Molecular Analysis and Chemical Evaluation of *Ephedra* Plants in Mongolia

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Ephedrae herba has been used in traditional Chinese and Japanese (Kampo) medicine from ancient times, with the primary resource being in China. In the present study, a field survey as well as molecular and chemical assessments were conducted on *Ephedra* plants in Mongolia to clarify whether they could be an alternative resource of the *Ephedrae herba* used in Japanese Kampo medicine. *Ephedra sinica*, *E. equisetina*, *E. przewalskii*, *E. regeliana*, *E. monosperma* and an unknown taxon (ESP) collected in Mongolia were divided into 9 genotypes on the basis of nucleotide sequences of 18S ribosomal RNA (rRNA) gene and *trnK* gene. *E. sinica*, *E. equisetina*, and *E. monosperma* presented completely identical sequences to the corresponding species from China. The sequences of *trnK* gene and 18S rRNA gene provide a useful index for identification and taxonomic classification of Mongolian *Ephedra* plants. Quantitative analysis of 5 ephedrine alkaloids revealed that almost all Mongolian *Ephedra* plants contained high amounts of total ephedrine alkaloids (TAs, 1.86–4.90%) and a high percentage of pseudoephedrine in TAs differed obviously from the Chinese. *E. sinica* and *E. equisetina* found in eastern and central Mongolia, showing total contents of ephedrine and pseudoephedrine higher than 1.43%, were potential new resources of Japanese Pharmacopoeia grade *Ephedrae herba*.

Key words *Ephedra*; Mongolia; molecular identification; ephedrine alkaloid; HPLC analysis

Ephedrae herba, called “Mahuang” in Chinese and “Mao” in Japanese, has been used in traditional Chinese medicine and Kampo medicine for inducing perspiration, healing a cold, relieving cough and asthma and is included in a number of popular Kampo formulations such as Kakkonto and Shoseiryuto. In 1887, the main constituent, ephedrine, was first isolated by Dr. Nagayoshi Nagai and further developed as an effective medicine for asthma, opening a way to reaffirm the value and significance of traditional medicines.¹⁾ Besides ephedrine, other alkaloids such as pseudoephedrine, norephedrine and methylephedrine are also included in *Ephedrae herba* as active constituents.²⁾ Pharmacological studies revealed central nervous system (CNS) stimulation, anti-tussive, decongestion, anti-inflammatory activities of ephedrine and its analogue,^{3–6)} providing the scientific basis underlying its traditional uses. Besides traditional usage in Asia, it has also been used as a dietary supplement in U.S.A. and Europe in recent years. However, misuse or abuse of ephedrine-containing dietary supplement for the purpose of weight loss or euphoric stimulation has resulted in various adverse effects, such as headache, insomnia, heart palpitations, and even death. Therefore, U.S.A. and many western countries have imposed regulations or banned the sale of ephedrine-containing dietary supplements since 1994.⁷⁾

Ephedra plants are in high demand for both traditional medicinal use and also as raw materials for ephedrine extraction all over the world. In Japanese Pharmacopoeia (JP), *Ephedrae herba* is prescribed as the dried aerial parts of *Ephedra sinica* STAPF, *E. intermedia* SCHRENK et C. A. MEYER and *E. equisetina* BUNGE with no less than 0.7% of total alkaloids (the total of ephedrine and pseudoephedrine). In Japan, a high demand of about 500 tons/year of *Ephedrae herba* used in Kampo medicine has relied heavily on importation

from China.⁸⁾ However, overharvesting has induced desertification and caused the Chinese government to curtail the exporting of *Ephedra* plants since 1999.⁹⁾ It is urgent to investigate the growth status in neighboring regions not only for new resources of *Ephedrae herba* but also for its protection and the development of further cultivation.

About 40 species belonging to the genus *Ephedra* are distributed in arid regions of east and north Africa, Asia, Europe, North America and South America, and 14 species in China.¹⁰⁾ In Mongolia, the northern neighbor of China, 9 *Ephedra* species are reported and 8 of them are common with those growing in China.^{11,12)} Therefore, we estimated that Mongolian *Ephedra* might be useful as an alternative resource of *Ephedrae herba*. Aiming to investigate the state of *Ephedra* plants in Mongolia, we carried out field surveys from 2002 to 2004, during which wide distribution of several *Ephedra* species was observed. Some populations in southern areas showed curved or twisted stems and other populations lacked clear characters for botanical classification. It is commonly known that *Ephedra* plants are quite difficult to identify because the morphological characters are very simple and easily change adapting to their growth environment. Molecular analysis and chemical fingerprinting methods¹³⁾ have been used to identify *Ephedra* species, and molecular analyses of Chinese *Ephedra* plants based on chloroplast *chlB* gene,¹⁴⁾ *trnL/trnF* region and nuclear ribosomal DNA (rDNA) internal transcribed spacer 1 (ITS1), ITS2 sequence^{15,16)} have been reported. No genetic investigation has been done, however, on Mongolian *Ephedra* plants. Our previous studies demonstrated that comparing sequences of nuclear 18S rRNA gene and chloroplast *trnK* gene was a powerful tool to authenticate the botanical origin of herbal drugs and assist taxonomic classification.^{17,18)} Therefore, in the

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Fig. 1. Distribution Map of *Ephedra* Species and Their Genotypes in Mongolia

Collection points of *Ephedra* plants are indicated by "E No." Genotypes are shown by corresponding colors. *E. equisetina* and *E. monosperma* have the same genotype, thus the abbreviation of species name is added in parentheses. Marks indicate: white circle, genotype Si-I; cross in white circle, genotype Si-II; black circle, genotype E-I (*E. equisetina*); white diamond, genotype E-I (*E. monosperma*); cross in gray circle, genotype Esp; white box, genotype Pr-I; gray box, genotype Pr-II; black box, genotype Pr-III; white triangle, genotype Rg-I; gray triangle, genotype Rg-II.

