Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3497

Publication history
Received: 2015-09-05
Accepted: 2016-04-22
Published: 2016-06-13

Handling editor
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Authors' contributions
LXX and YSZ equally contributed to this study; YSZ designed the study and analyzed the data; LXX performed the lab and wrote the manuscript; FYM, ZC, WPW, QPZ, and XHZ collected field data and collaborated with statistics and results interpretation; FYM and ZC analyzed *matK* data; WPW, QPZ, and XHZ analyzed *ITS1* data

Funding
This study was supported by a grant (No. 81160505) from the National Natural Science Foundation of China.

Competing interests
No competing interests have been declared.

Citation
Zhao YS, Xie LX, Mao FY, Cao Z, Wang WP, Zhao QP, et al. Identification of *Ephedra* species by phylogenetic analyses using *matK* and *ITS1* sequences. Acta Soc Bot Pol. 2016;85(2):3497. http://dx.doi.org/10.5586/asbp.3497

ORIGINAL RESEARCH PAPER

Identification of *Ephedra* species by phylogenetic analyses using *matK* and *ITS1* sequences

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Abstract
In this study, the species identifications of seven *Ephedra* plants, including three medicinal plants from the *Pharmacopoeia of the People's Republic of China*, were conducted using phylogenetic analyses, and the method's validity was verified. The phylogenetic trees constructed from the maturase-coding gene (*matK*) and internal transcribed spacer 1 (*ITS1*) sequences showed that the former could be used for identifying five *Ephedra* plants, *Ephedra intermedia*, *E. equisetina*, *E. antisyphilitica*, *E. major*, and *E. aphylla*, but it had less power to discriminate *E. sinica* and *E. przewalskii*, while the latter could distinguish five *Ephedra* plants, *E. przewalskii*, *E. equisetina*, *E. antisyphilitica*, *E. major*, and *E. aphylla*, but it had less power to discriminate *E. sinica* and *E. intermedia*. However, when the two genes were combined, the seven species could be completely distinguished from each other, especially the medicinal plants from the others, which is significant in developing their pharmaceutical uses and in performing quality control assessments of herbal medicines. The method presented here could be applied to the analysis of processed *Ephedra* plants and to the identification of the botanical origins of crude drugs. Additionally, we discovered that *E. equisetina* and *E. major* were probably closely related to each other, and that *E. sinica*, *E. intermedia*, and *E. przewalskii* also had a close genetic relationship.

Keywords
molecular identification; *Ephedra*; molecular phylogeny; *matK*; *ITS1*

Introduction

The genus *Ephedra*, which is distributed in the arid and semiarid regions of Asia, Europe, northern Africa, southwestern North America and South America, belongs to the family Ephedraceae and includes ~50 species [1]. In China, there are 13 species [2], and three species, *Ephedra sinica*, *E. intermedia*, and *E. equisetina* have long been used in traditional medicines according to the *Pharmacopoeia of the People's Republic of China* [3]. The medicinal *Ephedra* plants have been used primarily to treat asthma or bronchitis, but they are also prescribed for cold and flu symptoms, including nasal congestion, cough, fever, and chills [4].

Now, processed *Ephedra* herbs with vernacular names are distributed in the markets, making the identification of their species of origin more difficult. Moreover, some adulterants of *Ephedra* species are confused with the medicinal plants. To maintain quality control, it is essential that the medicinal *Ephedra* plants are identifiable. Therefore, the identification of the plant sources is critical for their use as herbal medicines.

Many *Ephedra* plants are morphologically similar, making their identification based on morphology very difficult. For example, *E. intermedia* and *E. przewalskii* not only have three-lobed leaves, but also have two-lobed leaves [2], which increases the difficulty to distinguish between them. Moreover, identifying the botanical origin of the processed *Ephedra* herbs is more difficult because during processing, the natural resource is cut into sections and dried, or broiled with honey.

