Studies on Cultivated *Ephedra* Plants in Inner Mongolia Autonomous Region and Ningxia Hui Autonomous Region

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Progression of the desertification in northern China has been causing damage to wild *Ephedra* plants on which we depend for most of supply of the traditional herbal medicine, "Ma huang." The Chinese government encourages the cultivation of *Ephedra* plants, and *Ephedra* fields have been reclaimed in the original *Ephedra* habitats in recent years. We surveyed 7 *Ephedra* fields that have been recently developed in the Inner Mongolia Autonomous Region and Ningxia Hui Autonomous Region to collect information on *Ephedra* plant cultivation, especially pertaining to crop species. Specimens taken from those *Ephedra* fields were genetically and morphologically analyzed, and their ephedrine alkaloid content was examined. DNA analyses of *Ephedra* specimens, including DNA sequencing of ITS (internal transcribing sequence of nuclear ribosomal DNA) and *trn L/F* (intron of *trn*L and intergenic spacer between the *trn*L and *trn*F of chloroplast DNA) region and species-specific amplification of *trn L/F* were conducted to identify *Ephedra* species. Based on the results of DNA sequencing and morphological determination, the crops grown in 6 fields ware identified as *Ephedra sinica*, while co-planting of *E. sinica* and *E. intermedia* was found in one field where a higher appearance rate of plants with varied morphology from wild *Ephedra* plants was observed. Furthermore, direct sequencing of the PCR product of the *trn L/F* region of some specimens from the field and their species-specific PCR showed ambivalent result. Cloning and sequencing of the PCR product of the *trn L/F* region of those specimens DNA suggested their heteroplasmy, containing both *E. sinica*- and *E. intermedia*-type chloroplasts. On the other hand, the profile of the ephedrine alkaloid content was clearly correlated with the result of direct sequencing of the *trn L/F* region; the specimens showing the *E. sinica*-type sequence contained more ephedrine than pseudoephedrine, and the specimens of the *E. intermedia*-type more pseudoephedrine.

Key words *Ephedra* species; cultivation; nuclear ribosomal DNA (nrDNA); internal transcribing sequence (ITS); chloroplastic DNA

The Japanese demand for *Ephedra* plants is supplied mostly by imported products from China, which consist of wild plants grown in the northern and western provinces. Aiming at the restoration of the *Ephedra* resource and the prevention of desertification, the Chinese government has promoted the development of *Ephedra* fields in the original *Ephedra* habitats in recent years. Intending to collect information of the preceding practice of *Ephedra* cultivation in China, which provides us key knowledge of *Ephedra* cultivation in Japan, we have surveyed several *Ephedra* fields since 2002.[1)] Here, we report the result of the surveys conducted in 2003 about the fields in the Inner Mongolia Autonomous Region and Ningxia Hui Autonomous Region. In previous papers, we reported the phylogenetic relationship of the 8 wild *Ephedra* species in China by DNA sequence analysis of nuclear ribosomal DNA, ITS1 and ITS2, as well as chloroplastic DNA, *trn L/F*.[2,3)] Based on the ITS1 and 2 sequences, 8 species could be divided into 3 groups: Group 1 (*Ephedra intermedia*, *E. sinica*, *E. przewalskii*), Group 2 (*E. equisetina*, *E. monosperma*, *E. gerardiana*), and Group 3 (*E. likiangensis*, *E. minuta*). We found that *E. sinica* and *E. intermedia*, as well as *E. przewalskii*, were genetically close. We developed a simple PCR method for the classification of those 3 groups based on the DNA sequence variation in ITS 1. We also proposed a method of discrimination of *E. sinica* and *E. intermedia*, which have different sequences in the *trn L/F* region. Using these methods, we examined the cultivated *Ephedra* species.

MATERIALS AND METHODS

Plant Materials The collection sites and date, and specimen number and ID of the cultivated *Ephedra* plants are listed in Table 1.

DNA Extraction The plant stem was cut into 2 mm-pieces, frozen in liquid nitrogen and ground into powder. Using the DNeasy Plant Mini Kit (QIAGEN, Germany), the DNA was extracted according to the manufacturer's protocol.