Table 1. Plant Materials from China

Species	Voucher No. ^{a)}	Genotype ^{b)}	Locality	Date of collection	GenBank accession No.	
					18S rRNA gene	trnK gene
<i>Ephedra intermedia</i> SCHRENK. et C. A. MEYER	KANP02309	In	Guinan, Qinghai Prov., China	2002, Jul. 31		AB453798
<i>Ephedra intermedia</i> SCHRENK. et C. A. MEYER	KANP02363	In	Yuzhong, Gansu Prov., China	2002, Aug. 13	AB453786	AB453797
<i>Ephedra intermedia</i> SCHRENK. et C. A. MEYER	KANP02369	In	Longxi, Gansu Prov., China	2002, Aug. 14		
<i>Ephedra sinica</i> STAPF	KANP02109	Si-II	E-er-duo-si, Inner Mongolia, China	2002, Jun. 5	AB453792	AB453807
<i>Ephedra sinica</i> STAPF	KANP02133	Si-II	Datong, Shanxi Prov., China	2002, Jun. 8		
<i>Ephedra przewalskii</i> STAPF var. <i>przewalskii</i>	KANP02321	Pr-I	Dulan, Qinghai Prov., China	2002, Aug. 4		
<i>Ephedra przewalskii</i> STAPF var. <i>przewalskii</i>	KANP02340	Pr-I	Xi-meng-gu-zu-zi-zhi-zhou, Qinghai Prov., China	2002, Aug. 7	AB453790	AB453802
<i>Ephedra equisetina</i> BUNGE	KANP02314	E-I	Xining, Qinghai Prov., China	2002, Aug. 1	AB453784	AB453795
<i>Ephedra likiangensis</i> FLORIN	KANP0101001	E-I	Kangding Xian, Sichuan Prov., China	2001, Jul. 27	AB453787	AB453799
<i>Ephedra monosperma</i> GMELIN ex C. A. MEYER	KANP02378	E-I	Qilianshan, Qinghai Prov., China	2002, Aug. 1	AB453789	AB453801
<i>Ephedra gerardiana</i> WALLICH ex C. A. MEYER	KANP0101117	E-I	Shiqu Xian, Sichuan Prov., China	2001, Aug. 6	AB453785	AB453796
<i>Ephedra minuta</i> FLORIN	KANP0101039	Mi	Kangding Xian, Sichuan Prov., China	2001, Jul. 28	AB453788	AB453800

a) Voucher No. is specimen reference number of the Herbarium of the Medicinal Plants of the Faculty of Pharmaceutical Sciences in Kanazawa University. b) Genotype is determined on the basis of nucleotide sequences of 18S rRNA gene and trnK gene.

present study, we determined the nucleotide sequences of these two regions and compared them with 8 clearly identified Chinese *Ephedra* plants for objective identification and to aid in the taxonomic resolution of *Ephedra* plants in Mongolia. In addition, clarification of the chemical equality of Mongolian *Ephedra* plants and the available *Ephedra* herba is needed, because the constitutions of ephedrine alkaloids differ among species and there are also some species without these alkaloids.²⁾ Subsequently, quantitative analysis on five major ephedrine alkaloids was conducted by HPLC- photodiode array detector (PDA) method for quality evaluation.

MATERIALS AND METHODS

Materials Twelve samples of 8 *Ephedra* species from China and 47 samples collected from Mongolia were ana-

lyzed. The Chinese samples were identified by Prof. M. Mikage (Table 1). The vouchers are deposited in the Herbarium of Medicinal Plants, School of Pharmacy, College of Medical, Pharmaceutical and Health Science, Kanazawa University.

Mongolian samples were collected during our field investigations in 2002–2004 and 2006. Sample M874 was the only cultivated source in this study, which has been cultivated in Guulin, Gobi-Altay Prov., Mongolia by using the seeds collected in Inner Mongolia, China; otherwise, all were wild plants distributed in Mongolia. Most of them were carefully identified by Dr. C. Sanchir in the Institute of Botany, Mongolian Academy of Science and the authors themselves as *Ephedra sinica* STAPF, *E. equisetina* BUNGE, *E. monosperma* GMELIN ex C. A. MEYER, *E. regeliana* FLORIN and *E. przewalskii* STAPF. However, specimens collected in southern Mongo-

Fig. 2. Morphology of Specimen M929

Specimen M929 (genotype Esp) which was collected in Ih Bogdyn Nuruu, Bayan-hongor Prov. shows a typically allophoric feature. The left part shows general morphology of ESP and the right part resembles *E. przewalskii*.

lia showed curved or twisted stems and some had allophoric features as shown in Fig. 2. Here, we tentatively treated these samples as *Ephedra* sp. (ESP) and included all of them in the following molecular and chemical analyses. It is noteworthy that due to fewer morphological characteristics being available in "Flora of Mongolia"¹¹⁾ and the "Key to the vascular plants of Mongolia,"¹²⁾ floristic literature with valuable morphological information on *Ephedra* plants of neighboring regions were used as reference in morphological identification, such as "Flora Xinjiangensis,"¹⁹⁾ "Flora of China" and "Flora of Siberia."²⁰⁾

All the vouchers and samples from Mongolia are stored in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan (Table 2).

Isolation of Total DNA and PCR Amplification Total DNA was extracted from 50–100 mg of dry stems by DNeasy Plant Mini Kit (Qiagen, Germany). Using it as template, 18S rRNA gene and two fragments with partial overlap of *trnK* gene (fragments A and B) regions were amplified by polymerase chain reaction (PCR), respectively (Figs. 3, 4). The PCR was performed using 10–100 ng of total DNA as a template in 50 μ l of reaction mixture consisting of 1 $\times$ GoTag Flexi Buffer (pH 8.5, Promega, U.S.A.), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 U *Taq* polymerase (Promega, U.S.A.), and 2.5 mM of each primer. Three pairs of primers were used for PCR amplification, that is, 18S 5'F and ITS 57R for 18S rRNA gene; *trnK* 3914F and *trnK* In 3R for the fragment A of *trnK* gene; *trnK* In 2F and *trnK* 2R for the fragment B of *trnK* gene (Table 3). PCR amplifications were carried out in a Thermal Controller PTC-100 (MJ Research Inc., U.S.A.) with cycling conditions as shown below. 18S rRNA gene: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 67 °C for 50 s, extension at 72 °C for 2 min, and then final extension at 72 °C for 20 min; fragment A of *trnK* gene: initial denaturation at 94 °C for 3 min, followed by 35 cycles of de-

naturation at 94 °C for 50 s, annealing at 56 °C for 50 s, extension at 72 °C for 1 min 45 s, and then final extension at 72 °C for 20 min; fragment B of *trnK* gene: 94 °C for 3 min, 35 cycles of 94 °C for 50 s, 54 °C for 50 s, 72 °C for 1 min 45 s, and then 72 °C for 20 min. The 4 μ l of PCR product was resolved by 1.0% agarose gel electrophoresis and the remaining part was purified using a Montage PCR Centrifugal Filter Device (Millipore, U.S.A.).