Recently, molecular systematics in plants, as well as other organisms, has been widely used for species identification and the determination of phylogenetic relationships [5]. In plants, chloroplast genes, including the maturase-coding gene (*matK*), the large subunit of ribulose 1,5-bisphosphate carboxylase-coding gene (*rbcL*), the non-coding plastid *trnH-psbA* intergenic spacer region and encoding subunit B of light-independent protochlorophyllide reductase (*chlb*), are usually used for molecular phylogenetic analyses [6–10]. For example, Lahaye et al. [11] used the *matK* sequences of 1566 orchid specimens representing 1084 species in Costa Rica to identify species and reconstruct a phylogeny. In another study, the use of *rbcL* gene sequences enabled the majority of the samples (92%) to be identified to the genus level [12]. In addition, nuclear ribosomal DNA (nrDNA) containing the internal transcribed spacer (*ITS*) region is also used in plant species identification. For instance, the *ITS*/*ITS2* regions could accurately and efficiently distinguish Corni Fructus and its adulterants, and provided a reference for the molecular identification of other Chinese herbal medicines [13].

Similarly, molecular systematics has also been used for *Ephedra* identification. Peng et al. [14] distinguished the Chinese *Ephedra* herb from other related species using *ITS2* sequences. A novel method to authenticate the *Ephedra* herb, based on the chloroplast *chlB* gene and *ITS* sequence of nrDNA genes was developed and successfully applied to identify the ingredients of crude drugs obtained at a Chinese market [10]. The method distinguished medicinal *Ephedra* plants from *E. przewalskii*, but their relationships were not recovered. Although the phylogenetic relationships in *Ephedra* were constructed from the chloroplast *matK* gene, *rbcL* gene and nrDNA *ITS1* to study the geographic range and morphological diversity of the genus [15], some different *Ephedra* species were not distinguished, in particular *E. intermedia* and *E. sinica*. Recently, the studies on identifying *Ephedra* species growing in different locations in China based on the phylogenetic analyses of *matK* and *ITS1* sequences have not been reported.

In this study, seven *Ephedra* species, including three medicinal *Ephedra* plants, and data deposited in GenBank, were used for species identification based on phylogenetic analyses. We chose the *matK* and *ITS1* sequences to distinguish different species, especially medicinal from non-medicinal plants, and assess the intra- and inter-species relationships of *Ephedra*.

Material and methods

Plant materials

A total of 45 sequences belonging to seven species of the genus *Ephedra* and one outroup *Gnetum montanum* (Tab. 1) were used in this study. Eleven sequences representing four species were from GenBank and the others generated in this study from plants were collected from different locations of China, including Ningxia, Gansu, Inner Mongolia, Shanxi, Xinjiang, and Shaanxi and plant vouchers were deposited in the Ningxia Research Center of Modern Hui Medicine Engineering and Technology, China.

Polymerase chain reaction (PCR), amplification, and DNA sequencing

The DNA extracts from 1.5 g of leaf tissue were obtained by the modified cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle [16]. The genomic DNA was dissolved in TE to a final concentration of 10 ng/μL to avoid any

Tab. 1 Plant material and sequence data of *Ephedra* species examined in this study.

