Regular Article

Field Survey of *Ephedra* Plants in Central Asia (1). Characterization of *Ephedra equisetina*, *Ephedra intermedia*, and Their Putative Hybrids Collected in the Zaravshan Mountains of Tajikistan

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Field surveys of *Ephedra* plants were conducted in the Zaravshan Mountains of Tajikistan. *E. equisetina*, *E. intermedia*, and their putative hybrids were collected. They were identified based on their phenotypes and their sequences of nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) region. Sequencing and species-specific PCR analyses of their ITS1 sequences revealed six putative hybrids of *E. equisetina* and *E. intermedia*. The total ephedrine and pseudoephedrine content of most of the *Ephedra* samples collected in Tajikistan were higher than the 0.7% lower limit prescribed by the Japanese pharmacopoeia, 17th edition (JP17), and varied from 0.34 to 3.21% by dry weight. The total alkaloid level of *E. intermedia* (11E08-1) cultivated in Japan varied from 1.77 to 2.30% by dry weight, which was much higher than the 0.7% lower limit prescribed by JP17.

Key words *Ephedra equisetina*; *Ephedra intermedia*; hybrid; ephedrine; pseudoephedrine; internal transcribed spacer 1

INTRODUCTION

Ephedra herb contains ephedrine (**Ep**) and pseudoephedrine (**PEp**), which are effective in treating asthma and nasal congestion.¹⁾ *Ephedra* herb is also an important ingredient in traditional Japanese Kampo prescriptions used to treat influenza, cough, nasal congestion, and obesity.^{2–4)} Three *Ephedra* species, *E. sinica* STAPF., *E. intermedia* SCHRENK et C. A. MEY., and *E. equisetina* BUNGE, have been recorded in the Japanese pharmacopoeia 17th edition (JP17).⁵⁾ All *Ephedra* herbs used in Japanese Kampo prescriptions are imported from China. However, the natural Chinese *Ephedra* resources have been rapidly depleted because of habitat destruction.⁶⁾

Tajikistan is a landlocked Central Asian country. Among the three *Ephedra* species recorded in JP17, *E. intermedia* and *E. equisetina* are distributed in Tajikistan.^{7–10)} *Ephedra* herb was collected in Tajikistan during the Soviet Union era for pharmaceutical production. In 1981, Japan imported 68 t of *Ephedra* herb from the former Soviet Union.¹¹⁾ Currently, however, *Ephedra* herb is no longer harvested in Tajikistan. Although Tajikistan is one of the most important habitats of *Ephedra* plants worldwide, the characteristics of *Ephedra* plants growing here have not yet been closely investigated. Thus, in the present study, field surveys were performed to characterize the *Ephedra* plants of Tajikistan.

MATERIALS AND METHODS

Plant Materials Field surveys of *Ephedra* plants were conducted in the Zaravshan Mountains of Tajikistan (Fig. 1). The herbaceous stems and seeds used in the present study were collected as shown in Fig. 1 and Table 1. *E. equisetina*

(Fig. 2A), *E. intermedia* (Fig. 2B), and their putative hybrids were collected and identified in field surveys. The plants were identified by Hiroaki Hayashi according to their phenotypic traits based on the descriptions of the Flora of the former U.S.S.R.,⁷⁾ Tajikistan,^{8,9)} and China.¹⁰⁾ The *Ephedra* specimens collected in the present study were deposited in the Herbarium of the Institute of Botany, Plant Physiology, and Genetics of the Academy of Science of Tajikistan.

Cultivation of *Ephedra* Plants Seeds of *Ephedra* plants (11E08 and 12E37) from Tajikistan were germinated and planted in pots containing vermiculite. They were fertilized with a 1000× dilution of liquid nutrients (Hanakoujou, Sumitomo Chemical Garden Products, Tokyo, Japan) and raised indoors under artificial light for >1 year. They were then transferred to the Herbal Garden of Iwate Medical University and grown outdoors for >3 years (11E08-1) and >1 year (12E37-1), respectively. A strain of *E. sinica* obtained from Tsumura & Co. (Tokyo, Japan) was also raised at the Herbal Garden of Iwate Medical University for >9 years. The herbal stems of these cultivated *Ephedra* plants were harvested on October 25, 2016 for HPLC analysis.

Chemicals Authentic **Ep** and **PEp** samples were obtained from Alps Pharmaceutical Industry Co., Ltd., Gifu, Japan.

Amplification and Sequencing of Nuclear Ribosomal DNA ITS1 Region DNA was extracted from dried herbaceous stems using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The 5'-terminal of the nuclear internal transcribed spacer 1 (ITS1) sequence was amplified by PCR using the template DNA from herbaceous stems, *Taq*-DNA polymerase (New England Biolabs Inc., Ipswich, MA, U.S.A.), anti-*Taq* high (Toyobo Co., Ltd., Osaka, Japan), and two reported primers for ITS1,^{12,13)} namely, 5'-GAC GTC GCG AGA AGT TCA

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Fig. 1. *Ephedra* Collection Sites in Tajikistan

Dotted lines on the map indicate the field survey route in Tajikistan.

TT-3' (Eph-F) and 5'-ACC ATA GAT AGG GGA AGC GTG TTA-3' (Eph-ITS 649R). After initial denaturation (2 min at 94°C), 30 cycles of 15 s at 94°C, 30 s at 55°C, and 15 s at 68°C, and a final elongation of 5 min at 68°C were performed on a thermocycler. The amplified fragments were treated with ExoSAP-It (Affymetrix/USB, Alfa Aesar, Tewksbury, MA, U.S.A.). The purified fragments were directly sequenced using the dideoxy chain termination method (3130xl Genetic Analyzer, Applied Biosystems, Foster City, CA, U.S.A.) and the two primers used in PCR amplification. To obtain the full ITS1 sequence, the 3'-terminal of the ITS1 sequence was amplified and sequenced using two reported primers for ITS1,^{12,13} namely, 5'-ATT TGA GAC AAA CGT CTC CC-3' (Eph-ITS 405F) and 5'-CGG GAT TCT GCA ATT CAC AC-3' (5.8S-R), as described above.