PCR Amplification Total DNA was used as a template for amplifying the ITS1 and 2, and the *trn L/F* by PCR. The primers were designed based on 18S, 5.8S and 26S nuclear ribosomal DNA and the *trn L/F* region of chloroplast DNA sequences from the Genbank. The primers, Eph-F (GAC GTC GCG AGA AGT TCA TT) and Eph-R (GTA AGT TTC TCT TCC TCC GC) were used for amplification of the ITS region (ITS1, 5.8S and ITS2) (PCR 1). The primers, Aco-1F (CGA AAR CGG TAG ACG CTA CG) and Aco-2R (ATT TGA ACT GGT GAC ACG AG), were used to amplify the region of *trn L/F*. Standard PCR was performed in 50 μl of reaction mixture containing 5 μl of 10×PCR buffer for KOD-Plus, 0.2 mM each of dNTP, 1 mM $MgSO_4$, 0.4 μM of each primer, approximately 100 ng of template and 1 unit of KOD-Plus DNA polymerase (TOYOBO). PCR was carried out as follows: hot start at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and elongation at 68 °C for 45 s, and a final elongation at 68 °C for 5 min. Five microliters of PCR product was checked by

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Table 1. The Collection Sites and Date, and Sample Number and ID of the Cultivated *Ephedra* Plants

Site ID	Collection site	Sample ID	Collecting date
1	Zhang-gu-tai Cun, Dao-de Xiang, Kai-lu Prefecture, Tong-liao City, Inner Mongolia	804-3-1—15	August 4, 2003
2	Da-ling-pu, Wu-fen-di Zhen, Wen-niu-te Qi, Chi-feng City, Inner Mongolia	803-41-1—13	August 3, 2003
3	Dang-pu-di Xiang, Song-shan Qu, Chi-feng City, Inner Mongolia	803-12-1—3	August 3, 2003
4	Da-fang-long-zhuang Cun, Dang-pu-di Xiang, Song-shan Qu, Chi-feng City, Inner Mongolia	803-11-1—5	August 3, 2003
5	Tu-ge-tu-ge-cha Cun, Bu-la-ge Xiang, E-tuo-ke Qianqi, E-er-duo-si City, Inner Mongolia	912-1-1—13	September 12, 2003
6	Farm of Ningxia Luyuan Company, Ci-yao-pu-zhen coal mine, Ling-wu City, Ning-xia Province	911-1-1—29	September 11, 2003
7	Ephedra cultivation base of Guangxia company, Jin-sha Xiang,Yong-ning Prefecture, Yin-chuan City, Ning-xia Province	913-1-1—9	September 13, 2003

agarose gel electrophoresis and the remaining product was purified using the QIA quick PCR Purification Kit (QIAGEN, Germany).

Sequencing The purified PCR product was subjected to direct sequencing using a Bigdye Terminator Cycle Sequencing Kit (Applied Biosystem) with ABI PRISM 310 (Applied-Biosystem). The primers Aco-1F and Aco-2R were used for sequencing the region covering deletions in *trn L/F*. The DNA sequences were aligned using 'DNASIS' version 3.0 (Hitachi).

Differentiation of the 3 Groups of *Ephedra* Species by PCR (PCR 2) Differentiation of the 3 groups of *Ephedra* species by PCR was conducted using a primer set of ITS-B-R (GTG AGC GGC AAG TAA GAT CC) and ITS-1A (GCG GGG ACG TGG ACG GTC TT) to amplify ITS region (ITS 1, 5.8S and ITS2). The annealing temperature of PCR was set at 65 °C for the detection of Group 1. The chain reaction was repeated 25 times. The other PCR conditions were the same as PCR 1.

Discrimination of *E. sinica* and *E. intermedia* by PCR (PCR 3) Discrimination of *E. sinica* and *E. intermedia* by PCR was performed using a primer set of trnL-2R (CCG GCC GGT AAC ACG AAT TT) and Aco-1F to amplify *trn L/F* region. PCR conditions were the same as the standard PCR 1, except for the annealing temperature (60 °C) and reaction cycles (26 cycles).

Cloning of PCR Product PCR for amplification of *trn L/F* region was performed using a set of tagged-primers, BH Aco-1F (TCT TGG ATC CGA AAR CGG TAG ACG CTA CG) and HD Aco-2R (CTC CAA AGC TTT GAA CTG GTG ACA CGA G). The PCR product and a plasmid, Bluescript SKII (−), were digested with restriction enzymes, *Bam* HI and *Hind* III and ligated using a DNA ligation kit (Takara Shuzo Ltd, Kyoto). The ligation reaction mixture was used for the transformation of an *E. coli* strain, DH5α. Thirty clones from each transformation were picked up and plasmids of 20 clones were isolated using the alkaline method. After treatment with RNase A and then polyethylene glycol precipitation, the plasmid was used for the template of PCR amplification with the primers, Aco-1F and Aco-2R. The PCR product was sequenced by using the primer of either Aco-2R or *trn L/F* exon F (TCC CTC TAT CCC CAA GTT TG).