Species	Symbol	Source	Longitude	Latitude	Altitude (m)	Accession No.	
						matK	*ITS*
E. intermedia	A1	Ningxia Pengyang	E 106°49′59.38″	N 35°52′07.59″	1486	KT286779	KT286813
	A2	Gansu Ningxian	E 107°54′05.86″	N 35°31′25.09″	1034	KT286780	KT286814
	A3	Ningxia Guyuan	E 106°27′36.73″	N 36°19′06.57″	1877	KT280781	KT280815
	A4	Ningxia Longde	E 106°08′12.63″	N 35°37′57.64″	2261	KT280782	KT280816
	A5	Gansu Qinan	E 105°62′37.60″	N 34°98′57.06″	1600	KT280783	KT280817
	A6	Gansu kongtong	E 106°39′24.15″	N 35°33′53.99″	1462	KT280784	KT280818
	A7	Gansu Longxi	E 104°38′21.01″	N 35°00′00.07″	1760	KT280785	KT280819
	A8	Gansu Yongchang	E 101°97′46.09″	N 38°24′10.82″	2913	KT280786	KT280820
	A9	Gansu Huining	E 104°29′	N 35°24′	1944	KT280787	KT280821
	A10	Gansu Jingtai	E 104°07′20.72″	N 36°49′49.54″	1734	KT280788	KT280822
	A11	Gansu Wushan	E 104°59′56.78″	N 34°44′52.00″	1584	KT280789	KT280823
	A12	Gansu Anningbao	E 103°40′58.32″	N 36°08′00.58″	1620	KT280790	KT280824
	A13	Gansu Jingchuan	E 107°37′04.29″	N 35°23′29.86″	1094	KT280791	KT280825
	A14	Gansu Anning	E 103°72′	N 36°10′	1540	KT280792	KT280826
	A15	Gansu Wuwei	E 102°88′69.63″	N 37°96′42.30″	1450	KT280793	KT280827
	A16	Gansu Tianzhu	E 103°01′51.08″	N 36°97′67.75″	2800	KT280794	KT280828
E. sinica	A17	Ningxia Qingtongxia	E 106°09′21.10″	N 38°21′06.63″	1123	KT280795	KT280829
	A18	Ningxia Lingwu	E 106°24′27.80″	N 37°53′51.81″	1250	KT280796	KT280830
	A19	Shanxi Youyu	E 111°53′26″	N 39°27′54″	1547	KT280797	KT280831
	A20	Shanxi Datong	E 113°25′26″	N 40°08′5″	1170	KT280798	KT280832
	A21	Shanxi Tianzhen	E 113°54′32″	N 40°16′37″	1672	KT280799	KT280833
	A22	Inner Mongolia, Chifengbalin	E 119°35	N 43°52	740	KT280800	KT280834

Tab. 1 Continued

Species	Symbol	Source	Longitude	Latitude	Altitude (m)	Accession No.	
						matK	*ITS*
	A23	Inner Mongolia, Etuokeqianqi	E 107°30′43.58″	N 38°29′51.58″	1349	KT280801	KT280835
	A24	Inner Mongolia, Keerqin	E 122°14′08″	N 43°17′22″	202	KT280802	KT280836
	A25	Inner Mongolia, Naimanqi	E 120°37′17″	N 43°08′33″	337	KT280803	KT280837
	A26	Inner Mongolia, Xinganmeng	E 121°29′30″	N 45°10′4″	322	KT280804	KT280838
	A27	Inner Mongolia, Wengniuteqi	E 118°59′16″	N 42°58′24″	670	KT280805	KT280839
	A28	Gansu Gulang	E 103°06′48.50″	N 37°37′51.53″	1736	KT280806	KT280840
	A29	Shaanxi Pucheng	E 109°72′74.84″	N 34°92′78.81″	423.1	KT280807	KT280841
E. przewalskii	A30	Xinjiang Hami	E 91°34′17″	N 43°22′33″	1030	KT280808	KT280842
	A31	Xinjiang Hejingbaluntai	E 86°16′21″	N 42°29′54″	1487	KT280809	KT280843
	A32	Xinjiang Tulufa, ntuokexun	E 88°29′00″	N 42°18′25″	1330	KT280810	KT280844
	A33	Xinjiang Heshu, ochengjiao	E86°54′02″	N42°17′17″	1131	KT280811	KT280845
	A34	Xinjiang Hejingxian	E86°16′21″	N42°29′54″	1400	KT280812	KT280846
E. antisyphilitica	*E. antisyphilitica* 1					JX217645.1	AY599152.1
	E. antisyphilitica 2					JX217644.1	AY599148.1
	E. antisyphilitica 3					AY492008.1	AF429442.1
E. aphylla	*E. aphylla* 1					AY492009.1	AF429449.1
	E. aphylla 2					KP997306.1	AY599128.1
	E. aphylla 3					KP788845.1	AY599127.1
E. major	*E. major* 1					LC010493.1	HQ882785.1
	E. major 2					LC010494.1	HQ882783.1
E. equisetina	*E. equisetina* 1					KP788851.1	HQ882770.1
	E. equisetina 2					AY492014.1	HQ876928.1