Species-Specific PCR Analysis A partial region of the nuclear ITS1 sequence was amplified by PCR using the template DNA from herbaceous stems, *Taq*-DNA polymerase (New England Biolabs), anti-*Taq* high (Toyobo Co., Ltd.), and two species-specific primers, namely, 5'-GCT CTC CGT AGA AGG AAC CGG ATG-3' and 5'-GGG AGA CGT TTG TCT CAA ATA TTT TTT TGA-3' for *E. equisetina* or 5'-GCT CTC CGT TGA AGG AAC CGG AAA-3' and 5'-GGG AGA CGT TTG TCT CAA ATA TTT TTT TAT-3' for *E. intermedia*. After initial denaturation (2 min at 94°C), 30 cycles of 15 s at 94°C, 30 s at 55°C, and 15 s at 68°C, and a final elongation of 5 min at 68°C were performed using a thermocycler.

HPLC Analysis of Ep and PEP The **Ep** and **PEP** content were determined using the method described in earlier studies, with a slight modification.¹⁴ Dried stem samples were ground with a mortar and a pestle, and 40 mg of each powdered sample was added to 4 mL of a mixture of MeCN, H₂O, and H₃PO₄ (400:600:0.4) with 0.4% sodium dodecyl sulfate

(SDS) and extracted ultrasonically for 60 min. An aliquot (2 µL) of the extract was analyzed by photodiode-array HPLC as follows: Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan) was used; column, Inertsil ODS-3 (3 µm, 2.1 mm i.d. × 150 mm, GL Sciences, Tokyo, Japan); solvent, MeCN, H₂O, and H₃PO₄ (400:600:0.4) with 0.4% SDS; flow rate, 0.2 mL/min; column temperature, 40°C. The quantities of **Ep** and **PEP** were determined on the basis of their peak areas of UV absorption at 210 nm. The calibration curves for **Ep** and **PEP** were as follows: **Ep** content (%) = Peak Area of **Ep**/1975817, **PEP** content (%) = Peak Area of **PEP**/1967978. The linearity was good in the 0–5% range for **Ep** and **PEP**. The identity of each constituent was verified by comparing its retention time and UV spectrum to that of its respective authentic sample.

Nucleotide Sequence The nucleotide sequence data reported in this paper are deposited in the DDBJ, EMBL, and GenBank under the accession numbers: LC342287 (*E. equisetina*, 11E02), LC342288 (*E. intermedia*, 11E03), LC342289 (*E. equisetina*, ITS1-EE1), LC342290 (*E. equisetina*, ITS1-EE2), LC342291 (*E. equisetina*, ITS1-EE3), LC342292 (*E. equisetina*, ITS1-EE4), LC342293 (*E. equisetina*, ITS1-EE5), and LC342294 (*E. intermedia*, ITS1-EI1).

RESULTS

Field Surveys of *Ephedra* Plants in the Northwestern Mountain Range of Tajikistan Field surveys of *Ephedra* plants were carried out in the Zaravshan Mountains of Tajikistan as shown in Fig. 1. This region was the *Ephedra* production center of the former Soviet Union. In total, 73 *Ephedra* samples from 50 plants were collected as shown in Table 1 and Fig. 1. These plants were identified as *E. equisetina*