Sequencing and Molecular Analysis Sequencing reaction of purified PCR products was carried out using ABI PRISM BigDye Terminator v3.1 Cycle sequencing kits (Applied Biosystems, U.S.A.) with the primers shown in Table 3. Sequence was determined directly by an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, U.S.A.). The analyzed sequences were assembled, and a consensus sequence of each sample was finally constructed. Then the obtained DNA sequences were aligned and compared by BioEdit program (ver. 7.0.8.2.).

HPLC Analysis of Ephedrine Alkaloids Quantitative analysis of five ephedrine alkaloids (ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine and methylephedrine, Fig. 5) was conducted by the reported method²¹⁾ with modification. It was performed by a Jasco HPLC system composed of a PU-1580 pump, a LC-1580-02 ternary gradient unit, and a MD-1510 multiwavelength detector. As for molecularly identified plants, the middle part of the stem except nodes was pulverized and then dried in a desiccator for 24 h. An aliquot (ca. 150 mg) was extracted two times with 5 ml of a mixed solvent of H₂O:acetonitrile:phosphoric acid=65:35:0.04 with 0.4% sodium lauryl sulfate (SDS) by ultrasonic extraction for 30 min and centrifuged 3000 *g* for 10 min. The divided supernatants were combined and made up to a final volume of 10 ml. An aliquot of 10 μ l filtered through a 0.45 μ m filter was subjected to HPLC analysis under the following conditions: column, Waters Symmetry C₁₈ column (4.6 mm $\times$ 150 mm i.d.); mobile phase, the same solvent as used in the extraction; flow rate, 0.8 ml/min; column temperature, 30 °C. Detection was performed at 210 nm.

Ephedrine and pseudoephedrine used as chemical standards were purchased from Daiichi Fine Chemical Co., Ltd. (Japan). Norephedrine was kindly provided by Dr. Yukiko Makino (Kanto-Shinetsu Regional Bureau of Health and Welfare, Japan). Methylephedrine standard was purified from methylephedrine parenteral solution (Fuso Pharmaceutical Industry, Ltd., Japan). Content of norpseudoephedrine was calculated using the calibration curve of norephedrine.

RESULTS

18S rRNA Gene and *trnK* Gene Sequences of *Ephedra* Plants from China The result of DNA analysis of Chinese *Ephedra* plants is shown in Table 4. The determined sequences of 18S rRNA gene were 1792 bp in length and 4 types of sequences were observed among the 8 species. *E. sinica*, *E. intermedia* and *E. przewalskii* possessed similar sequences with only 1 site difference. At nucleotide position 1709 from upstream, *E. sinica* presented double peaks of thymine (T)/cytosine (C) where the peak of T was obviously higher than that of C (T>C), whereas *E. intermedia* possessed T and *E. przewalskii* had C at this position. The other 5 species (*E. equisetina*, *E. likiangensis*, *E. monosperma*, *E.*