Tab. 1 Continued

Species	Symbol	Source	Longitude	Latitude	Altitude (m)	Accession No.	
						matK	*ITS*
Outgroup	*E. equisetina* 3					LC010496.1	HQ876922.1
	Gnetum montanum					AF280994.1	KP256672.1

variation in PCR due to DNA concentration differences. Two primer pairs were designed based on the conservative coding sequences of *matK* and *ITS1*. The primer pairs, matK 1L (5′-AATCCAGAGCATTCTGCTGTTT-3′) and matK 1R (5′-TCGGTTCMAGCTAGATTGTACT-3′); ITS 1L (5′-CCGCYGAGTAAGTTCGCTCTC-3′) and ITS 1R (5′-CCRTTGCCAGATTGCTTCCT-3′), obtained from Sangon Biotech (Shanghai, China), were used to amplify the complete *matK* gene and *ITS1* regions. For the amplification of the entire *matK* or *ITS1* regions, PCR was performed in 50 μL reaction mixture consisting of 19 μL sterile water, 25 μL 2× Power Taq PCR MasterMix (Bio Teke), 2 μL of each primer (10 μM) and 2 μL of template DNA. Amplification conditions for *matK* consisted of one cycle of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 56–58°C for 40 s and extension at 72°C for 1.5 min, with a final amplification of 72°C for 10 min. Amplification program for *ITS1* consisted of an initial step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58–63°C for 40 s, and 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. Amplifications were performed in the T100TM Thermal Cycler (BIO-RAD). PCR products were separated by agarose gel electrophoresis and purified with the Axyprep DNA Gel Extraction Kit (Axygen) according to the manufacturer's instructions. The purified PCR products were sequenced at Beijing Genomics Institution in China. Sequence data were submitted to GenBank and were assigned accession numbers ranging from KT286779 to KT286846.

DNA sequence data analysis

DNA sequences obtained for *matK* and *ITS1*, were aligned using Clustal X [17]. The maximum likelihood (ML) and Bayesian inference (BI) methods were selected for the construction of phylogenetic trees. The sequence of *Gnetum montanum* Markgraf was used as the outgroup. Maximum likelihood analyses were processed using the MEGA 4 [18] program with the Kimura-2-Parameter model. The reliability of each branch was tested by a bootstrap analysis with 1000 replications. Bayesian inference analyses were performed using MrBayes 3.0b4 [19]. Evolutionary models were chosen with MrModeltest 1.0b [20] in combination with PAUP 4.0 b10 [21]. Each analysis consisted of two independent runs with four chains for 2 000 000 generations, sampling one tree every 100 generations.

Results

For the 34 samples containing three *Ephedra* species, the PCR-amplified fragments of both the *matK* gene and *ITS1* regions were sequenced. The sequenced *matK* gene was 1408 base pairs (bp), and *ITS1* was 918 bp. When the 11 sequences from GenBank were combined, the alignment of the 1141-bp regions of 45 different *matK* sequences revealed that 1122 bp (98.33%) were conserved, 19 bp (1.67%) were variable, and 14 bp (1.23%) were parsimony informative sites. The estimated transition/transversion ratio was found to be 2.1. The alignment of the 918 bp region of 45 different *ITS1* sequences revealed that 848 bp (92.37%) were conserved sites, 70 bp (7.63%) were variable sites, and 66 bp (7.19%) were parsimony informative sites. The estimated transition/transversion ratio was 3.0.

In the BI analyses, the best-fit model (GTR + I) was selected based on the results of the Akaike information criterion or hierarchical likelihood ratio tests.

Phylogenetic trees of the *matK* and *ITS1* sequences were created using both the ML and BI methods. The phylogenetic tree of the *matK* sequences (Fig. 1) showed that the seven species formed four clades, in which *E. aphylla* (*E. aphylla* 1, *E. aphylla* 2, and *E. aphylla* 3), *E. major* (*E. major* 1 and *E. major* 2), and *E. antisyphilitica* (*E. antisyphilitica* 1, *E. antisyphilitica* 2, and *E. antisyphilitica* 3) formed one clade each, while the other four species, *E. intermedia* (A1–A16), *E. sinica* (A17–A19), *E. przewalskii* (A30–A34), and *E. equisetina* (*E. equisetina* 1, *E. equisetina* 2, and *E. equisetina* 3) belonged to the same clade. In the last large clade, the three medicinal plants, *E. intermedia*, *E. sinica*, and *E. equisetina*, were clustered with *E. przewalskii*, which indicated that the