Table 1. *Ephedra* Plants Collected in Tajikistan

Plant No.	Herbarium accession No.	Collection site coordinates		ITS1 genotype by sequencing	Species-specific PCR		Species
		GPS data	Altitude (m)		<i>E. equisetina</i>	<i>E. intermedia</i>	
11E01	68559	39°07'32.01"N/68°51'46.56"E	2390m	ITS1-EE2	○		<i>E. equisetina</i>
11E02	68560	39°07'46.14"N/68°51'49.56"E	2380m	ITS1-EE1	○		<i>E. equisetina</i>
11E03	68611	39°07'52.60"N/68°51'46.73"E	2270m	ITS1-EI1		○	<i>E. intermedia</i>
11E04	68612	39°10'18.30"N/68°47'32.21"E	2090m	ITS1-EI1		○	<i>E. intermedia</i>
11E05	68613	39°11'43.78"N/68°38'48.78"E	1870m	ITS1-EI1		○	<i>E. intermedia</i>
11E06	68614	39°11'33.76"N/68°37'56.15"E	2020m	ITS1-EI1		○	<i>E. intermedia</i>
11E07	68615	39°11'17.54"N/68°37'41.17"E	2100m	ITS1-EI1		○	<i>E. intermedia</i>
11E08	68616	39°11'18.34"N/68°37'40.90"E	2100m	ITS1-EI1		○	<i>E. intermedia</i>
11E09	68561	39°11'09.85"N/68°37'43.18"E	2130m	ITS1-EE3	○		<i>E. equisetina</i>
11E10	68617	39°10'30.36"N/68°37'40.53"E	2270m	ITS1-EI1		○	<i>E. intermedia</i>
12E13	68625	39°5'13.41"N/68°23'40.54"E	2380m	ITS1-EI1		○	<i>E. intermedia</i>
12E14	68563	39°3'3.84"N/68°18'18.61"E	2400m	ITS1-EE3	○		<i>E. equisetina</i>
12E15	68564	39°3'0.93"N/68°18'20.49"E	2400m	ITS1-EE3	○		<i>E. equisetina</i>
12E16	68626	39°03'27.97"N/68°20'35.48"E	2250m	ITS1-EI1		○	<i>E. intermedia</i>
12E17	68668	39°03'30.55"N/68°20'50.79"E	2210m	MIX	○	○	Putative hybrid
12E18	68565	39°03'30.55"N/68°20'50.79"E	2210m	ITS1-EE3	○		<i>E. equisetina</i>
12E19	68566	39°03'30.55"N/68°20'50.79"E	2210m	ITS1-EE1	○		<i>E. equisetina</i>
12E20	68567	39°5'10.71"N/68°22'13.24"E	2220m	ITS1-EE4	○		<i>E. equisetina</i>
12E21	68568	39°5'9.84"N/68°22'17.55"E	2220m	ITS1-EE1	○		<i>E. equisetina</i>
12E22	68569	39°05'3.43"N/68°23'28.84"E	2380m	ITS1-EE3	○		<i>E. equisetina</i>
12E27	68572	39°10'18.59"N/68°47'33.06"E	2090m	ITS1-EE5	○		Putative hybrid
12E31	68631	39°08'28.29"N/68°39'43.15"E	2460m	ITS1-EI1		○	<i>E. intermedia</i>
12E32	68632	39°08'29.31"N/68°39'43.47"E	2460m	ITS1-EI1		○	<i>E. intermedia</i>
12E37	68574	39°06'15.03"N/68°24'29.79"E	2020m	ITS1-EE3	○		<i>E. equisetina</i>
12E39	68575	39°05'03.36"N/68°23'29.11"E	2380m	ITS1-EE1	○		<i>E. equisetina</i>
12E41	68577	39°05'03.52"N/68°23'28.89"E	2380m	ITS1-EE1	○		<i>E. equisetina</i>
12E44	68580	39°02'57.57"N/68°18'20.85"E	2400m	MIX	○	○	Putative hybrid
12E45	68581	39°02'56.86"N/68°18'19.90"E	2400m	ITS1-EE1	○		<i>E. equisetina</i>
12E46	68582	39°02'57.12"N/68°18'20.05"E	2400m	ITS1-EE3	○		<i>E. equisetina</i>
12E47	68583	39°02'55.93"N/68°18'19.65"E	2400m	ITS1-EE5	○		<i>E. equisetina</i>
12E48	68636	39°03'31.53"N/68°20'29.80"E	2320m	ITS1-EI1		○	<i>E. intermedia</i>
12E51	68584	39°03'30.73"N/68°20'50.96"E	2210m	MIX	○	○	Putative hybrid
12E53	68586	39°03'30.55"N/68°20'50.79"E	2210m	ITS1-EE3	○	○	Putative hybrid
12E54	68638	39°03'31.03"N/68°20'51.46"E	2210m	ITS1-EI1		○	<i>E. intermedia</i>
12E55	68670	39°03'30.93"N/68°20'51.41"E	2210m	MIX	○	○	Putative hybrid
12E56	68639	39°08'17.22"N/68°27'45.48"E	1820m	ITS1-EI1		○	<i>E. intermedia</i>
12E57	68640	39°08'17.17"N/68°27'45.76"E	1820m	ITS1-EI1		○	<i>E. intermedia</i>
12E58	68641	39°11'13.78"N/68°35'08.17"E	1720m	ITS1-EI1		○	<i>E. intermedia</i>
13E06	68591	39°03'21.21"N/68°20'39.21"E	2210m	ITS1-EE1	○		<i>E. equisetina</i>
13E07	68592	39°03'20.85"N/68°20'39.48"E	2210m	ITS1-EE1	○		<i>E. equisetina</i>
13E08	68593	30°01'58.42"N/68°16'19.52"E	2470m	ITS1-EE3	○		<i>E. equisetina</i>
13E09	68594	39°04'00.16"N/68°17'24.14"E	2420m	ITS1-E1	○		<i>E. equisetina</i>
13E10	68595	39°03'03.81"N/68°18'19.08"E	2390m	ITS1-E1	○		<i>E. equisetina</i>
13E11	68642	39°10'43.26"N/68°31'17.81"E	1700m	ITS1-EI1		○	<i>E. intermedia</i>
13E29	68647	39°11'30.34"N/69°07'02.74"E	2460m	ITS1-EI1		○	<i>E. intermedia</i>
13E30	68605	39°12'59.74"N/69°00'36.22"E	2350m	ITS1-EE3	○		<i>E. equisetina</i>
13E31	68649	39°11'02.49"N/68°43'15.44"E	2070m	ITS1-EI1		○	<i>E. intermedia</i>
13E32	68650	39°10'59.70"N/68°42'46.71"E	2060m	ITS1-EI1		○	<i>E. intermedia</i>
13E33	68606	39°01'55.77"N/68°16'17.99"E	2470m	ITS1-EE3	○		<i>E. equisetina</i>
13E35	68608	39°02'00.88"N/68°16'20.99"E	2470m	ITS1-EE3	○		<i>E. equisetina</i>

(Fig. 2A), *E. intermedia* (Fig. 2B), and their putative hybrids (*E. sp.*), based on their morphologies and DNA analyses. *E. equisetina* plants collected in the present study were 1–2 m in height, and their woody stems were well developed. Their internodes were 1.5–3 cm in length and 1–2 mm in diameter. Their seed cones contained one seed, fleshy red bracts, and slightly curved integument tubes (Fig. 3A). In contrast, *E. in-*

termedia plants collected in the present study were 0.5–1 m in height. Their internodes were 3–6 cm in length and 1.5–3 mm in diameter. Their seed cones contained two seeds, fleshy red bracts, and spirally twisted, long integument tubes (Fig. 3B).

Nineteen *Ephedra* species, namely, *E. strobilacea* BUNGE, *E. ciliata* FISCH. et C. A. MEY., *E. aitchisonii* V. NIKITIN, *E. heterosperma* V. NIKITIN, *E. glauca* REGEL, *E. tesquorum* V.