Table 2. Plant Materials Collected in Mongolia

Species	Voucher No. ^{a)}	Genotype ^{b)}	Point ^{c)}	Locality	Altitude (m)	Date of collection	GenBank accession No.	
							18S rRNA gene	trnK gene
<i>Ephedra sinica</i>	M874	Si-I	E11	Guulin, Govi-Altay Prov. (Cultivated; Seeds from Inner Mongolia)	1829	Aug. 5, 2002	AB453793	
<i>Ephedra sinica</i>	M1157	Si-I	E28	Ondorhaan–Batnorov, Hentiy Prov.	1060	Jul. 14, 2004		
<i>Ephedra sinica</i>	M969	Si-II	E16	Ulaanbaatar–Bayan-Onjuul, Tov Prov.	1429	Jul. 23, 2003		
<i>Ephedra sinica</i>	M990	Si-II	E17	Bayan-Onjuul, Tov Prov.	1437	Jul. 24, 2003		
<i>Ephedra sinica</i>	M1054	Si-II	E24	Ih Gazryn chuluu, Dundgovi Prov.	1430	Jul. 28, 2003		
<i>Ephedra sinica</i>	M1056	Si-II	E24	Ih Gazryn chuluu, Dundgovi Prov.	1430	Jul. 28, 2003		
<i>Ephedra sinica</i>	M1069	Si-II	E25	Mandalgovi–Ulaambaatar	1508	Jul. 28, 2003		
<i>Ephedra sinica</i>	M1071	Si-II	E26	Bornuur–Bayangol, Selenge Prov.	977	Jul. 30, 2003		
<i>Ephedra sinica</i>	M1156	Si-II	E27	Jargalthaan–Moron, Hentiy Prov.	1203	Jul. 13, 2004	AB453792	AB453807
<i>Ephedra sinica</i>	M1210	Si-II	E29	Sergelen–Choybalsan, Dornod Prov.	828	Jul. 17, 2004		
<i>Ephedra sinica</i>	M1211	Si-II	E30	Choybalsan–Matad, Dornod Prov.	836	Jul. 18, 2004		
<i>Ephedra sinica</i>	M1213	Si-II	E31	Choybalsan–Matad, Dornod Prov.	831	Jul. 18, 2004		
<i>Ephedra sinica</i>	M1215	Si-II	E32	Sangiyn Dalaynuul–Tamsagiyn hooloy, Dornod Prov.	697	Jul. 19, 2004		
<i>Ephedra sinica</i>	M1227	Si-II	E33	Matad–Erdenetsagaan, Suhbaatar Prov.	822	Jul. 20, 2004		
<i>Ephedra sinica</i>	M1229	Si-II	E34	Matad–Erdenetsagaan, Suhbaatar Prov.	822	Jul. 20, 2004		
<i>Ephedra sinica</i>	M1242	Si-II	E35	Dariganga, Suhbaatar Prov.	1058	Jul. 21, 2004		
<i>Ephedra equisetina</i>	M995	E-I	E18	Delgerhangay, Dundgovi Prov.	1642	Jul. 24, 2003	AB453784	AB453795
<i>Ephedra equisetina</i>	M998	E-I	E18	Delgerhangay, Dundgovi Prov.	1642	Jul. 24, 2003		
<i>Ephedra monosperma</i>	M581	E-I	E2	Hovsgol, Hovsgol Prov.	1845	Aug. 23, 2002	AB453789	AB453801
<i>Ephedra regeliana</i>	M707	Rg-I	E4	Songino–Jingiin halzan, Uvs Prov.	1489	Jul. 28, 2002		
<i>Ephedra regeliana</i>	M778	Rg-I	E6	Bayannuur, Uvs Prov.	983	Jul. 28, 2002		
<i>Ephedra regeliana</i>	M1301	Rg-I	E7	Talyn Har uul, Uvs Prov.	1888	Jul. 31, 2002	AB453791	AB453805
<i>Ephedra regeliana</i>	M1302	Rg-I	E7	Talyn Har uul, Uvs Prov.	1888	Jul. 31, 2002		
<i>Ephedra regeliana</i>	M872	Rg-I	E10	Altay–Sharga, Govi-Altay, Prov.	1498	Aug. 4, 2002		
<i>Ephedra regeliana</i>	M779	Rg-II	E6	Bayannuur, Uvs Prov.	983	Jul. 30, 2002		AB453806
<i>Ephedra przewalskii</i>	M871	Pr-II	E10	Altay–Sharga, Govi-Altay, Prov.	1498	Aug. 4, 2002		AB453803
<i>Ephedra przewalskii</i>	M931	Pr-III	E14	Ih Bogdyn Nuruu–Baga Bogdyn Nuruu, Bayanhongor Prov.	1489	Aug. 8, 2002	AB453790	AB453804
<i>Ephedra przewalskii</i>	M1028	Pr-III	E20	Bulgan–Hongor, Omnogovi Prov.	1618	Jul. 25, 2003		
<i>Ephedra przewalskii</i>	M1348-1	Pr-I	E42	Shinejinst–Ehiyn gol, Bayanhongor Prov.	1460	Jul. 25, 2006		AB453802
<i>Ephedra przewalskii</i>	M1348-2	Pr-I	E42	Shinejinst–Ehiyn gol, Bayanhongor Prov.	1460	Jul. 25, 2006		
<i>Ephedra</i> sp.	M928-1	Esp	E13	Ih Bogdyn Nuruu, Bayanhongor Prov.	2186	Aug. 8, 2002		
<i>Ephedra</i> sp.	M928-2	Esp	E13	Ih Bogdyn Nuruu, Bayanhongor Prov.	2186	Aug. 8, 2002		
<i>Ephedra</i> sp.	M928-3	Esp	E13	Ih Bogdyn Nuruu, Bayanhongor Prov.	2186	Aug. 8, 2002		
<i>Ephedra</i> sp.	M929	Esp	E13	Ih Bogdyn Nuruu, Bayanhongor Prov.	2186	Aug. 8, 2002		
<i>Ephedra</i> sp.	M930	Esp	E13	Ih Bogdyn Nuruu, Bayanhongor Prov.	2186	Aug. 8, 2002		
<i>Ephedra</i> sp.	M950	Esp	E15	Baruunbayan–Ulaan–Baga Bogdyn Nuruu, Ovorhangay Prov.	1961	Aug. 9, 2002		
<i>Ephedra</i> sp.	M951	Esp	E15	Baruunbayan–Ulaan–Baga Bogdyn Nuruu, Ovorhangay Prov.	1961	Aug. 9, 2002		
<i>Ephedra</i> sp.	M952	Esp	E15	Baruunbayan–Ulaan–Baga Bogdyn Nuruu, Ovorhangay Prov.	1961	Aug. 9, 2002	AB453794	AB453808
<i>Ephedra</i> sp.	M953	Esp	E15	Baruunbayan–Ulaan–Baga Bogdyn Nuruu, Ovorhangay Prov.	1961	Aug. 9, 2002		
<i>Ephedra</i> sp.	M954	Esp	E15	Baruunbayan–Ulaan–Baga Bogdyn Nuruu, Ovorhangay Prov.	1961	Aug. 9, 2002		
<i>Ephedra</i> sp.	M1027-1	Esp	E19	Bulgan–Hongor, Omnogovi Prov.	1471	Jul. 25, 2003		
<i>Ephedra</i> sp.	M1027-2	Esp	E19	Bulgan–Hongor, Omnogovi Prov.	1471	Jul. 25, 2003		
<i>Ephedra</i> sp.	M1029	Esp	E20	Bulgan–Hongor, Omnogovi Prov.	1618	Jul. 25, 2003		
<i>Ephedra</i> sp.	M1030	Esp	E21	Bulgan–Hongor, Omnogovi Prov.	1783	Jul. 25, 2003		
<i>Ephedra</i> sp.	M1036	Esp	E22	Uliin Davaa, Omnogovi Prov.	2035	Jul. 26, 2003		
<i>Ephedra</i> sp.	M1037	Esp	E22	Uliin Davaa, Omnogovi Prov.	2035	Jul. 26, 2003		
<i>Ephedra</i> sp.	M1039	Esp	E22	Uliin Davaa, Omnogovi Prov.	2035	Jul. 26, 2003		
<i>Ephedra</i> sp.	M1051	Esp	E23	Hanhongor–Huld, Omnogovi Prov.	1264	Jul. 27, 2003		
<i>Ephedra</i> sp.	M1052	Esp	E23	Hanhongor–Huld, Omnogovi Prov.	1264	Jul. 27, 2003		

a) Voucher No. cited here is the abbreviation of Komatsu *et al.* M-No. b) Genotype is determined on the basis of nucleotide sequences of 18S rRNA gene and *trnK* gene. c) Locality is shown in Fig. 1 with point number.

gerardiana and *E. minuta*) were of the same sequence which differed from that of *E. sinica* at nucleotide positions 625, 1381, 1683 and 1709.

The length of determined *trnK* gene sequences ranged

from 2307 to 2383 bp, and 13 sites of nucleotide differences were observed among the 8 species. *E. intermedia* had a long insertion consisting of 1 or 2 repeats of a 38 bp sequence unit, which resulted in this sequence being the longest among

the 8 *Ephedra* species. *E. sinica*, *E. intermedia*, *E. przewalskii* and *E. minuta* showed their own specific sequences in *trnK* gene and the remaining 4 species possessed the same sequence. Therefore, based on sequences of both genes, 8 Chinese *Ephedra* species were of 5 genotypes assigned as follows: Si-II type: *E. sinica*, In type: *E. intermedia*, Pr-I type: *E. przewalskii*, E-I type: *E. equisetina*, *E. likiangensis*, *E. monosperma* and *E. gerardiana*, Mi type: *E. minuta* (Table 4).

