Ephedra resource in Sichuan and Yunnan Provinces 2007

Note

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Summery

Wild *Ephedra* plants growing near the Tibetan border of Yunnan and Sichuan Provinces and north-central Sichuan were surveyed and their DNA and ephedrine alkaloids content were analyzed. By analysis of ITS 1 DNA, *E. likiangensis* was found to be the dominant species in these regions, which clustered into 2 major groups in the phylogenic tree. Most *Ephedra* plants in these regions of ordinal size contained ephedrine and pseudoephedrine of more than 0.7 %, the requirement for Japanese Pharmacopoeia 15th edition, suggesting that they have potential for crude drug production of Ephedra herbs.

Keywords

Ephedra likiangensis, ephedrine, phylogeny, Yunnan, Sichuan

Introduction

Ephedra herb, the stem of the *Ephedra* plant of the family *Ephedraceae*, is a main Chinese herbal drug, which is a component of the most frequently used Kampo prescriptions. Today, *Ephedra* plants prescribed in the Chinese and Japanese Pharmacopoeia are *E. sinica*, *E. intermedia*, and *E. equisetina*, while other *Ephedra* plants were also used in the past in both countries. *E. likiangensis* is one of the *Ephedra* plants growing in mainly Yunnan and Sichuan Provinces. “Maozhou Mahuang” described as a high-quality Ephedra herb of the Chinese classic herbals since the Song Dynasty, seems to be *E. likiangensis*[1)]. Apart from historic evidence, *E. likiangensis* contains as much ephedrine as the official *Ephedra* plants, and is expected to have similar pharmaceutical properties. With the aim of developing new *Ephedra* resources, we investigated wild *Ephedra* plants, especially *E. likiangensis*, in Yunnan and Sichuan Provinces in 2007.

Materials and Methods

Plant materials

Plant materials were collected in Yunnan and Sichuan, as described in Table 1, in July 2007.

DNA analysis

Total DNA was extracted, and the DNA sequence of the internal transcribed spacer 1 (ITS 1) of nuclear ribosomal DNA was amplified by polymerase chain reaction (PCR). The DNA sequences of PCR products were analyzed as previously reported [2) 3)]. ITS nucleotide sequences were aligned by ClustalX software. Phylogenic analysis was performed by PAUP* 4.0.

Analysis of ephedrine alkaloids content by HPLC

HPLC conditions: Analysis was performed on a Hitachi Elite LaChrom HPLC equipped with an L-2130 pump, an L-2200 auto sampler, an L-2400 UV detector, and an ODS column (4.6 mm, 250 mm). The composition of the mobile phase was CH_3CN: H_2O: H_3PO_4 =390: 610: 0.8 with 0.48 % of sodium dodecylsulfate; the flow rate was 1 ml per min. The detection wavelength was set to 210 nm. Sample preparation: Herb samples were powdered and dried at 105°C to constant weight. One hundred milligrams of the herb powder was weighed exactly and 5 ml mobile phase was added. Extraction was performed by leaving at room temperature for 20 min followed by ultrasonication for 25 min. After centrifuging at 3000 rpm for 15 min,

supernatant fluid was removed and filtered through a 0.45 um membrane filter. Ten microliters of sample solution was injected into the HPLC system.

Results and discussion

We conducted surveys at sites near the Tibetan border of Yunnan and Sichuan Provinces, Baimangxueshan, Deqin, Derong, Batang, and Meerkong as well as north-central part Sichuan Province, Lixian and Maoxian (Fig. 1). The majority of *Ephedra* specimens collected were morphologically identified as *E. likiangensis*. In addition, a few *E. gerardiana*, *E. minuta*, and several unidentified *Ephedra* plants were found (Table 1). The ITS 1 sequences of specimens were analyzed for identification and confirmation of species. A nucleotide difference at 83 sites was identified among these specimens. Table 2 shows 16 of these differences in comparison with our previous data of 2001 specimens from Kangding and Xinglong, Sichuan [2)]. The nucleotide difference in ITS 1 was further identified by neighbor-joining phylogenic analysis using *E. sinica*[2)] as an outgroup (Fig. 2). From these nucleotide analyses, the unidentified specimens, specimens 53, 55, 59, 61 and 62, were found to be similar to *E. likiangensis*, and could be identified as species. These specimens, together with *E. likiangensis* specimens collected around Batang and Moxian, specimens 47 and 63, made a cluster in the phylogenic tree. Furthermore, ITS 1 data of the specimens identified morphologically as *E. gerardiana*, specimens 22, 23, 24, 25 and 26, were more similar to *E. likiangensis* rather than *E. gerardiana*, and made a cluster with *E. likiangensis* specimens collected Yunnan, specimens 28, 29, 31 and 32, and the southern border of Sichuan Province, specimens 35, 38, 44, and 45, in the tree. Thus, these specimens were concluded to be *E. likiangensis*. These unidentified and misidentified specimens grew at a relatively higher altitude and were small, which may be why their morphology was not typical of the species. The specimens morphologically identified as *E. minuta*, specimens 41, and 43, were positioned in the *E. likiangensis* and *E. minua* groups. Previously, we observed that ITS 1 of *E. minua* was very similar to that of *E. likiangensis*[2)]. Together with the morphological data, specimens 42, and 43 were confirmed as *E. minua*.