NIKITIN, *E. tibetica* V. NIKITIN, *E. microsperma* V. NIKITIN, *E. ferganensis* V. NIKITIN, *E. persica* V. NIKITIN, *E. intermedia*, *E. regeliana* FLORIN, *E. minuta* FLORIN, *E. equisetina*, *E. valida* V. NIKITIN, *E. gerardiana* WALLICH et C. A. MEY., *E. pulvinaris* V. NIKITIN, *E. fedtschenkoi* PAULSEN, and *E. lomatolepis* SCHRENK are listed in the Flora of Tajikistan.^{8,9)} Furthermore, *E. glauca*, *E. tesquorum*, *E. tibetica*, *E. microsperma*, *E. ferganensis*, and *E. persica* have been recorded as synonyms of *E. intermedia* in the Flora of China.¹⁰⁾ In addition, *E. heterosperma*, a newly defined *Ephedra* species in the Flora of Tajikistan, has a spirally twisted integument tube,⁸⁾ characteristic of *E. intermedia*.¹⁰⁾ Thus, in the present study, *Ephedra* plants collected in Tajikistan were identified based on the classification of *Ephedra* in the Flora of China.¹⁰⁾

Characterization of *Ephedra* Plants Based on ITS1 Se-

Fig. 2. *Ephedra equisetina* (A, 11E02) and *E. intermedia* (B, 11E03) in Tajikistan

quences The *Ephedra* plants collected from Tajikistan were characterized by determining their nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) sequences. Figure 4 shows the alignment of the ITS1 sequences of the *E. equisetina* (11E02) and *E. intermedia* (11E03) collected in the present study. The 3'-terminal part of the ITS1 region is rich in substitutions and deletions. Therefore, it was difficult to analyze the 3'-terminal ITS1 regions of some of the *Ephedra* samples collected in Tajikistan by direct sequencing. For this reason, we focused on the 5'-terminal part of the ITS1 region. The partial ITS1 sequences of 50 *Ephedra* plants were analyzed. All *E. intermedia* plants identified by their morphological traits showed the same ITS1 sequence. In contrast, the *E. equisetina* plants identified by their morphological characteristics were divided by ITS1 genotype (Table 2). Putative hybrids were also identified by direct sequencing. They showed mixed sequences derived from both *E. equisetina* and *E. intermedia* (Table 2). Two *Ephedra* plants (12E17 and 12E55) had a nearly 50% mixed ratio of *E. intermedia* sequences. Plants 12E44 and 12E51 showed almost 25% mixed ratio of *E. intermedia* sequences.

The morphological traits of these plants, showing mixed sequences by direct sequencing, differed from those of *E. equisetina* and *E. intermedia*. Therefore, these plants may be hybrids of the two species. Two putative hybrids (12E17 and 12E55), which had neither seed cones nor pollen cones, were shrubs of 1 m height, and their woody stems were well developed, similar to that in *E. equisetina*. However, their internodes were 3–6 cm in length and 2–4 mm in diameter, which were similar to those of *E. intermedia*. Another putative hybrid (12E44) measured 1 m in height, and its woody stem was also well-developed, like that in *E. equisetina*. This plant had seed cones with one seed, fleshy red bracts, and slightly curved integument tubes, which were similar to those of *E. equisetina*. In contrast, internodes of 12E44 were 1.5–2.5 cm in length and 1.5–2 mm in diameter, which were thicker than those of *E. equisetina*. The morphological traits of 12E51 were similar to those of *E. equisetina*, although 12E51 showed almost 25% mixed ratio of *E. intermedia* sequences.

To confirm hybridization of the plants collected in the present study, PCR was conducted using species-specific primers (Fig. 4) for the ITS1 sequences of *E. equisetina* and *E. intermedia*, respectively (Fig. 5). Four putative hybrids (12E17,

Fig. 3. Seed Cones of *Ephedra equisetina* (A, 12E37) and *E. intermedia* (B, 13E11) Collected in Tajikistan