18S rRNA Gene and *trnK* Gene Sequences of Mongolian *Ephedra* Plants and Comparison with Those of Chinese Plants *Ephedra* plants collected in Mongolia were morphologically identified as 5 species (*E. sinica*, *E. equisetina*, *E. monosperma*, *E. przewalskii* and *E. regeliana*) and an unknown *Ephedra* sp. (ESP). Forty-nine samples were analyzed for sequences of 18S rRNA gene and *trnK* gene in the same way as Chinese samples. The results divided Mongo-

Fig. 5. Structures of 5 Ephedrine Alkaloids Used in Quantitative Analysis

Table 3. Primers of 18S rRNA Gene and *trnK* Gene for PCR and Sequencing Reaction

Primer	Sequence (5'→3')	Length (bp)
18S rRNA gene		
Forward primer		
18S 5' ^{a)}	CAA CCT GGT TGA TCC TGC CAG T	22
FZ 418	TCC AAG GAA GGC AGC AGG	18
18S 891F	GTA TTT CAT TGT CAG AGG TG	20
FZ 1308	TTT GTC TGG TTA ATT CCG TT	20
Reverse primer		
RZ 376	CCC TCT CCG GAA TCG AAC CC	20
RZ 905	ATC CAA GAA TTT CAC CTC T	19
RZ 1310	AAC GGA ATT AAC CAG ACA	18
ITS 57R ^{a)}	GAG AGC GAA CTT ACT CAG GGG TT	23
<i>trnK</i> gene		
Forward primer		
<u>trnK 3914F^{a)}</u>	TGG GTT GCT AAC TCA ATG G	19
<u>trnK In 1F</u>	ATG ACG ATT GAG ATT CTT TC	20
<u>trnK In 2F^{a)}</u>	AAT TTT CAA TCA GTC TTT TCT CTA	24
<u>trnK In 3F</u>	ATT GAT TTT GCT GGG AAC TAC TAT	24
<u>trnK In 4F</u>	GCA TGG CTT AGT TTT ACC GAC AAT	24
Reverse primer		
<u>trnK In 5R</u>	CTT AGC AAC CAG GCG TTC TC	20
<u>trnK In 4R</u>	AGA TAG TTT TGT TGA TGT AAT AGC	24
<u>trnK In 3R^{a)}</u>	CTT TAG CAA TCT TAT CGT GTC TTT	24
<u>trnK In 2R</u>	ACG TTT ATT TAT TGC TTC ACT GTA	24
<u>trnK In 1R</u>	TCA CAA AAA TTG ATC TAA CA	20
<u>trnK 2R^{a)}</u>	AAC TAG TCG GAT GGA GTA G	19

a) Primers for PCR amplification. New designed primers for *Ephedra* species are underlined.

Fig. 3. Approach for Sequence Determination of 18S rRNA Gene

The positions of primers used in PCR amplification and sequence determination of 18S rRNA gene are indicated by arrows and mountain-shaped arrows, respectively.

Fig. 4. Approach for Sequence Determination of *trnK* Gene

Two fragments with partial overlap were amplified by PCR to obtain the whole sequence of *trnK* gene. The positions of primers used in PCR amplification and sequence determination of *trnK* gene are indicated by arrows and mountain-shaped arrows, respectively.

Table 4. Nucleotide Differences in 18S rRNA Gene and *trnK* Gene of *Ephedra* Plants from China

		Nucleotide position															
		Nuclear 18S rRNA gene								Chloroplast <i>trnK</i> gene							
Species	Genotype	6	3	6	7	1	2	5	6	—	0	7	8	0	1	8	9
		2	8	8	0	8	5	3	8	5	8	2	2	2	6	5	4
		5	1	3	9	2	0	6	(units No.) ^{a)}	7	5	7	7	7	6	5	7
<i>Ephedra sinica</i>	Si-II	A	T	T	Y	T	C	C	(1)	G	G	C	G	G	G	G	C
<i>E. intermedia</i>	In	*	*	*	T	A	*	*	(2 or 3)	*	A	*	*	*	*	*	*
<i>E. przewalskii</i>	Pr-I	*	*	*	C	A	*	*	(1)	*	*	*	*	*	*	*	*
<i>E. equisetina</i>	E-I	G	C	C	C	A	G	T	(1)	*	*	T	*	A	*	T	C
<i>E. monosperma</i>	E-I	G	C	C	C	A	G	T	(1)	*	*	T	*	A	*	T	C
<i>E. likiangensis</i>	E-I	G	C	C	C	A	G	T	(1)	*	*	T	*	A	*	T	C
<i>E. gerardiana</i>	E-I	G	C	C	C	A	G	T	(1)	*	*	T	*	A	*	T	C
<i>E. minuta</i>	Mi	G	C	C	C	A	G	T	(1)	A	*	T	A	A	C	T	*

Numerals above sequence are nucleotide positions of *E. sinica*, which correspond to all other species except *E. intermedia*. Asterisk indicates the identical nucleotide to which of *E. sinica*. Y=C and T. a) Sequence of a repeat unit is GTGTTATTTTATTCTAAAAAGAAAGTTTAGGAGGTT (38 bp). Two or three of this sequence unit have been observed in *E. intermedia*.

Table 5. Nucleotide Differences in 18S rRNA Gene and *trnK* Gene of *Ephedra* Plants from Mongolia

		Nucleotide position																				
		Nuclear 18S rRNA gene								Chloroplast <i>trnK</i> gene												
Species	Genotype																					
		1	1	1																		
		6	3	6	7	1	2	5	7	8	8	9	0	3	7	8	0	1	1	2	2	
		5	8	8	0	8	5	3	5	8	—	8	9	2	2	8	5	7	1	3	4	8
		2	1	3	9	2	0	6	3	6	8	8	7	0	5	7	1	5	7	4	5	
<i>Ephedra sinica</i>	Si-I	A	T	T	T	T	C	C	T	G	T	G	T	C	C	C	G	C	G	G	C	G
	Si-II	*	*	*	Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
<i>E. equisetina</i>	E-I	G	C	C	C	A	G	T	*	*	*	*	*	T	*	*	A	*	T	C	T	A
<i>E. monosperma</i>	E-I	G	C	C	C	A	G	T	*	*	*	*	*	T	*	*	A	*	T	C	T	A
<i>E. przewalskii</i>	Pr-I	*	*	*	C	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Pr-II	*	*	*	C	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*
	Pr-III	*	*	*	C	A	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*
<i>E. regeliana</i>	Rg-I	*	*	*	C	A	*	*	*	C	A	C	C	*	*	T	A	*	*	*	*	*
	Rg-II	*	*	*	C	A	*	*	*	C	A	C	C	*	*	T	A	T	*	*	*	*
<i>Ephedra</i> sp.	Esp	*	*	*	Y	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Numerals above sequence are nucleotide positions of *E. sinica*, which correspond to all other species. Asterisk indicates the identical nucleotide to which of *E. sinica*. Y=C and T.