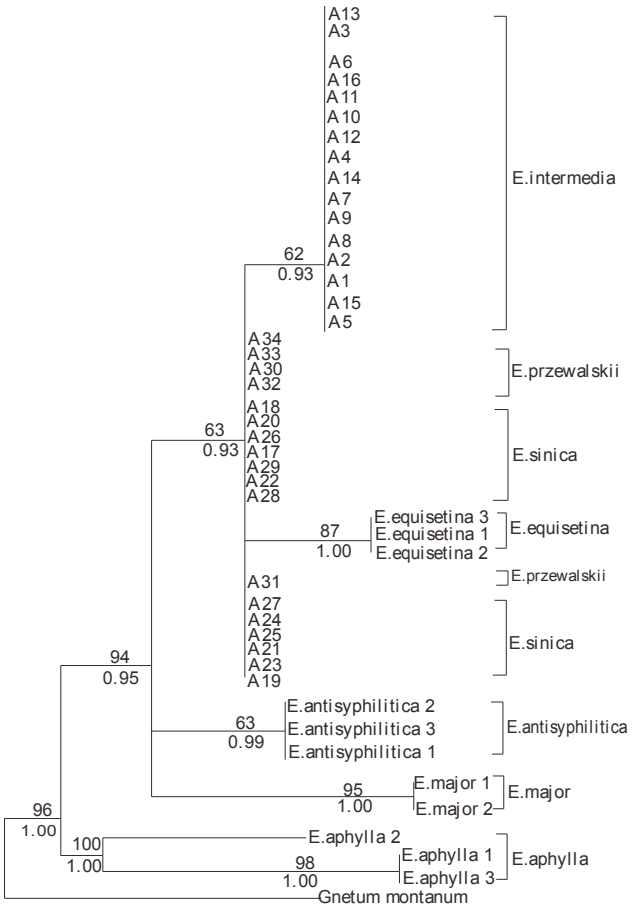

Fig. 1 Phylogenetic analysis of *Ephedra* species based on maximum likelihood and Bayesian inference analyses of *matK* sequence data. Numbers at the nodes indicate bootstrap values (% over 1000 replications) and Bayesian posterior probability.

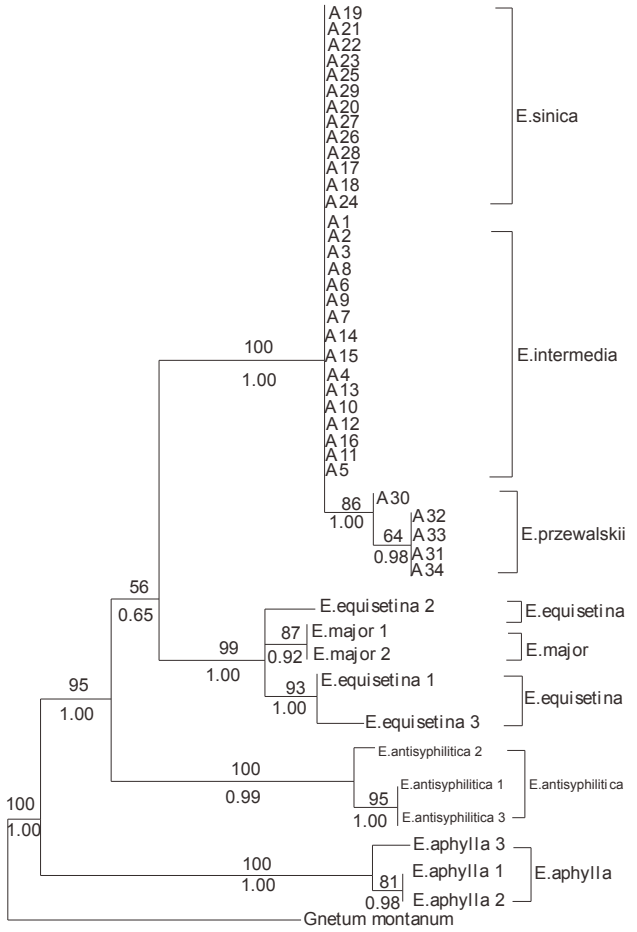

Fig. 2 Phylogenetic analysis of *Ephedra* species based on maximum likelihood and Bayesian inference analyses of *ITS1* sequence data. Numbers at the nodes indicate bootstrap values (% over 1000 replications) and Bayesian posterior probability.