<i>E. equisetina</i> 11E02	1	TATGTTTTTACAGGTACCCCAAGGTAGAGGACCGCGCTTCTTCCTGCTGAGCGGGTAACCGCTGAGTAAGTTCGCTCTCCGT	120
<i>E. intermedia</i> 11E03	1	TATGTTTTTACAGGTACCCCAAGGTAGAGGACCGCGCTTCTTCCTGCTGAGCGGGTAACCGCTGAGTAAGTTCGCTCTCCGT	120
		Equisetina-specific-F 5'-GCTCTCCGTAGAAGGAACCGGATG-3' →	
		Intermedia-specific-F 5'-GCTCTCCGTAGAAGGAACCGGAAA-3' →	
<i>E. equisetina</i> 11E02	121	CTGCAATCCCTCCGAGAA- GGGGGTCCATACGGTTTAATTCGTGGACGGGGAGAGTTCTCCCGTGTCTGCCAGAGGGCCATGGGCGTTCCTCTGCGGGACACCGGTGAAGCGGGA	239
<i>E. intermedia</i> 11E03	121	CTGCAATCCCTCCGAGAA- GGGGGTCCATACGGTTTAATTCGTGGACGGGGAGAGTTCTCCCGTGTCTGCCAGAGGGCCATGGGCGTTCCTCTGCGGGACACCGGTGAAGCGGGA	240
<i>E. equisetina</i> 11E02	240	AAGAGGTGCGGGGAATCTGCCCGTCATTAC- GGTCCCTCCCTTTAGGGAAGGAGAGGATACGTTTGCGACACCGGTTGGCTTTTCGGGACCGTGACTGCCGGGAAGAAGTACGGGGAATCGCG	359
<i>E. intermedia</i> 11E03	241	AAGAGGTGCGGGGAATCTGCCCGTCATTAC- GGTCCCTCCCTTTAGGGAAGGAGAGGATACGTTTGCGACACCGGTTGGCTTTTCGGGACCGTGACTGCCGGGAAGAAGTACGGGGAATCGCG	360
<i>E. equisetina</i> 11E02	360	GGACGTGGACCGTCTTTGACCAAGTTTATAAATTA- AAAAAATATTGAGACAAACGCTCCGAGGGGTGTGAACATTATCGGCATGGGATGCTTGGCACATCATAGTA- STAGACAT	476
<i>E. intermedia</i> 11E03	361	GGACGTGGACCGTCTTTGACCAAGTTTATAAATTA- AAAAAATATTGAGACAAACGCTCCGAGGGGTGTGAACATTATCGGCATGGGATGCTTGGCACATCATAGTA- STAGACAT	480
		Eph-ITS405F 5'-ATTGAGACAAACGCTCCCC-3'	
		3'-AGTT---TTTTATAAAGCTGTTTTCGAGAGGG-5' Equisetina-specific-R	
		3'-TATTTTTTATAAAGCTGTTTTCGAGAGGG-5' Intermedia-specific-R	
<i>E. equisetina</i> 11E02	477	GGTGTGTCGACCCCGCTTCCCGAGCGAC- GGTGGTTCACGGTTAGGTGGGTGTTCT- GATAACAG- CCCCCTGAGTGGCGC- CCGGGGCGGTGGAT- GGCTCACTCGTTGGGG	596
<i>E. intermedia</i> 11E03	481	GGTGTGTCGACCCCGCTTCCCGAGCGAC- GGTGGTTCACGGTTAGGTGGGTGTTCT- GATAACAG- CCCCCTGAGTGGCGC- CCGGGGCGGTGGAT- GGCTCACTCGTTGGGG	600
<i>E. equisetina</i> 11E02	597	TGCTCCGCGCGGCCCTTCCCGGTGAACACGCTTCCCTATCTATGGTCT- CCGGACGGTGTGGCGTAGGTTGCGGAGGGGAACGTTGGGACGCGCTATGGACCGCGCTATTTCCT	716
<i>E. intermedia</i> 11E03	601	TGCTCCGCGCGGCCCTTCCCGGTGAACACGCTTCCCTATCTATGGTCT- CCGGACGGTGTGGCGTAGGTTGCGGAGGGGAACGTTGGGACGCGCTATGGACCGCGCTATTTCCT	720
		3'-ATTGTGCAAGGGATAGATACCA-5' Eph-ITS649R	
<i>E. equisetina</i> 11E02	717	TTCTACGTGCTGGGACGTTAAACCAACCAAGGTC- CTGGAATGC- TGGTGCAG- GGGGGCGGATCT- C- CGCTCA- TGGGCTTCCGCCCCCTCCCGTCCGCGCGG	831
<i>E. intermedia</i> 11E03	721	TTCTACGTGCTGGGACGTTAAACCAACCAAGGTC- CTGGAATGC- TGGTGCAG- GGGGGCGGATCT- C- CGCTCA- TGGGCTTCCGCCCCCTCCCGTCCGCGCGG	840
<i>E. equisetina</i> 11E02	832	ACGCGGTGTGTCG- SATGGGGCGGATCT- C- CGCTCA- TGGGCTTCCGCCCCCTCCCGTCCGCGCGG	940
<i>E. intermedia</i> 11E03	841	ACGCGGTGTGTCG- SATGGGGCGGATCT- C- CGCTCA- TGGGCTTCCGCCCCCTCCCGTCCGCGCGG	959
<i>E. equisetina</i> 11E02	941	CATGGGCTTCCGCCCCCTCCGTCGTC- CCTCTCTTCCGGGG- GCCATATCCAAATTTT- CTGATAGAGAAAACITGGGCGGGAGTTCCGCGCAAGAGGGCGGAGTACCGCA	1060
<i>E. intermedia</i> 11E03	960	CATGGGCTTCCGCCCCCTCCGTCGTC- CCTCTCTTCCGGGG- GCCATATCCAAATTTT- CTGATAGAGAAAACITGGGCGGGAGTTCCGCGCAAGAGGGCGGAGTACCGCA	1079
<i>E. equisetina</i> 11E02	1061	AGGAAGCAATCTGGCAACGGGC- CGCGGGGTGATTCCTG- GCGCCGCC- G- CAACGAAA	1120
<i>E. intermedia</i> 11E03	1080	AGGAAGCAATCTGGCAACGGGC- CGCGGGGTGATTCCTG- GCGCCGCC- G- CAACGAAA	1139

Fig. 4. Alignment of the ITS1 Sequences of *Ephedra equisetina* (11E02) and *E. intermedia* (11E03) Collected in TajikistanTable 2. Nucleotide Differences of Nuclear Ribosomal DNA ITS1 Region of *Ephedra* Plants Collected in Tajikistan

Species	Genotype	Nucleotide position of nuclear ribosomal DNA ITS1 region of <i>E. equisetina</i>																		
		33	47	83	96	97	138–139	180	216	228	268	310	391–392	468	474	507	534	544	563	579
<i>E. equisetina</i>	HQ882770	C	C	A	T	G	AG	C	C	C	T	C	TC	C	C	C	G	A	C	T
	AY 394073	Y	C	A	T	G	AG	C	C	C	T	C	TC	C	C	C	G	A	C	T
	ITS1-EE1	C	C	A	T	G	AG	C	C	C	T	C	TC	C	C	C	G	A	C	T
	ITS1-EE2	Y	C	A	T	G	AG	C	C	Y	T	C	TC	C	C	C	G	A	C	T
	ITS1-EE3	C	C	A	T	G	AG	C	C	C	T	C	TC	Y	C	C	G	A	C	T
	ITS1-EE4	C	C	A	T	G	AG	C	C	C	T	Y	TC	C	C	C	G	A	C	T
	ITS1-EE5	C	Y	A	T	G	AG	C	C	C	T	C	TC	C	C	C	G	A	C	T
<i>E. intermedia</i>	AY 394070	C	C	T	A	A	ATG	T	T	C	C	C	TAAAT	T	T	T	A	C	A	A
	ITS1-EI1	C	C	T	A	A	ATG	T	T	C	C	C	TAAAT	T	T	T	A	C	A	A
Putative hybrids	12E17	C	C	W	W	R	nd.	nd.	nd.	nd.	nd.	nd.	nd.	Y	Y	Y	R	M	M	W
	12E55	C	C	W	W	G	nd.	nd.	nd.	nd.	nd.	nd.	nd.	Y	Y	Y	R	M	M	W
	12E44	C	Y	A	W	R	nd.	nd.	nd.	nd.	nd.	nd.	nd.	C	C	Y	R	M	M	W
	12E51	C	Y	W	W	R	nd.	nd.	nd.	nd.	nd.	nd.	nd.	C	C	Y	R	M	C	A