lian *Ephedra* plants into 9 genotypes (Table 5). *E. sinica*, *E. equisetina* and *E. monosperma* presented completely identical sequences in both gene regions to those of corresponding species from China (genotypes Si-II and E-I). However, two samples of *E. sinica* collected at points E11 and E28 showed a single peak of T (genotype Si-I) at position 1709 in 18S rRNA gene sequence, differing from others which showed obviously double peaks (T>C) as that of Chinese ones except one sample (M1069) with almost equal intensity of T and C (T=C). *E. przewalskii* was of the same 18S rRNA gene sequence as the Chinese congener, whereas three types of *trnK* gene sequences were observed. One type was the same as that of the same species from China (genotype Pr-I) and the other two differed from Pr-I type at 1 nucleotide site (genotypes Pr-II and Pr-III). *E. regeliana* collected in western Mongolia around Hyargas Lake, possessed its own specific nucleotide sequence, differing considerably from other Mongolian species at 1–4 nucleotide sites in 18S rRNA gene and at 6–13 sites in *trnK* gene region. The unknown ESP had identical sequence to genotype Si-II in the 18S rRNA gene region and to genotype Pr-I in the *trnK* gene region, and so was assigned as genotype Esp. The relative intensities of C and T at position 1709 in 18S rRNA gene sequence varied among samples, approximately two-thirds of the samples were T>C and one-third were C>T or T=C.

Contents of Ephedrine Alkaloids in Mongolian Samples In the HPLC chromatograms, the five ephedrine alkaloids were detected sequentially as norpseudoephedrine (retention time, 25.0±0.8 min), norephedrine (26.8±0.8 min), pseudoephedrine (28.5±0.9 min), ephedrine (31.4±0.9 min) and methylephedrine (33.8±1.0 min). Contents of the five ephedrine alkaloids in the stem of Mongolian *Ephedra* plants were quantitatively analyzed (Table 6).

Except for *E. przewalskii* (genotypes Pr-I, Pr-II and Pr-III), all samples contained high amounts of total ephedrine alkaloids (TAs), ranging from 1.86 to 4.90% of dry weight (Table 6). In regard to the relative contents of the two main constituents, ephedrine (Eph) and pseudoephedrine (PEph), a high percentage of PEph in TAs was observed in Mongolian

Ephedra plants, differing from the Chinese.²²⁾

E. sinica (genotypes Si-I and Si-II) contained 1.95–4.16% TAs and showed a relatively higher percentage of Eph in TAs (10.6–77.8%) than other species (≤35.8%). Within the species, the samples collected from eastern grassland areas showed quite a bit higher content of Eph (33.2–77.8% of TAs) than those from central areas (10.6–47.3% of TAs, Table 6, Fig. 6). *E. equisetina* showed the highest content of TAs (3.98–4.90%) and more than 90% of TAs was PEph. The two species satisfied the criterion in Japanese Pharmacopoeia that Eph and PEph comprise no less than 0.7% of the total: the sum contents were 1.43–3.68% in *E. sinica* and 3.81–4.59% in *E. equisetina* (Table 6). Therefore, these two species growing in Mongolia were found applicable as a new resource of “Mao” for Kampo medicine.

E. regeliana contained a similar amount of TAs as *E. sinica*, but the content of PEph accounted for more than 70% of TAs. Caveney *et al.*²⁾ reported that this species collected in Karakoram, Pakistan contained a comparable amount of ephedrine alkaloids to *E. intermedia*, but Long *et al.*¹⁶⁾ did not detect ephedrine alkaloids in their samples of this species collected in Xinjiang, China. These differences might be attributable to the growth environments or to taxonomic divergence in this species.

ESP (genotype Esp) showed higher TAs content (2.06–4.78%) and higher percentage of PEph in TAs (45.0–95.7% of TAs) than *E. sinica*. In addition, some samples presented allophoric features as shown in Fig. 2. The left part showed general morphology of ESP and the right part was similar to *E. przewalskii*. We analyzed separately the ESP parts and the *E. przewalskii*-resembling parts to check whether there were any differences in alkaloid content between these two parts. The results showed that generally the ESP part had higher ephedrine alkaloid content, but the relative compositions of the five alkaloids were similar.

DISCUSSION

Mongolia is one of the main habitats of *Ephedra* plants.