four species had a close genetic relationship, but *E. equisetina* had a distant relationship with the species of the other clades. Additionally, in the last clade, *E. intermedia* and *E. equisetina* formed one sub-cluster, while *E. sinica* and *E. przewalskii* formed another. Thus, the *matK* gene could be used for identifying five *Ephedra* species, *E. intermedia*, *E. equisetina*, *E. antisyphilitica*, *E. major*, and *E. aphylla*, but it had a low ability to discriminate *E. sinica* and *E. przewalskii*.

The phylogenetic tree based on the *ITS1* sequences (Fig. 2) revealed that the seven species formed four clades, in which *E. antisyphilitica* (*E. antisyphilitica* 1, *E. antisyphilitica* 2, and *E. antisyphilitica* 3) and *E. aphylla* (*E. aphylla* 1, *E. aphylla,* 2 and *E. aphylla* 3) each formed a clade, while *E. equisetina* (*E. equisetina* 1, *E. equisetina* 2, and *E. equisetina* 3) and *E. major* (*E. major* 1 and *E. major* 2) belonged to the same clade, in which the two species formed three sub-clusters. Additionally, *E. intermedia* (A1–A16), *E. sinica* (A17–A29), and *E. przewalskii* (A30–A34) were clustered into one clade, in which *E. przewalskii* formed a sub-cluster and the other two species were clustered together. Therefore, the phylogenetic tree of the *ITS1* sequences showed that *E. intermedia*, *E. sinica*, and *E. przewalskii* had close genetic relationships, which was consistent with the *matK*-based phylogenetic tree. Thus, the *ITS1* regions could be used to identify five *Ephedra plants*, *E. przewalskii*, *E. equisetina*, *E. antisyphilitica*, *E. major*, and *E. aphylla*, but it had a low ability to discriminate *E. sinica* and *E. intermedia*.

When the use of the *matK* gene and the *ITS1* region were combined (Fig. 3), the seven *Ephedra* species could be clearly distinguished from each other. More importantly, the three medicinal plants could be distinguished from the other non-medicinal plants.