12E55, 12E44, and 12E51) were identified by species-specific PCR as well as direct sequencing. In addition, two *Ephedra* plants (12E27 and 12E53), both morphologically identified as *E. equisetina*, generated PCR products specific to both *E. intermedia* and *E. equisetina*. Therefore, these two plants may also be derived from hybrids of the two species.

Ep and PEp Content of *Ephedra* Plants Collected in Tajikistan HPLC analysis was performed to determine the **Ep** and **PEp** content in the *Ephedra* samples collected in the present study. Table 3 shows the **Ep** and **PEp** levels in the herbaceous stems of *E. equisetina* collected in Tajikistan. The total alkaloid content of **Ep** and **PEp** (**TAC**) varied from 0.34 to 2.69% by dry weight. The **TAC** was >0.7% by dry weight in 91% of the *E. equisetina* samples, where 0.7% is the lower limit prescribed by JP17. The relative alkaloid ratios (**RAR**, **Ep**/[**Ep** + **PEp**]) of the *E. equisetina* samples ranged from 0 to 0.99. Table 4 shows the content of **Ep** and **PEp** in the herbaceous stems of *E. intermedia* collected in the pres-

ent study. The **TAC** of the *E. intermedia* samples varied from 1.06 to 3.21% by dry weight, which were higher than the 0.7% lower limit prescribed by JP17. The **RAR** of the *E. intermedia* samples ranged from 0.02 to 0.19. Table 5 shows the content of **Ep** and **PEp** in the stems of the putative hybrids identified by species-specific PCR. The **TAC** of the putative hybrids varied from 0.73 to 2.92% by dry weight, which were also higher than the 0.7% lower limit prescribed by JP17. The **RAR** of the putative hybrids ranged from 0.34 to 0.70.

Figure 6 compares the **TAC** and **RAR** of *E. equisetina*, *E. intermedia*, and their putative hybrids. Most of *E. equisetina* were plotted in the *E. equisetina* group except for 13E08 and 13E10, which were plotted in the *E. intermedia* group. Many putative hybrids were plotted in the *E. equisetina* group but 12E17 and 12E55 were located between *E. equisetina* and *E. intermedia*.

Ep and PEp Content of *Ephedra* Plants Cultivated in Japan The **Ep** and **PEp** content of the *Ephedra* plants col-

Fig. 5. Identification of Putative Hybrids by PCR Using Species-Specific Primers for ITS1

E: PCR using *E. equisetina*-specific primers; I: PCR using *E. intermedia*-specific primers.Table 3. Contents of Ephedrine (**Ep**) and Pseudoephedrine (**PEp**) in Herbaceous Stems of *Ephedra equisetina* Collected in Tajikistan

Plant No.	Sample No.	Collection date	Content (% of dry weight) of			RAR Ep/(Ep + PEp)
			Ep	PEp	TAC (Ep + PEp)	
11E01	1	Oct. 9, 2011	0.91	0.81	1.72	0.53
	2	Jun. 3, 2012	0.55	0.46	1.01	0.54
11E02	1	Oct. 9, 2011	1.10	0.56	1.66	0.66
	2	Jun. 3, 2012	0.88	0.53	1.41	0.63
11E09	1	Oct. 9, 2011	1.16	0.93	2.09	0.55
	2	Jun. 2, 2012	1.01	0.56	1.57	0.64
12E14	1	Jun. 3, 2012	1.48	0.98	2.46	0.60
	2	Oct. 14, 2012	1.10	0.68	1.78	0.62
12E15	1	Jun. 3, 2012	0.26	0.37	0.63	0.41
	2	Oct. 14, 2012	0.38	0.61	0.99	0.38
12E18	1	Jun. 3, 2012	0.88	0.24	1.12	0.78
12E19	1	Jun. 3, 2012	1.28	0.46	1.74	0.73
	2	Oct. 14, 2012	1.45	0.53	1.98	0.73
12E20	1	Jun. 3, 2012	1.09	0.28	1.37	0.79
12E21	1	Jun. 3, 2012	0.27	0.07	0.34	0.79
12E22	1	Jun. 3, 2012	0.81	0.32	1.13	0.72
	2	Oct. 13, 2012	1.07	0.49	1.56	0.68
12E37	1	Oct. 13, 2012	0.64	0.00	0.64	0.99
12E39	1	Oct. 13, 2012	0.56	0.40	0.96	0.58
12E41	1	Oct. 13, 2012	1.16	1.16	2.32	0.50
12E45	1	Oct. 14, 2012	0.73	0.57	1.30	0.56
12E46	1	Oct. 14, 2012	0.70	0.70	1.40	0.50
12E47	1	Oct. 14, 2012	1.11	0.89	2.00	0.55
13E06	1	Jul. 27, 2013	0.95	0.49	1.44	0.66
13E07	1	Jul. 27, 2013	1.67	1.02	2.69	0.62
13E08	1	Jul. 27, 2013	0.00	1.17	1.17	0.00
	2	Oct. 19, 2013	0.00	1.15	1.15	0.00
13E09	1	Jul. 28, 2013	1.29	0.60	1.89	0.68
13E10	1	Jul. 28, 2013	0.31	1.26	1.57	0.20
13E30	1	Oct. 13, 2013	0.60	0.67	1.27	0.47
13E33	1	Oct. 19, 2013	0.89	0.50	1.39	0.64
13E35	1	Oct. 19, 2013	0.60	0.57	1.17	0.51

