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 68t 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′-GTC GGC AGA AGT TCA
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, 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 CAG AGG AAC CGG ATG-3' and 5'-GGG AGA AGG CGT TTG TCT CAA ATA TTT TGA-3' for E. equisetina or 5'-GCT CTC CAG AGG AAC CGG AAA-3' and 5'-GGG AGA AGG CGT TTG TCT CAA ATA TTT TGA-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 on 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. 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: Prominance HPLC system (Shimadzu Corporation, Kyoto, Japan) was used; column, Inertsil ODS-3 (5 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-EE1).

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.
slightly curved integument tubes (Fig. 3A). In contrast, \(E\). intermedia plants collected in the present study were 0.5–1 m in height. 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, \(E.\) intermedia 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., \(E.\) atchissonii 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. regelianana 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. 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. In addition, E. heterosperma, a newly defined Ephedra species in the Flora of Tajikistan, has a spirally twisted integument tube, characteristic of E. intermedia. Thus, in the present study, Ephedra plants collected in Tajikistan were identified based on the classification of Ephedra in the Flora of China.

Characterization of Ephedra Plants Based on ITS1 Sequences

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 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,
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 present 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-
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-ESI) 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-ESI) 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, Mongolia, Pakistan, and Nepal. 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

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**Table 3. Contents of Ephedrine (Ep) and Pseudoephedrine (PEp) in Herbaceous Stems of *Ephedra equisetina* Collected in Tajikistan**

| Plant No. | Sample No. | Collection date | Ep (% of dry weight) | PEp (% of dry weight) | TAC (Ep + PEp) (%) | RAR Ep/(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              |
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, Pakistan, and Mongolia.

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 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/(Ep + PEP) |
|-----------|------------|-----------------|-----------------------------|------------------|
|           |            |                 | Ep  | PEP | TAC (Ep + PEP) | Ep/(Ep + PEP) |
| 11E03     | 1          | Oct. 9, 2011    | 0.64| 1.29| 1.93          | 0.35          |
|           | 2          | Jun. 3, 2012    | 0.07| 1.86| 2.13          | 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/(Ep + PEP) |
|-----------|------------|-----------------|-----------------------------|------------------|
|           |            |                 | Ep  | PEP | TAC (Ep + PEP) | Ep/(Ep + PEP) |
| 12E17     | 1          | Jun. 3, 2012    | 0.64| 1.19| 1.83          | 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          |
| 12E51     | 1          | Oct. 14, 2012   | 0.47| 0.26| 0.73          | 0.64          |
| 12E55     | 1          | Oct. 14, 2012   | 0.61| 1.13| 1.74          | 0.35          |
| 12E53     | 1          | Oct. 14, 2012   | 1.26| 0.54| 1.80          | 0.70          |
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, and hybrids between *E. gerardiana* and *E. intermedia* were observed in Nepal. A hybrid of *E. likiangensis* and *E. gerardiana* was also found among the Ephedra plants cultivated in Japan. 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. 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%), Mongolia (0.00–4.59%), and Pakistan (0.00–1.87%). The RAR were reported to vary with Ephedra species and strain. The RAR may depend on genetic factors. 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. **PEp** was shown to have more potent anti-inflammatory effects than **Ep**. It has been recommended that *E. intermedia* with higher **PEp** levels should be used in anti-inflammatory Ephedra preparations. 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 China and Mongolia. 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. 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.

REFERENCES

1. Abourashed EA, El-Alfy AT, Khan IA, Walker L. Ephedra in perspective—a current review. Phytother. Res., 17, 703–712 (2003).
2. Hioki C, Arai M. Bofutsushosan use for obesity with IGT: search for scientific basis and development of effective therapy with Kampo medicine. J. Trad. Med., 24, 115–127 (2007).
3. Nabeshima S, Kashihagi K, Ajisaka K, Kitajima K, Masui S, Ike-
matsu H, Kashiwagi S. A comparison of oseltamivir with maoto, a traditional herbal medicine, for the treatment of adult seasonal influenza A. *J. Trad. Med.*, 27, 148–156 (2010).

4) Shimada T, Kondoh M, Motonaga C, Kitamura Y, Cheng L, Shi HB, Enomoto T, Tsuruta D, Ishii M, Kobayashi H. Enhancement of anti-allergic effects mediated by the Kampo medicine sho-seryuto (xiao-qing-long-tang in Chinese) with lysed *Enterococcus faecalis* FK-23 in mice. *Asian Pac. J. Allergy Immunol.*, 28, 59–66 (2010).