The ephedrine alkaloid content of the specimens was analyzed as shown in Table 3. Most Yunnan specimens met the standard for JP regulation, containing more than 0.7 % of the sum of ephedrine and pseudoephedrine, while most morphologically unidentified specimens of Sichuan did not. This also seems to be due to their dwarfism.

In conclusion, *Ephedra* plants at the Tibetan border of Yunnan and Sichuan Provinces, especially *E. likiangensis*, have potential as medicinal resources. The ITS 1 variation, depending on the locality, can be useful for locality identification; however, neither the content nor ephedrine/pseudoephedrine ratio seems to be correlated with the nucleotide clusters. Furthermore, individuals not showing characteristic morphology of the species, most of which were small, tended to have poor ephedrine alkaloid content. The morphology of *Ephedra* plants could be an indicator of quality as the crude drug.

Acknowledgements

This study was supported by Grant-in-Aids from the Japan Society for the Promotion of Science (No.13575010 and 20255005) and a grant from the Minister of Education, Culture, Sport Science and Technology for the Kanazawa University 21st Century COE Program. The authors are also grateful for the opportunity given by JSPS Asian core program headed by Prof. M. Shoyama.

References

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2) Long, C.F., Kakiuchi, N., Takahashi, A., Komatsu, K., Cai, S.Q., Mikage, M. *Planta Med*., **70**, 1080-1085 (2004)

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Legends for Tables and Figures

Table 1. List and collection sites of *Ephedra* specimens

Table 2. Variation of DNA sequence in ITS 1

Nucleotide numbers are based on the data of specimen 63. EL, EM and EG, indicate ITS data of previously reported *E. likiangensis*, *E. minuta* and *E. gerardiana*, respectively [2)]. Specimens numbered in bold/italics were collected in Yunnan. 59-1 and -2 indicate 2 types of overlapping sequences of specimen 59, with (59-1) or without (59-2) deletion at nucleotide 145. 28-1 and -2, and 35-1 and -2 are also 2 types of overlapping sequence with deletion differences for specimen 28 and 35, respectively. Abbreviations: * = same nucleotide as in the top column, - = deletion, M=A/C, R=A/G, W=A/T, Y=C/T.

a) GenBank Accession No. AY394075

b) GenBank Accession No. AY394076

c) GenBank Accession No. AY394074

d) GenBank Accession No. FJ868729

e) GenBank Accession No. FJ868730

Fig. 1. Map of collection sites

Fig. 2. Neighbor-joining phylogenic analysis using *E. sinica* as an outgroup.

Specimens numbered in bold/italics were collected in Yunnan. EG, EL, EM and ES indicate ITS data of previously reported *E. gerardiana*[c)], *E. likiangensis*[a)], *E. minuta*[b)] and *E. sinica*[f)], respectively [2)]. Numbers above lines indicate substitution per site.

a) GenBank Accession No. AY394075

b) GeneBank Accession No. AY394076

c) GeneBank Accession No. AY394074

f) GeneBank Accession No. AY394071

Fig. 3 Sum of ephedrine (black bars) and pseudoephedrine (grey bars) content in aerial parts of *Ephedra* plant specimens. Specimens numbered in bold/italics were collected in Yunnnan.