lected in Tajikistan were determined to be very high. Therefore, we cultivated *Ephedra* plants using seeds derived from those plants. Table 6 shows the **Ep** and **PEp** content in the stems of the *Ephedra* plants cultivated in Japan. The **TAC** of *E. intermedia* (11E08-1) varied from 1.77 to 2.31% by dry weight, which was much higher than the 0.7% lower limit prescribed by JP17. The **RAR** of *E. intermedia* (11E08-1) ranged from 0.16 to 0.19 and was similar to those of the *E. intermedia* collected in Tajikistan (Table 4). In contrast, the **TAC** of *E. equisetina* (12E37-1) varied from 0.44 to 0.88% by dry weight, and the **RAR** ranged from 0.89 to 0.92. In addition, the **TAC** of *E. sinica* (IMU-ES1) varied from 0.14 to 0.30% by dry weight, which were lower than the 0.7% lower limit prescribed by JP17. The **RAR** of *E. sinica* (IMU-ES1) ranged from 0.46

to 0.51.

DISCUSSION

Ephedra plants is one of the most important medicinal plants used in traditional Kampo prescriptions. Therefore, field surveys of *Ephedra* plants have been undertaken in many countries such as China,^{6,14,15} Mongolia,^{13,16} Pakistan,¹⁷ and Nepal.¹⁸ However, the characteristics of the *Ephedra* plants of Central Asia, including Tajikistan, have not yet been explored in depth. In the present study, *E. equisetina*, *E. intermedia*, and their putative hybrids were collected from the Zaravshan Mountains of Tajikistan. This region was the production center of *Ephedra* herb of the former Soviet Union. Numerous

Table 4. Contents of Ephedrine (**Ep**) and Pseudoephedrine (**PEp**) in Herbaceous Stems of *Ephedra intermedia* Collected in Tajikistan

Plant No.	Sample No.	Collection date	Content (% of dry weight) of			RAR
			Ep	PEp	TAC (Ep + PEp)	Ep/(Ep + PEp)
11E03	1	Oct. 9, 2011	0.06	1.29	1.35	0.04
	2	Jun. 3, 2012	0.07	1.86	1.93	0.04
11E04	1	Oct. 9, 2011	0.08	2.53	2.61	0.03
	2	Jun. 3, 2012	0.10	2.45	2.55	0.04
11E05	1	Oct. 9, 2011	0.12	3.03	3.15	0.04
	2	Jun. 2, 2012	0.09	2.07	2.16	0.04
11E06	1	Oct. 9, 2011	0.32	2.45	2.77	0.12
	2	Jun. 2, 2012	0.32	1.90	2.22	0.14
11E07	1	Oct. 9, 2011	0.47	2.74	3.21	0.15
	2	Jun. 2, 2012	0.40	2.80	3.20	0.13
11E08	1	Oct. 9, 2011	0.34	2.25	2.59	0.13
	2	Jun. 2, 2012	0.29	1.85	2.14	0.14
11E10	1	Oct. 9, 2011	0.20	2.51	2.71	0.07
	2	Jun. 2, 2012	0.23	2.68	2.91	0.08
12E13	1	Jun. 2, 2012	0.42	1.77	2.19	0.19
	2	Oct. 13, 2012	0.24	1.15	1.39	0.17
12E16	1	Jun. 3, 2012	0.28	2.13	2.41	0.12
	2	Oct. 14, 2012	0.23	1.66	1.89	0.12
12E31	1	Sep. 30, 2012	0.21	1.16	1.37	0.15
12E32	1	Sep. 30, 2012	0.09	1.95	2.04	0.05
12E48	1	Oct. 14, 2012	0.13	1.86	1.99	0.07
12E54	1	Oct. 14, 2012	0.10	0.96	1.06	0.10
	2	Oct. 19, 2013	0.10	1.13	1.23	0.08
12E56	1	Oct. 14, 2012	0.04	1.88	1.92	0.02
12E57	1	Oct. 14, 2012	0.39	1.93	2.32	0.17
12E58	1	Oct. 14, 2012	0.14	2.74	2.88	0.05
13E11	1	Jul. 28, 2013	0.23	1.95	2.18	0.11
13E29	1	Oct. 13, 2013	0.11	1.73	1.84	0.06
13E31	1	Oct. 13, 2013	0.12	1.83	1.95	0.06
13E32	1	Oct. 13, 2013	0.36	2.42	2.78	0.13

Table 5. Contents of Ephedrine (**Ep**) and Pseudoephedrine (**PEp**) in Herbaceous Stems of Putative Hybrids of *Ephedra* Plants Collected in Tajikistan

Plant No.	Sample No.	Collection date	Content (% of dry weight) of			RAR
			Ep	PEp	TAC (Ep + PEp)	Ep/(Ep + PEp)
12E17	1	Jun. 3, 2012	0.64	1.19	1.83	0.35
	2	Oct. 14, 2012	0.55	1.04	1.59	0.34
	3	Oct. 19, 2013	0.66	1.23	1.89	0.35
12E27	1	Sep. 29, 2012	1.14	0.95	2.09	0.55
12E44	1	Oct. 14, 2012	1.37	0.84	2.21	0.62
	2	Oct. 19, 2013	1.62	0.92	2.54	0.64
12E51	1	Oct. 14, 2012	0.47	0.26	0.73	0.64
	2	Oct. 19, 2013	0.80	0.55	1.35	0.59
12E55	1	Oct. 14, 2012	0.61	1.13	1.74	0.35
	2	Oct. 19, 2013	1.03	1.89	2.92	0.35
12E53	1	Oct. 14, 2012	1.26	0.54	1.80	0.70

Ephedra habitats were observed during this field survey, and the *Ephedra* resources of the Zaravshan Mountains may potentially supply *Ephedra* herb worldwide.