Table 6. Ephedrine Alkaloids Content of Mongolian *Ephedra* Plants

Point	Species	Genotype	Voucher No.	Contents (%)					TAs content (%)	Indication content in JP (%) ^{a)}	Eph/TAs (%)	PEph/TAs (%)
				Eph	PEph	NEph	NPEph	MEph				
E28	<i>Ephedra sinica</i>	Si-I	M1157	0.99	0.84	0.15	0.29	tr	2.26	1.82	43.7	37.1
E16	<i>Ephedra sinica</i>	Si-II	M969	0.39	2.24	0.33	0.69	tr	3.65	2.63	10.6	61.4
E17	<i>Ephedra sinica</i>	Si-II	M990	0.52	1.57	tr	0.15	0.12	2.36	2.10	22.1	66.8
E24	<i>Ephedra sinica</i>	Si-II	M1054	0.64	0.97	0.17	0.13	0.07	1.98	1.61	32.2	48.9
E24	<i>Ephedra sinica</i>	Si-II	M1056	1.97	1.66	0.22	0.11	0.20	4.16	3.63	47.3	39.9
E25	<i>Ephedra sinica</i>	Si-II	M1069	1.04	1.83	0.10	0.42	0.14	3.53	2.87	29.5	51.8
E26	<i>Ephedra sinica</i>	Si-II	M1071	0.38	1.62	0.18	0.25	0.10	2.53	2.00	15.0	64.0
E27	<i>Ephedra sinica</i>	Si-II	M1156	1.35	1.31	0.09	0.25	0.20	3.20	2.66	42.2	40.9
E29	<i>Ephedra sinica</i>	Si-II	M1210	0.90	1.45	tr	0.14	0.22	2.71	2.35	33.2	53.5
E30	<i>Ephedra sinica</i>	Si-II	M1211	2.14	0.22	tr	0.07	0.32	2.75	2.36	77.8	8.0
E31	<i>Ephedra sinica</i>	Si-II	M1213	1.28	1.12	0.07	0.26	0.19	2.92	2.40	43.9	38.4
E32	<i>Ephedra sinica</i>	Si-II	M1215	1.15	0.47	tr	0.12	0.12	1.86	1.62	61.8	25.1
E33	<i>Ephedra sinica</i>	Si-II	M1227	1.64	1.17	0.34	0.73	0.12	4.00	2.81	41.0	29.3
E34	<i>Ephedra sinica</i>	Si-II	M1229	1.00	0.88	tr	0.25	0.11	2.24	1.88	44.7	39.2
E35	<i>Ephedra sinica</i>	Si-II	M1242	1.13	0.30	0.14	0.27	0.11	1.95	1.43	57.9	15.4
E18	<i>Ephedra equisetina</i>	E-I	M995	0.14	4.45	0.12	0.12	0.07	4.90	4.59	2.8	90.8
E18	<i>Ephedra equisetina</i>	E-I	M998	0.15	3.66	tr	0.07	0.10	3.98	3.81	3.7	91.9
E10	<i>Ephedra przewalskii</i>	Pr-II	M871	—	tr	—	—	tr	tr	tr	—	—
E14	<i>Ephedra przewalskii</i>	Pr-III	M931	tr	tr	tr	—	tr	tr	tr	—	—
E20	<i>Ephedra przewalskii</i>	Pr-III	M1028	—	tr	—	—	—	tr	tr	—	—
E42	<i>Ephedra przewalskii</i>	Pr-I	M1348-1	tr	tr	tr	—	—	tr	tr	—	—
E42	<i>Ephedra przewalskii</i>	Pr-I	M1348-2	tr	tr	tr	—	—	tr	tr	—	—
E6	<i>Ephedra regeliana</i>	Rg-II	M779	0.41	1.44	tr	tr	0.10	1.95	1.85	21.1	74.0
E7	<i>Ephedra regeliana</i>	Rg-I	M1301	tr	2.78	tr	0.35	tr	3.13	2.78	—	88.8
E7	<i>Ephedra regeliana</i>	Rg-I	M1302	0.59	2.25	0.13	0.14	0.08	3.19	2.84	18.5	70.6
E13	<i>Ephedra</i> sp.	Esp	M928-1	0.48	2.87	0.25	0.66	tr	4.27	3.35	11.2	67.3
E13	<i>Ephedra</i> sp.	Esp	M928-2	0.53	3.15	0.31	0.79	tr	4.77	3.68	11.1	66.0
E13	<i>Ephedra</i> sp.	Esp	M928-3	0.41	3.10	0.25	0.76	tr	4.52	3.51	9.0	68.6
E15	<i>Ephedra</i> sp.	Esp	M950	1.01	3.19	tr	0.20	0.18	4.58	4.20	22.1	69.5
E15	<i>Ephedra</i> sp.	Esp	M951	0.14	2.73	tr	0.10	tr	2.97	2.87	4.6	91.9
E19	<i>Ephedra</i> sp.	Esp	M1027-1	1.13	2.58	0.18	0.74	tr	4.61	3.70	24.4	55.9
E19	<i>Ephedra</i> sp.	Esp	M1027-2	0.59	1.67	0.63	0.82	tr	3.72	2.27	15.9	45.0
E20	<i>Ephedra</i> sp.	Esp	M1029	0.37	3.00	tr	0.23	tr	3.60	3.37	10.3	83.4
E22	<i>Ephedra</i> sp.	Esp	M1036	1.03	1.98	tr	0.34	0.08	3.43	3.01	30.0	57.8
E22	<i>Ephedra</i> sp.	Esp	M1037	0.83	2.08	tr	0.37	tr	3.28	2.91	25.2	63.5
E22	<i>Ephedra</i> sp.	Esp	M1039	0.35	1.28	0.18	0.26	tr	2.06	1.62	16.8	62.0
E23	<i>Ephedra</i> sp.	Esp	M1051	1.31	1.71	tr	0.51	0.13	3.66	3.02	35.8	46.8
E23	<i>Ephedra</i> sp.	Esp	M1052	0.41	2.52	tr	0.07	0.16	3.16	2.93	13.0	79.6
E13	<i>Ephedra</i> sp.	Esp	M929	**	0.68	3.16	tr	0.17	4.16	3.83	16.3	75.8
				***	0.42	1.90	tr	0.19	2.52	2.33	16.8	75.6
E13	<i>Ephedra</i> sp.	Esp	M930	**	0.77	3.49	tr	0.19	4.61	4.26	16.7	75.7
				***	0.59	2.14	tr	0.27	3.00	2.73	19.6	71.4
E15	<i>Ephedra</i> sp.	Esp	M952	**	1.06	3.36	tr	0.19	4.78	4.42	22.2	70.4
				***	0.73	2.20	tr	0.17	3.10	2.93	23.5	71.1
E15	<i>Ephedra</i> sp.	Esp	M953	**	0.43	3.40	tr	0.13	4.09	3.83	10.5	83.3
				***	0.88	1.84	tr	0.24	2.95	2.71	29.7	62.3
E15	<i>Ephedra</i> sp.	Esp	M954	**	0.79	2.68	tr	0.11	3.74	3.47	21.2	71.7
				***	0.52	2.32	tr	0.08	2.91	2.83	17.7	79.5
E21	<i>Ephedra</i> sp.	Esp	M1030	**	tr	3.00	tr	0.23	3.23	3.00	—	93.0
				***	tr	2.30	tr	0.10	2.40	2.30	—	95.7

Eph: ephedrine, PEph: pseudoephedrine, NEph: norephedrine, NPEph: norpseudoephedrine, MEph: methylephedrine. Indication content in JP: sum contents of Eph and PEph. TAs content: total content of Eph, PEph, NEph, NPEph and MEph. a) JP: Japanese Pharmacopoeia, tr: <0.07%, —: not detected. Sample with allopathic features was analyzed separately and contents of ESP part (**) and *E. przewalskii*-resembling part (***) are shown.

Nine species are reported to distribute throughout the country. The characters of seed, integument tube, bracts of seed cone, etc. were particularly important for morphological identification of these plants.^{10–12,19,20)} We made great efforts to conduct field investigations in summer, the season of blossoming and fruiting of *Ephedra* plants. But in many habitats, reproductive organs had not been observed, which made morphological identification difficult. Moreover, it has been pointed out that even the cut products of *Ephedra* herba were impossible to be identified by morphological features.