Analysis of Ephedrine Alkaloids The content of ephedrine alkaloid was determined using a previously reported procedure.[3)]

RESULTS

Morphological Identification of the Cultivated *Ephedra* Plants The surveys were conducted twice in 2003, from July 29 through August 12 in the eastern part of Inner Mongolia (site 1—4), and from September 9 through 23 in the western part of Inner Mongolia and the northern part of Ninxia (site 5—7). The result of field research was reported earlier.[1)] Three to 19 plant specimens were taken from each *Ephedra* field for identification. The morphology of sexual organs, such as seeds and flowers, is the principal criteria for the identification of *Ephedra* species.[4)] Most of the specimens taken from sites 1 and 7 bore seeds at the time of investigation. Judging from the fleshy seed cones with straight integument tubes, as well as the size and shape of aerial part, the species growing in fields 1 and 7 could be identified as *E. sinica*. On the other hand, the plants grown in the other *Ephedra* fields were bearing neither flowers nor seed cones. Considering the economical importance and our previous investigation of the wild *Ephedra* plants habitat in Inner Mongolia and Ningxia, *E. sinica* and *E. intermedia* were the most probable species for cultivation. According to the Flora of China,[4)] the two species can be distinguished by the size and color of the aerial part, as well as the number and shape of the leaf. *E. sinica* is small shrub, growing up to 40 cm, with greenish branchlets and has opposite leaves. *E intermedia* is taller shrub growing up to 1 m, yellowish or bluish green branchlets and whorls of 3 or opposite leaves. From these criteria, we assumed that the specimens taken from the site 2 through 5 to be *E. sinica* (Table 2). On the other hand, of the specimens collected in the field 6, *E. intermedia* seemed to be planted together with *E. sinica* and the morphologically unidentified *Ephedra* species. Two examples are seen in Fig 1. 911-1-1 and -18 have an *E. sinica*-type morphology, a long rootstock, opposite leaves with sharply pointed apexes, and that of *E. intermedia*, a whitish stem color.

DNA Analyses of the Cultivated *Ephedra* Plants As we reported in the previous paper, the wild *Ephedra* plants collected in China showed positive PCR result with a product of around 1700 bp by PCR 1. According to PCR 2, only group 1 *Ephedra* plants (*E. sinica*, *E. intermedia* and *E. przewalskii*) were positive, showing a PCR product of 450 bp. Since DNA sequences of ITS 1 and 2 of *E. sinica* and *E. intermedia* were identical, they should be discriminated by the *trn L/F* sequence on which PCR 3 was designed. All *Ephedra* plants except *E. sinica* showed a PCR product of 400 bp by PCR 3. Using these PCR analyses, the *Ephedra* specimens were examined. As summarized in Table 2, the specimens collected in the site 1 through 5 and site 7 showed positive

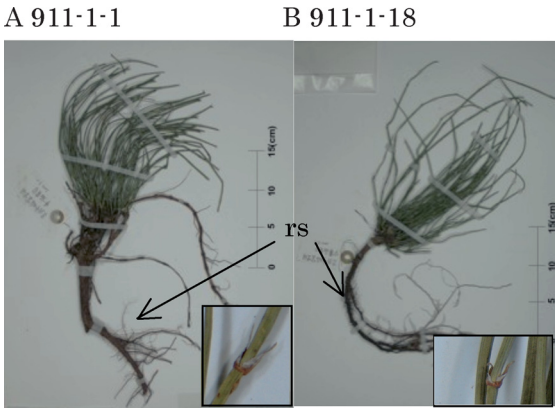

Fig. 1. Cultivated *Ephedra* Plants Specimens Collected from Site 6
rs: rootstock, in the insets are shown the magnified view of the leaves.

Table 2. Summary of Morphological Typing and Result of DNA Analyses

Site ID	Number of samples	Morphological typing	DNA typing deduced from PCR 1, 2 and 3	Result of *trn L/F* sequencing
1	15	ES	ES	ES
2	13	ES	ES	ES
3	3	ES	ES	ES
4	5	ES	ES	ES
5	13	ES	ES	ES
6	29	ES+EI+ND	ES+EI+ND	ES+EI
7	9	ES	ES	ES

ES: *E. sinica*, EI: *E. intermedia*, ND: not determined.