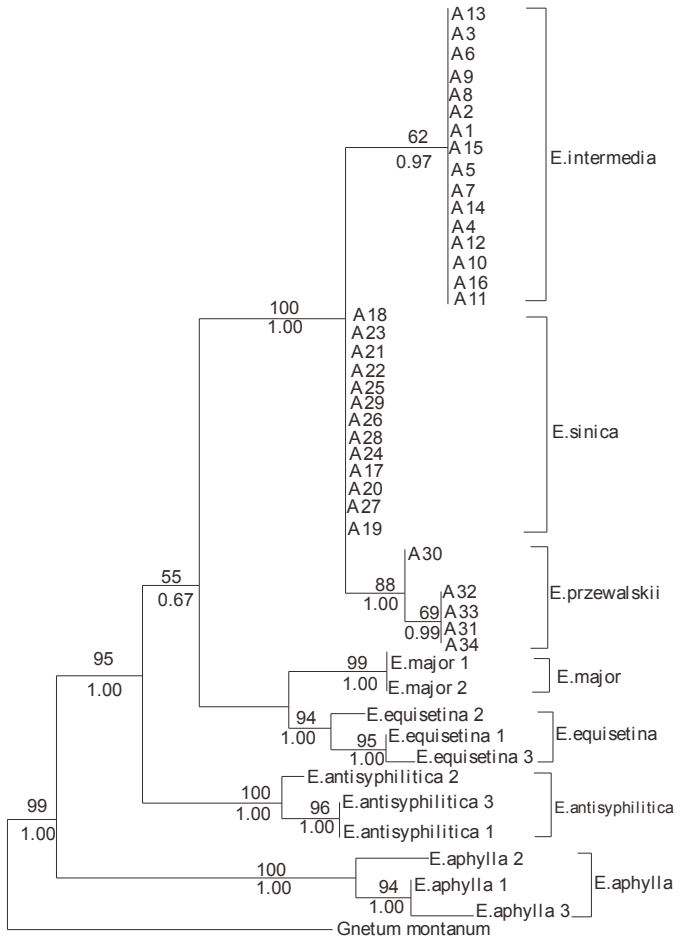

Fig. 3 Phylogenetic analysis of *Ephedra* species based on maximum likelihood and Bayesian inference analyses of *matK* + *ITS1* sequence data. Numbers at the nodes indicate bootstrap values (% over 1000 replications) and Bayesian posterior probability.

Discussion

The phylogenetic tree of the *ITS1* sequences indicated that *E. sinica* and *E. intermedia* were clustered together, which was consistent with the results of Peng et al. [14], who discovered that the two species belonged to the same clade using the *ITS2* sequences. Additionally, Wang et al. [22] found that *E. sinica* and *E. intermedia* clustered together using *matK* + *rbcL* and *18S* + *ITS* sequences, indicating that the two species were closely related. *Ephedra przewalskii* formed a sub-cluster in the same clade as *E. sinica* and *E. intermedia*. Similarly, the phylogenetic tree of *matK* sequences showed that *E. sinica*, *E. intermedia*, *E. przewalskii*, and *E. equisetina* formed a clade, but that *E. equisetina* had a distant relationship with the other three species. Combining the *matK* and *ITS1* sequence phylogenies, showed that *E. sinica*, *E. intermedia*, and *E. przewalskii* had a closer genetic relationships than *E. equisetina*. This was in agreement with Guo et al. [10] who showed that *E. sinica*, *E. intermedia*, and *E. przewalskii* were phylogenetically close to each other, while *E. equisetina* was an outgroup of the three *Ephedra* species. Similarly, Long et al. [23] also placed the three species (*E. sinica*, *E. intermedia*, and *E. przewalskii*) into one group based on *ITS* sequences.

Moreover, previous analyses of the *ITS1* and *ITS2* regions of nrDNA indicated that *E. sinica* and *E. intermedia* had identical sequences, while *E. przewalskii* had several nucleotide sites different from *E. sinica* and *E. intermedia* [23,24]. Our study also distinguished *E. przewalskii* from *E. sinica* and *E. intermedia*. Yamaji et al. (2001; cited by [10]) reported that *E. intermedia* and *E. przewalskii* had the identical chloroplast *rbcL* sequence, and Guo et al. [10] also found that *E. intermedia* had the identical *chlB* sequence as *E. przewalskii*. In contrast, the present investigation indicates that *E. intermedia* and *E. przewalskii* have some different nucleotide sites not only in their *matK* sequences, but also in their *ITS1* sequences, providing a simple method to identify the two species. Previous reports showed that *E. sinica* and *E. equisetina* could be identified based on the *chlB* sequence [10]. Similarly, in our report just using the *matK* or *ITS1* sequence, not only *E. sinica* and *E. equisetina* were identified, but *E. intermedia* and *E. equisetina* also were distinguishable. More importantly, *E. intermedia* could be separated from other species based on the *matK* gene, and *E. equisetina* could also be identified using either the *matK* or *ITS1* sequence, providing a brief and rapid method to identify *E. intermedia* and *E. equisetina*.

In addition, as was shown in Fig. 3, *E. equisetina* and *E. major* clustered together with strong support, and this result implies that the two species have a close relationship, while *E. antisyphilitica* and *E. aphylla* clearly formed two clades, as seen in Fig. 3, indicating that the two species are genetically distant from the other species.

In conclusion, when the *matK* gene was combined with the *ITS1* region, seven *Ephedra* species could be clearly distinguished from each other, including the medicinal and non-medicinal plants, which is significant for developing their pharmaceutical uses and is also important for the quality control of herbal medicines.

Acknowledgments

We are grateful to Junyu Liang and Hongling Tian for collecting the *Ephedra* plants. We thank Professor Minsheng Yan from Northwest Normal University for the morphological identification of plant species.

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