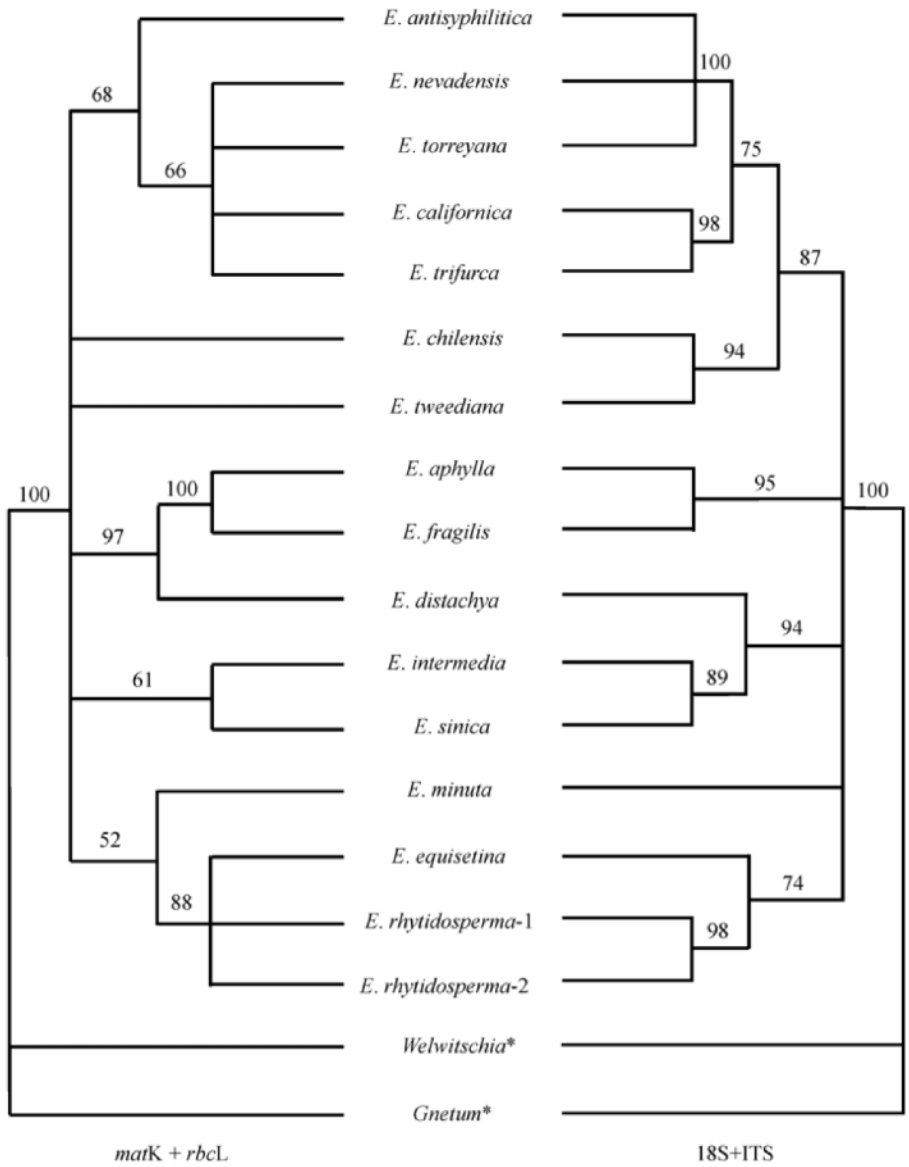

Fig. 1. Phylogenetic trees generated using *mat*K + *rbc*L (left) and 18S + ITS (right) sequences with ML method. Numbers indicate bootstrap support values (%) of the branches. * Represent the outgroups.

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