result in PCR 1 and 2, and PCR negativity in PCR 3, were thus concluded to be *E. sinica*. The sequencing result of the *trn L/F* region confirmed that all the specimens taken from-site 1 through 5 and site 7 had deletions at 458 and 459 of that region. The specimens from site 6, however, gave complicated results, as detail is given in Table 3. All 29 specimens were positive in PCR 1 and 2, meaning that they belonged to group 1 *Ephedra* plants. The morphology of *E. przewalskii*, larger in size and stout stems, is distinguishable from that of *E. intermedia* and *E. sinica*. The DNA sequences of ITS 1 and 2 of *E. przewalskii* were also different from those of *E. sinica* and *E. intermedia*.[3] The species is not recommended for medical use in Chinese and Japanese traditional medicine as described earlier, and the species is economically regarded poorly, because it does not contain ephedrine alkaloids. Thus *E. przewalskii* may be excluded from the possible species. The wild *E. intermedia* and *E. sinica* could be discriminated by PCR 3, and so were the cultivated samples 911-1-1 through 4 and 911-1-19 through 29, with some of their PCR results shown in Fig. 2. However, the result of PCR 3 of the remaining 14 specimens was ambivalent, meaning that a faint or even a clear band of PCR product (911-1-7, -8, -10, -11) was observed (Fig. 2, Table 3). DNA sequencing of the *trn L/F* region showed that half of the plant specimens in site 6 had guanine and thymine at 458 and 459, respectively. From the result, we concluded that they were *E. intermedia*. The other plants had deletions at these positions, and were identified as *E. sinica*.

Cloning and Sequencing Analysis of the PCR Product of the *trn L/F* Region of Some Specimens from Site 6 Since the result of direct sequencing of the PCR product of the *trn L/F* region and the species-specific PCR of 911-1-7, -8, -10 and -11 showed ambivalent results, their PCR product of the *trn L/F* region were cloned and the DNA sequence of each of the 20 clones were analyzed. As Table 4 shows, two

Table 3. Result of Morphological and DNA Identification of Samples Collected in the Site 6

Specimen ID	Morphological type	Result of PCR 3	*trn L/F* sequence
911-1-1	ND	+	EI
911-1-2	EI	+	EI
911-1-3	EI	+	EI
911-1-4	EI	+	EI
911-1-5	ND	−	ES
911-1-6	ND	−	ES
911-1-7	ES	+	ES
911-1-8	ND	+	ES
911-1-9	ND	±	ES
911-1-10	ND	+	ES
911-1-11	ND	+	ES
911-1-12	EI	+	EI
911-1-13	EI	+	EI
911-1-14	EI	+	EI
911-1-15	EI	+	EI
911-1-16	EI	+	EI
911-1-17	EI	±	EI
911-1-18	ND	±	EI
911-1-19	ES	−	ES
911-1-20	ES	−	ES
911-1-21	ES	−	ES
911-1-22	ES	−	ES
911-1-23	ND	−	ES
911-1-24	ND	−	ES
911-1-25	ES	−	ES
911-1-26	ES	−	ES
911-1-27	EI	+	EI
911-1-28	EI	+	EI
911-1-29	EI	+	EI

EI: *E. intermedia*, ES: *E. sinica*, ND: not determined.

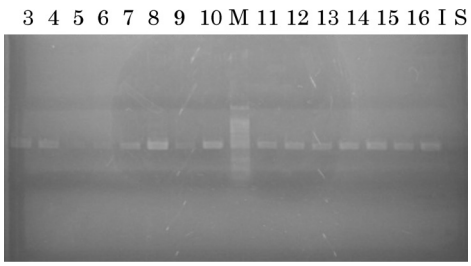

Fig. 2. Examples of PCR 3 Results
Lane 3: 911-1-3, lane 4: 911-1-4, lane 5: 911-1-5, lane 6: 911-1-6, lane 7: 911-1-7, lane 8: 911-1-8, lane 9: 911-1-9, lane 10: 911-1-10, lane M: size marker, lane 11: 911-1-11, lane 12: 911-1-12, lane 13: 911-1-13, lane 14: 911-1-14, lane 15: 911-1-15, lane 16: 911-1-16, lane I: *E. intermedia*, lane S: *E. sinica*.