The present study proposed genetic markers for objective authentication of *Ephedra* plants and *Ephedra* herba. Although intra-species variation was observed in *E. sinica*, *E. przewalskii* and *E. regeliana*, the five species and ESP collected in Mongolia could be discriminated from each other by the nucleotide sequences of the 18S rRNA-*trnK* genes. The distribution of *Ephedra* plants in Mongolia and their genotypes is shown in Fig. 1, integrating the results from field investigations and molecular analysis. *E. sinica* (genotypes Si-I and Si-II) was found in wide areas of eastern and central areas; *E.*

Fig. 6. Contents of Five Ephedrine Alkaloids in Mongolian *Ephedra* Species

Shaded column, ephedrine; white column, pseudoephedrine; sandy column, norephedrine; vertical line column, norpseudoephedrine; horizontal line column, methylephedrine. Contents are indicated as mean $\pm$ S.D.

equisetina was found in the mountainous region of the south-central area; *E. przewalskii* with genotypes Pr-I and Pr-III was found mainly in the southern area, growing together with *Ephedra* sp. (genotype Esp), and those with genotype Pr-II were at Sharga in the western area; *E. monosperma* was growing in a high mountain area (alt. 1845 m) near Hovsgol Lake in the northern area and *E. regeliana* (genotypes Rg-I and Rg-II) was found on western rocky slopes.

According to conventional morphological taxonomy, *E. przewalskii* is well separated from other *Ephedra* species by the texture of its cone bracts in mature seed.¹⁹⁾ *E. przewalskii* belongs to Section Alatae which is characterized by dry and winged bracts in mature seed, whereas others belong to Section Ephedra which possesses fleshy cone bracts. However, the present molecular data of 18S rRNA-*trnK* gene sequences did not support this classification, and *E. przewalskii* was genetically close to *E. sinica* and *E. intermedia*. Previous phylogenetic studies on *Ephedra* plants from China, Europe-Mediterranean, and America based on nucleotide sequences of ITS1, ITS2, *trnL/trnF*, *matK* and *rbcL* regions also suggest that the traditional classification on the basis of bracts texture might be unnatural.^{15,23)}

Although at first glance ESP was similar to *E. sinica* distributed in eastern and central areas, the characteristic features such as a height less than 25 cm, a stem often procumbent and with curved or twisted apex, etc. were different from *E. sinica*. These characters were clearly mentioned as discriminating *E. sinica* from *E. distachya* in "Flora of China"¹⁰⁾ and "Flora Xingiangensis"¹⁹⁾; that is, the morphology of ESP was thought to be consistent with *E. distachya* L. On the other hand, in "Flora of Siberia," *E. dahurica* TURZ. was also described as having a curved or twisted stem, and it was treated as a synonym of *E. distachya* auct. non L.²⁰⁾ In "Flora of Mongolia,"¹¹⁾ *E. dahurica* and *E. sinica* are listed separately, but *E. distachya* is not included, whereas in "Flora of China" and "Flora Xingiangensis," there is no *E. dahurica* citation. Due to these inconsistencies, further detailed investigation is needed to clarify the relation among the three species. In the present study genetic data suggested a close

relation of ESP to *E. sinica*. Some specimens of genotype Esp revealed allopheric features in the same plant. As shown in Fig. 2, the left part of the specimen shows general features of ESP, but the right part resembles *E. przewalskii* in color and stem roughness, number of scaly leaves, etc. We assumed that the very different features of these two parts might have resulted from growth periods, that is, the ESP part was the newer portion, younger than the *E. przewalskii*-resembling part. Higher ephedrine content of ephedrine alkaloids in the ESP parts was detected, which might be indirectly explained by the observation of Malla *et al.*,²⁴⁾ who found by tracing alkaloid content during 9 years of cultivation that ephedrine alkaloid in *E. gerardiana* decreased after 4 years. On the other hand, the nuclear rDNA sequence which is of biparental inheritance has been demonstrated to be effective in speculation of hybridization.²⁵⁾ The 18S rRNA gene sequence of the samples of allopheric features presented 1 site of double peaks (C and T) at position 1709 where high intensity of C (C>T or C $\approx$ T) differed from others, which possibly suggested a hybrid origin of these samples. Considering these genetic data (genotype Esp consisting of Si-II type 18S rRNA gene sequence and Pr-I type *trnK* gene sequence) as well as the distribution areas, the derivation of the allopheric specimens might be related to the pure line of ESP or its relatives and *E. przewalskii*. However, 18S rRNA gene presented hundreds or thousands of tandem repeats in the genome, and additive peaks at position 1709 resulting from one site of nucleotide difference among these repeated copies could not be absolutely excluded. Further studies, such as determination of other nuclear DNA regions as internal transcribed spacer or 5S rRNA spacer region together with detailed field investigations and even a crossing experiment, are needed to resolve the taxonomic position of these samples.

Almost all Mongolian *Ephedra* plants (except *E. przewalskii*) contained high amounts of TAs, 1.86–4.90% and a high percentage of pseudoephedrine in TAs (except *E. sinica* growing in the eastern area). On the other hand, it was reported that Chinese *Ephedra* plants contained more Eph than PEph, except for *E. intermedia*.²²⁾ The phenomenon that the content ratio of Eph to PEph varied considerably even in the same species was much attractive from the viewpoint of biosynthetic regulation of these two constituents. Further investigation of the regulatory mechanism on formation of these two compounds is ongoing. PEph was reported to have effect of anti-tussive, anti-inflammation and other effects,^{4,26)} suggesting the usefulness of Mongolian *Ephedra* plants to relieve rhinitis and nasal congestion.

In conclusion, through field investigations together with subsequent laboratory research, the present study assessed the molecular and chemical diversity of *Ephedra* plants in Mongolia. The determined nucleotide sequences of 18S rRNA gene and *trnK* gene provided a useful index for identification and taxonomic classification. Chemical assessment suggested that *E. sinica* and *E. equisetina* found in eastern and central Mongolia had potential as new resources of Ephedrae herba of Japanese Pharmacopoeia grade and that other species could be resources of ephedrine alkaloids. Except for *E. przewalskii*, other species were characterized by a relatively high ratio of pseudoephedrine. Moreover, the deterioration of the natural environment, especially the progress

of desertification in the southern area of Mongolia has been serious in recent years. It is necessary to promote the cultivation of selected species in selected suitable regions to secure a resource for *Ephedrae herba* and also for environmental preservation. Our results are believed to contribute much basic data to this effort.

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