The *Ephedra* plants collected in Tajikistan were divided into seven ITS1 genotypes including putative hybrids with mixed ITS1 sequences. All the ITS1 sequences of the *E. intermedia* plants had the same genotype, namely, ITS-EI1. However, those of the *E. equisetina* plants were divided into five different ITS1 genotypes, namely, ITS-EE1, ITS-EE2,

ITS-EE3, ITS-EE4, and ITS-EE5. Similar results were obtained for *Ephedra* plants collected in China^{12,14)} Pakistan,¹⁷⁾ and Mongolia.¹³⁾

Two putative hybrids of *E. equisetina* and *E. intermedia*, 12E17 and 12E55, were observed in the present study and showed identical mixed ITS1 sequences. Two other putative hybrids (12E44 and 12E51) were also identified. They showed mixed ITS1 sequences with a major *E. equisetina* component and a minor *E. intermedia* component. PCR analysis using

Table 6. Contents of Ephedrine (**Ep**) and Pseudoephedrine (**PEp**) in Herbaceous Stems of *Ephedra* Plants Cultivated in Iwate, Japan

Plant No.	Species	Collection date	Content (% of dry weight) of			RAR
			Ep	PEp	TAC (Ep + PEp)	Ep /(Ep + PEp)
IMU-ES1	<i>Ephedra sinica</i>	Oct. 25, 2016	0.14	0.15	0.29	0.47
			0.14	0.16	0.30	0.46
			0.07	0.07	0.14	0.51
11E08-1	<i>Ephedra intermedia</i>	Oct. 25, 2016	0.29	1.48	1.77	0.16
			0.31	1.66	1.97	0.16
			0.45	1.86	2.31	0.19
12E37-1	<i>Ephedra equisetina</i>	Oct. 25, 2016	0.45	0.05	0.50	0.90
			0.41	0.03	0.44	0.92
			0.78	0.10	0.88	0.89

Fig. 6. Relationship between Total Alkaloid Content (TAC, **Ep** + **PEp**) and Relative Alkaloid Ratios (RAR, **Ep**/(**Ep** + **PEp**)) of *Ephedra equisetina* (○), *E. intermedia* (●), and Putative Hybrids (▲) Collected in Tajikistan

species-specific ITS1 primers indicated that two additional plants (12E27 and 12E53) may also be hybrids of *E. equisetina* and *E. intermedia*. These plants (12E27 and 12E53) were identified as *E. equisetina* based on their morphological features and their ITS1 sequences by direct sequencing; however, these plants may have minor ITS1 sequences of *E. intermedia*, which were not detected by direct sequencing. Hybrids between *E. przewalskii* and *E. intermedia* (*E. glauca*) were previously reported among the *Ephedra* plants collected in Mongolia,¹³⁾ and hybrids between *E. gerardiana* and *E. intermedia* were observed in Nepal.¹⁸⁾ A hybrid of *E. likiangensis* and *E. gerardiana* was also found among the *Ephedra* plants cultivated in Japan.¹⁹⁾ The present study showed that hybridization of *E. equisetina* and *E. intermedia* is common in the Zaravshan Mountains of Tajikistan since both species grow in the same habitats.

HPLC analysis revealed that the TAC in the 73 *Ephedra* samples collected in Tajikistan varied from 0.34 to 3.21% by dry weight. The TAC of most of the *Ephedra* samples (70; 96%) was higher than the lower limit of 0.7% by dry weight, as prescribed by JP17.⁵⁾ The maximum TAC was 3.21% by dry weight in one *E. intermedia* specimen (11E07), and was similar to the levels determine for those collected in China (0.39–3.40%),¹⁵⁾ Mongolia (0.00–4.59%),¹⁶⁾ and Pakistan (0.00–1.87%).¹⁷⁾ The RAR were reported to vary with *Ephedra* species and strain.^{15–17)} The RAR may depend

on genetic factors.^{20,21)} In the present study, the RAR of *E. intermedia* collected in Tajikistan ranged from 0.02 to 0.19 and resembled those for most *E. intermedia* collected in other countries.^{15,16)} **PEp** was shown to have more potent anti-inflammatory effects than **Ep**.²²⁾ It has been recommended that *E. intermedia* with higher **PEp** levels should be used in anti-inflammatory *Ephedra* preparations.^{20,22)} In the present study, only *E. intermedia* plants were observed at altitudes <2000 m. Therefore, Tajikistan could serve as a production center of *E. intermedia*. In contrast, the RAR of *E. equisetina* collected from Tajikistan ranged from 0 to 0.99. This wide variation in alkaloid content was also observed in *E. equisetina* and *E. sinica* collected in China¹⁵⁾ and Mongolia.¹⁶⁾

Seeds of *E. equisetina* and *E. intermedia* collected in Tajikistan were germinated and raised in Iwate, Japan. The TAC of the cultivated *E. intermedia* strain 11E08-1 was much higher than the lower limit prescribed by JP17 (0.7% by dry weight) and those of other cultivated *Ephedra* species such as *E. sinica* strain IMU-ES1. The TAC of the cultivated *E. sinica* strains in Japan has been reported to be relatively low.²⁰⁾ Therefore, the *E. intermedia* strain 11E08-1 collected from Tajikistan could be used for the cultivation of *Ephedra* plants.

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Conflict of Interest The authors declare no conflict of interest.

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