Table 4. Sequencing Results of Clones

ID of specimen	Number of clone	
	Sinica type	Intermedia type
911-1-7	18	2
911-1-8	20	0
911-1-10	20	0
911-1-11	19	1

Table 5. Alkaloid Content of Specimens Collected from Site 6

Specimen ID	Alkaloid content (%)[a]		
	Ephedrine	Pseudo-ephedrine	Total[b]
911-1-1	0.77	0.87	1.80
911-1-2	0.07	1.07	1.18
911-1-3	0.08	1.52	1.65
911-1-4	0.07	1.43	1.46
911-1-5	1.97	t[c]	2.24
911-1-6	2.08	t	2.34
911-1-7	1.12	0.06	1.28
911-1-8	2.06	nd[d]	2.32
911-1-9	1.91	t	2.14
911-1-10	1.99	nd	2.24
911-1-11	2.03	0.01	2.33
911-1-12	t	1.99	2.01
911-1-14	nd	1.82	1.82
911-1-15	0.06	1.64	1.70
911-1-16	nd	2.17	2.17
911-1-17	nd	1.94	1.94
911-1-18	0.93	1.64	2.67
911-1-19	0.62	0.52	1.23
911-1-20	1.11	0.27	1.47
911-1-21	1.38	0.19	1.73
911-1-22	1.19	0.06	1.36
911-1-23	0.30	0.06	0.41
911-1-24	0.28	nd	0.32
911-1-25	0.29	0.32	0.63
911-1-26	0.30	0.17	0.52
911-1-27	0.44	1.08	1.69
911-1-28	0.11	2.10	2.24
911-1-29	0.44	1.08	1.69

a) Alkaloid content: calculated on a dry weight basis. *b*) Total: sum of ephedrine, methylephedrine, norephedrine and pseudoephedrine contents. *c*) t: trace. *d*) nd: not detected.

of those four specimens' DNA contained *E. intermedia*-type chloroplasts.

Ephedrine Alkaloids Content The sum of ephedrine and pseudoephedrine content of the site 6 specimens, except 911-1-23, -24, -25, -26, which were stunted, exceeded the minimum limit fixed by Japanese Pharmacopoeia, 0.7% (Table 5). The specimens determined as *E. intermedia* by sequencing analysis contained more pseudoephedrine than ephedrine. Likewise, the ephedrine content of the specimens determined as *E. sinica* was higher than pseudoephedrine.

DISCUSSION

Without flowers or seed cones, the morphological identification of *Ephedra* plants is hard to achieve. In this survey, therefore, we applied DNA analyses for the identification of the cultivated species. Combination of 3 PCR methods made it possible to analyze all of the plant specimens without sexual organs, except some samples from site 6, which were analyzed by DNA sequencing. From the detailed survey of cultivated *Ephedra* plant in the northeast provinces, Inner Mongolia and the northern part of Ningxia, we found that *E. sinica* was the major cultivation species. Nevertheless, co-planting of *E. sinica* and *E. intermedia* was detected in site 6, located in the northern part of Ningxia. In our previous paper, we showed that wild *E. sinica* habitats were located in the eastern part of Inner Mongolia and Qinghai and Xinjiang. The location of site 6 was nearer to the *E. intermedia* habitats than *E. sinica*. We presume that there may have been chances to obtain seed supply of *E. intermedia*. The fairly high percentage of specimens from site 6 did not show a typical morphology of *E. intermedia* or *E. sinica*. Furthermore, we failed to obtain a clear PCR 3 result of half of the site 6 specimens, we could identify them only by direct DNA sequencing. We speculated that plants grown in site 6 might be heteroplasmy,[5] and that the minority chloroplast was detectable by PCR 3. By direct DNA sequencing, on the other hand, only signals from the majority chloroplast could be detected. The further study including cloning of the PCR product of the *trn L/F* region confirmed that at least two specimens contained both *E. intermedia*- and *E. sinica*-type chloroplasts. Thus, the co-plantation of 2 species may affect their genetic and morphological identity. In the case of wild *Ephedra* plants, they seemed to be regenerated by sexual reproduction, during which the state of heteroplasmy is suppressed.[6] Although *E. intermedia* and *E. sinica* are both included in the Japanese and the Chinese Pharmacopoeia, the ephedrine alkaloid contents of the crude drugs prepared from wild plants of these 2 species were reported to be different, especially in the ratio of ephedrine to pseudoephedrine.[7] The difference in alkaloid content may be due to the difference in habitat, including climate, soil and growing state. As reported above, we observed similar results for cultivated plants that grew in the same conditions. Therefore, it can be concluded that the alkaloid content profile depends principally on their genetic background. The alkaloid content profile of specimens in heteroplasmy also showed a clear dependence on the direct sequencing result. The state of minor modifications of the genetic properties may not affect the secondary metabolite production, though it may influence the plant morphology. We were unable to find out whether the co-plantation was intended or accidental; however, we do think that the confusion over the cultivated species is not beneficial for its use as the crude drug or the raw material for ephedrine extraction. However, this confusion may occur more often for cultivated *Ephedra* plants in the future.

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