Table 1

Sample No.	Morphological identification	Voucher No.	Collection date	Collection site	Altitude (m)
22	*E. gerardiana*	707022	2007.7.15	Baimangxueshan Yunnan Prov.	4190
23		707023			4130
24		707024			
25		707025			
26		707026			
27	*E. likiangensis*	707027	2007.7.16	Deqin, Yunnan Prov.	3300-3240
28		707028			
29		707029			
31		707031			
32		707032			
35		707035	2007.7.20	Derong, Sichuan Prov.	3160
37		707037			
38		707038			
41	*E. minuta var. dioeca*	707041		Border between Batang, and Derong Sichuan Prov.	3870
43		707043			
44	*E. likiangensis*	707044			3880
45		707045			
47		707047			
48		707048		Batang, Sichuan Prov	2870
51		707051	2007.7.21	Dedaxiang, Batang, Sichuan Prov.	3630
52	*E. gerardiana var congesta*	707052			
53	undivided	707053	2007.7.21	Border between Batang and Litang, Sichuan Prov	4280
54		707054			
55		707055			
59		707059	2007.7.25	Border between Maerkang and Lixian, Sichuan Prov	4070
61		707061			3830
62		707062			3800
63	*E. likiangensis*	707063	2007.7.26	Maoxian, Sichuan Prov	2230

Table 2

	11	12	96	97	135	139	145	160	171	181	396-403	498	581	674	842	1139
EL[c)]	C	A	T	G	A	G	-	T	G	T	TAAAAAAA	T	A	T	G	T
EM[b)]	*	*	*	*	*	*	-	*	*	*	********	*	*	*	*	*
EG[c)]	*	*	*	A	*	*	-	*	*	*	C*******	*	*	*	*	C
37	*	*	*	*	*	*	-	*	*	C	********	*	*	*	*	*
48	*	*	*	*	*	*	-	*	*	C	********	*	*	*	*	*
47	*	*	*	*	*	*	-	*	M	*	****W***	*	*	*	*	*
63[d)]	T	*	*	*	G	T	G	A	T	*	********	*	*	*	*	*
53	T	*	A	A	G	T	G	A	*	*	*****---	*	*	*	*	*
55	T	*	A	A	G	T	G	A	*	*	********	*	*	*	*	*
59-1	T	T	A	A	G	T	G	A	*	*	********	*	*	*	*	*
59-2	T	W	A	A	G	T	G	A	*	*	********	*	*	*	*	*
62	T	T	A	A	G	T	G	A	*	*	********	*	*	*	*	*
61	T	W	A	A	*	*	-	A	T	*	****T***	*	*	*	*	*
22	*	*	*	*	*	*	-	*	*	C	****T***	*	*	*	R	C
23	*	*	*	*	*	*	-	*	*	C	****T***	*	G	*	R	C
31	*	*	*	*	*	*	-	*	*	C	****T***	*	G	*	A	C
32	*	*	*	*	*	*	-	*	*	C	****T***	*	G	*	A	C
29	*	*	*	*	*	*	-	*	*	C	********	*	G	G	A	C
24	*	*	*	*	*	*	-	*	*	C	********	*	*	Y	A	C
44	*	*	*	*	*	*	-	*	*	C	****T***	A	G	*	A	C
25	*	*	*	*	*	*	-	*	*	C	********	*	R	*	A	C
26[e)]	*	*	*	*	*	*	-	*	*	C	****T***	*	R	*	A	C
28-1	*	*	*	*	*	*	-	*	*	C	****T***	*	G	*	A	C
28-2	*	*	*	*	*	*	-	*	*	Y	--------	*	G	*	A	C
38	*	*	*	*	*	*	-	*	*	Y	****T***	*	G	*	A	C
35-1	*	*	*	*	*	G	-	*	*	Y	****T***	*	G	G	A	C
35-2	*	*	*	*	*	G	-	*	*	C	*****---	*	G	G	A	C
45	*	*	*	*	*	G	-	*	*	C	****T***	*	*	*	A	*
41	*	*	*	*	*	G	-	*	*	C	********	*	*	*	*	*
43	*	*	*	*	*	G	-	*	*	C	********	*	*	*	*	C
52	*	*	*	*	*	G	-	*	*	C	********	*	*	*	*	C