5) The Japanese Pharmacopoeia (17th ed.) (The Society of Japanese Pharmacopoeia ed.). Ministry of Health, Labour and Welfare of Japan, Tokyo, p. 1916 (2016).

6) Mikage M, Takahashi A, Chen HB, Li QS. Studies of *Ephedra* plants in Asia. Part I: On the resources of *Ephedra* plants in China. *Nat. Med.*, 57, 202–208 (2003).

7) Bobrov EG. Genus 45. *Ephedra* L. *Flora of the USSR*. (Komarov VL ed.) Vol. 1, Academy of Science of the USSR, Leningrad, pp. 195–204 (1934).

8) *Flora of SSR of Tajikistan*. (Ovchinnikov PN ed.) Vol. 1, Academy of Science of SSR of Tajikistan, Moscow-Leningrad, pp. 60–83 (1957).

9) *Flora of SSR of Tajikistan*. (Rasulova MR ed.) Vol. 10, Academy of Science of SSR of Tajikistan, Leningrad, p. 470 (1991).

10) Wu ZY, Peter HR. *Flora of China*. Vol. 4, Missouri Botanical Garden Press, St. Louis, pp. 97–101 (1999).

11) “The trade statistics of Japan,” Ministry of Finance of Japan: [http://www.customs.go.jp/toukei/info/index_e.htm](http://www.customs.go.jp/toukei/info/index_e.htm).

12) Long C, Kakiuchi N, Takahashi A, Komatsu K, Cai S, Mikage M. Phylogenetic analysis of the DNA sequence of the non-coding region of nuclear ribosomal DNA and chloroplast of *Ephedra* plants in China. *Planta Med.*, 70, 1080–1084 (2004).

13) Kitani Y, Zhu S, Batkhuu J, Sanchir C, Komatsu K. Genetic diversity of *Ephedra* plants in Mongolia inferred from internal transcribed spacer sequence of nuclear ribosomal DNA. *Biol. Pharm. Bull.*, 34, 717–726 (2011).

14) Long C, Kakiuchi N, Zhong G, Mikage M. Survey on resources of *Ephedra* plants in Xinjiang. *Biol. Pharm. Bull.*, 28, 285–288 (2005).

15) Hong H, Chen HB, Yang DH, Shang MY, Wang X, Cai SQ, Mikage M. Comparison of contents of five ephedrine alkaloids in three official origins of *Ephedra* Herb in China by high-performance liquid chromatography. *J. Nat. Med.*, 65, 623–628 (2011).

16) Kitani Y, Zhu S, Omote T, Tanaka K, Batkhuu J, Sanchir C, Fushimi H, Mikage M, Komatsu K. Molecular analysis and chemical evaluation of *Ephedra* plants in Mongolia. *Biol. Pharm. Bull.*, 32, 1235–1243 (2009).

17) Kakiuchi N, Inoue K, Kurita Y, Ohkubo K, Tsuda Y, Mikage M. Survey of *Ephedra* resources in the northern areas of Pakistan and their genetic diversity. *J. Nat. Med.*, 61, 357–365 (2007).

18) Hamanaka E, Ohkubo K, Mikage M, Kakiuchi N. Molecular genetic characteristics of Nepalese *Ephedra* plants (Ephedraceae). *Jpn. J. Bot.*, 86, 303–313 (2011).

19) Ando H, Kitamura M, Sasaki Y, Kitaoka F, Mikage M. Studies of cultivation of *Ephedra* plants (Part 8). Genetic study on the *Ephedra* plant labeled Ep-13, which had introduced by the former National Institute of Hygienic Science. *Jpn. J. Med. Resour.*, 38, 1–9 (2016).

20) Matsuamoto M, Hirayama M, Ohtomi N, Ohno T, Nomura Y, Iida O, Sugimura K, Kawahara N, Tsuchida T, Mikage M. Influence of genetic factors on the ephedrine alkaloid composition ratio of *Ephedra* plants. *J. Nat. Med.*, 69, 63–67 (2015).

21) Krizevski R, Bar E, Shalit O, Sitrit Y, Ben-Shabat S, Lewinsohn E. Composition and stereochemistry of ephedrine alkaloids accumulation in *Ephedra sinica* Stapf. *Phytochemistry*, 71, 895–903 (2010).

22) Hikino H, Konno C, Takata H, Tamada M. Antiinflammatory principle of *Ephedra* herbs. *Chem. Pharm. Bull.*, 28, 2900–2904 (1980).