Fig. 1

Collection sites

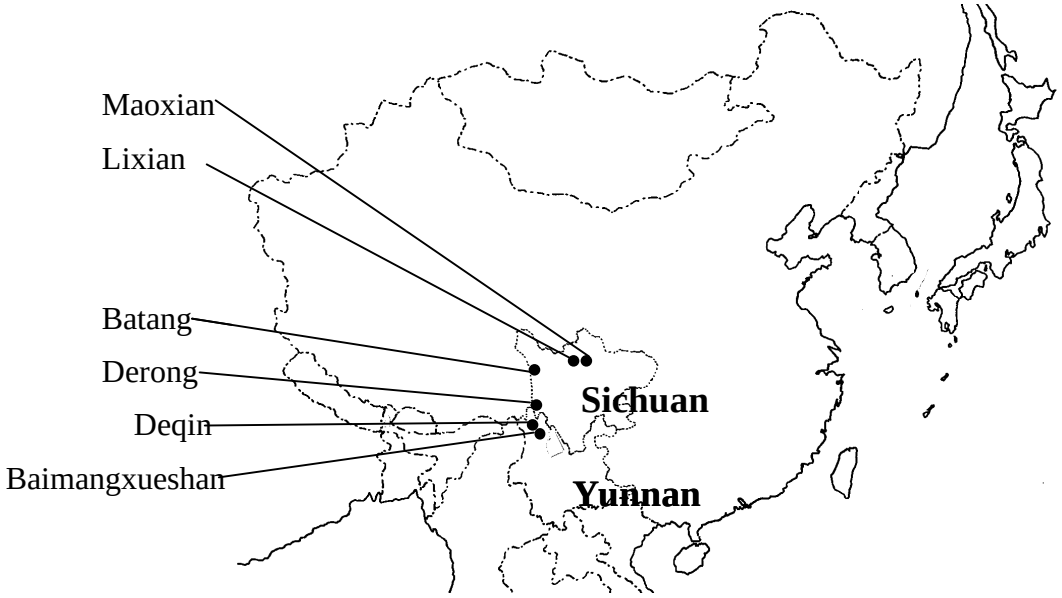


Fig. 2

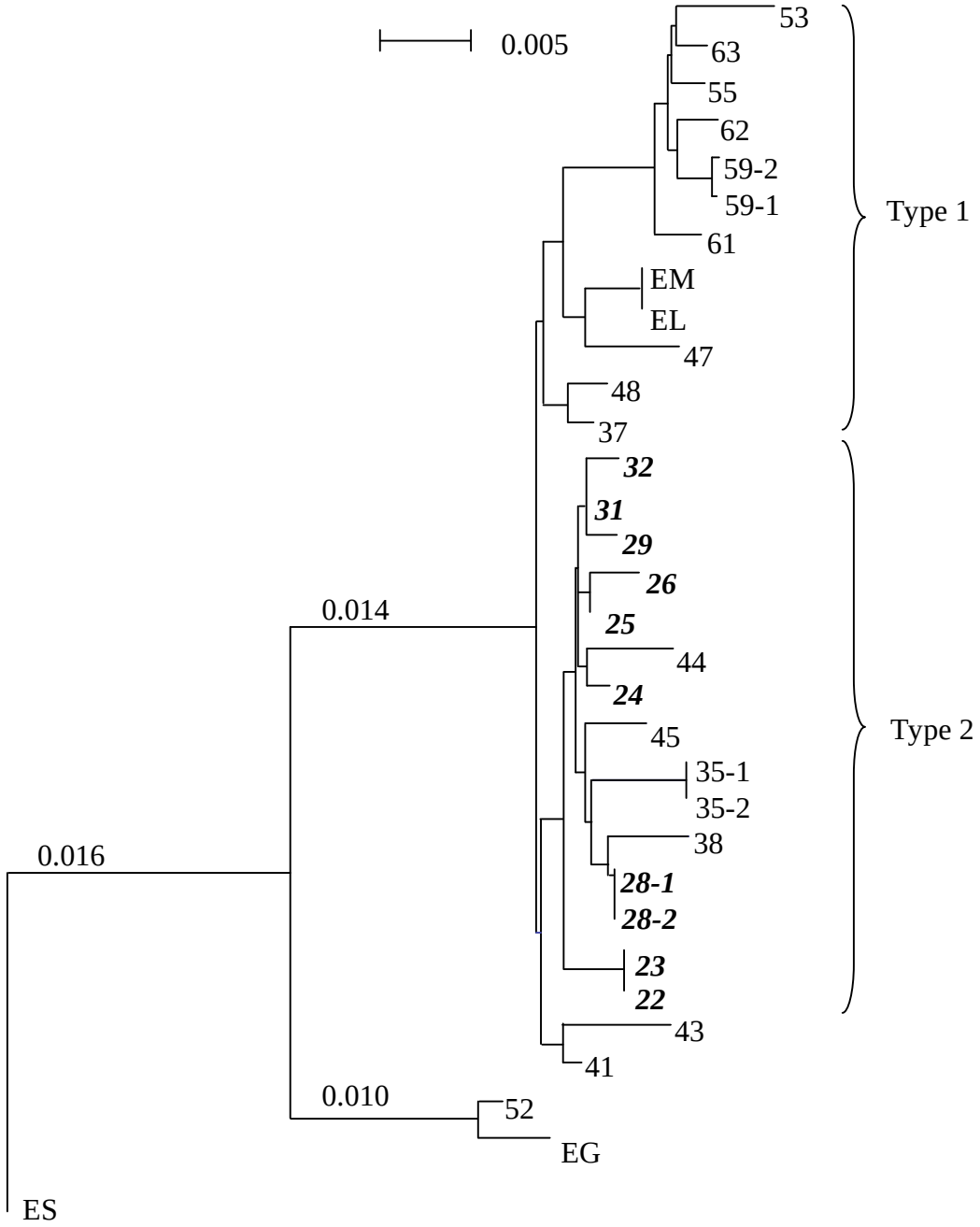
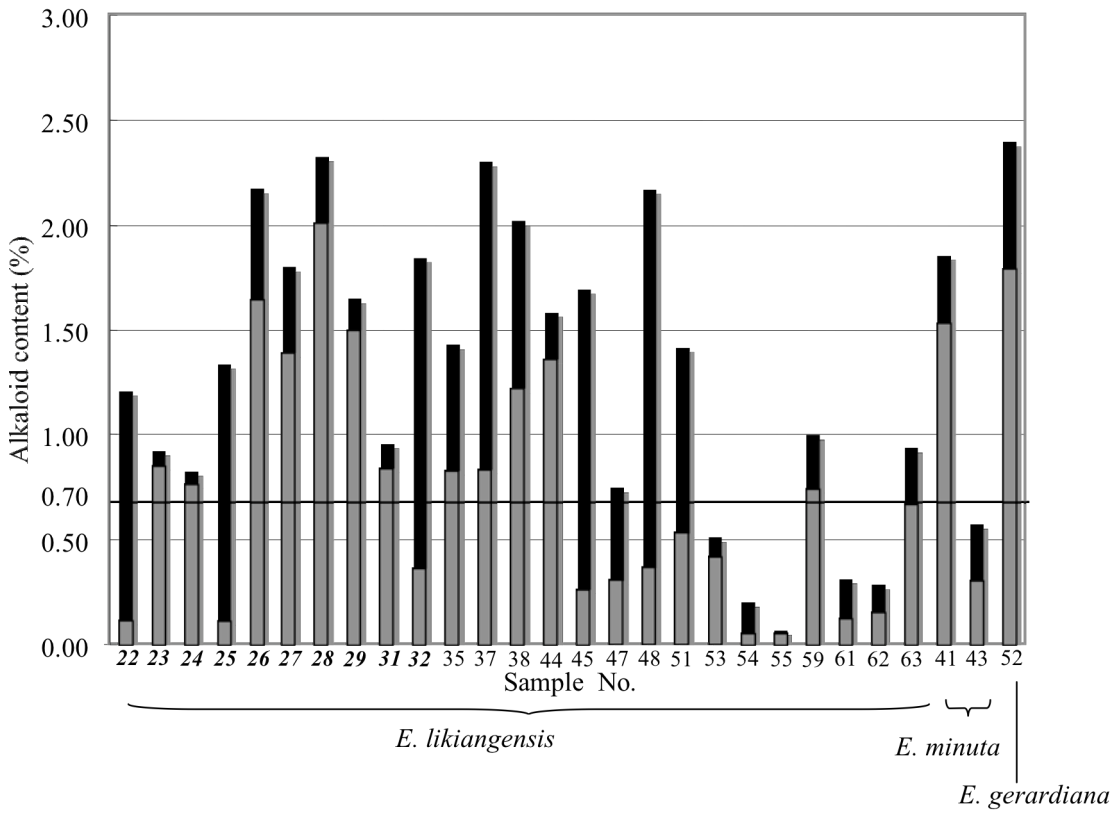

0.005
53
63
55
62
59-2
59-1
61
EM
EL
47
48
37
Type 1
32
31
29
26
25
44
24
45
35-1
35-2
38
28-1
28-2
23
22
43
41
Type 2
0.014
0.016
0.010
52
EG
ES

Fig. 3


3.00
2.50
2.00
1.50
1.00
0.70
0.50
0.00
Alkaloid content (%)
22 23 24 25 26 27 28 29 31 32 35 37 38 44 45 47 48 51 53 54 55 59 61 62 63 41 43 52
Sample No.
E. likiangensis
E. minuta
E. gerardiana