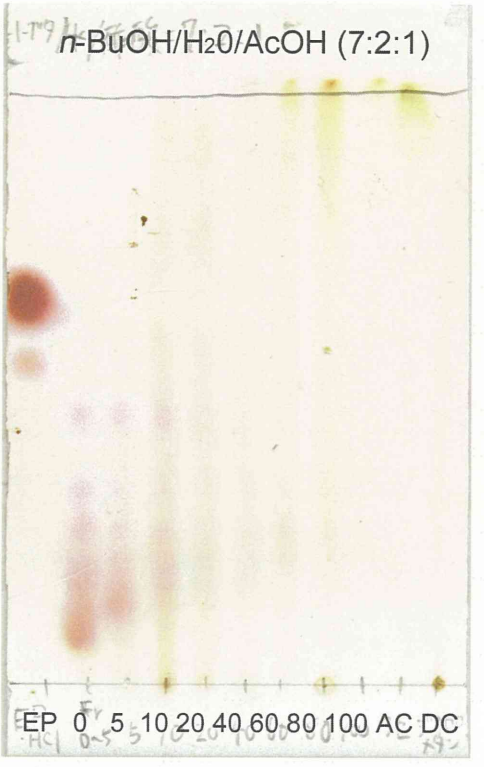

図５．イオン交換樹脂分画物のSi-TLCプロファイル（ニンヒドリン呈色）
EP：エフェドリン標準品
0〜100：0% MeOH Fr.〜100% MeOH Fr.
AC：Acetone Fr.
DC：CH_2Cl_2 Fr.

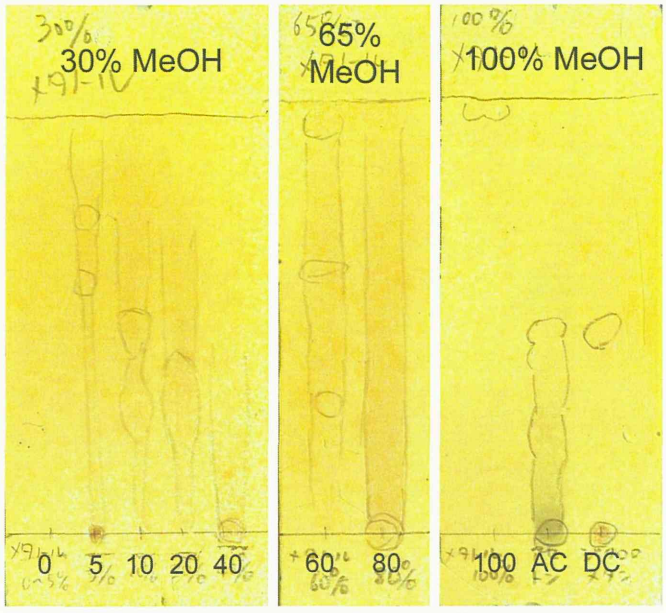

図６．イオン交換樹脂分画物のODS-TLCプロファイル（ドラーゲンドルフ試液呈色）
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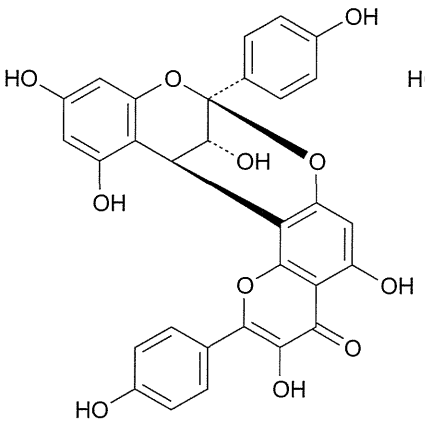
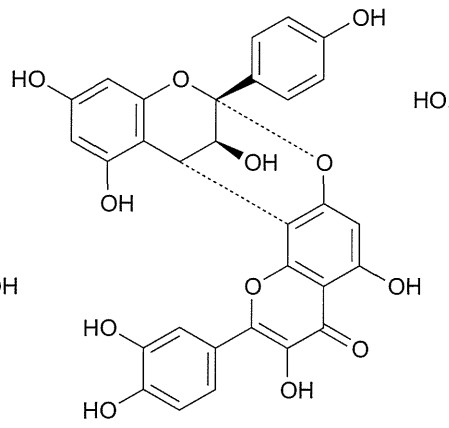
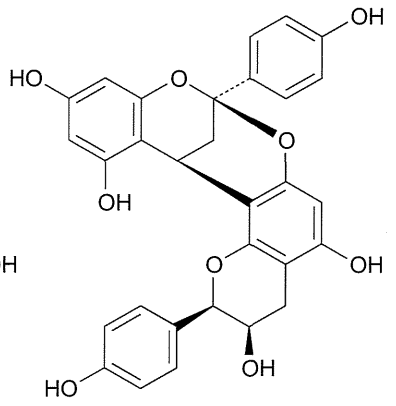
AC：Acetone Fr.
DC：CH_2Cl_2 Fr.



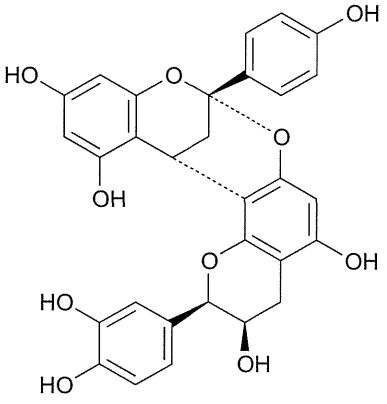
Compound 1



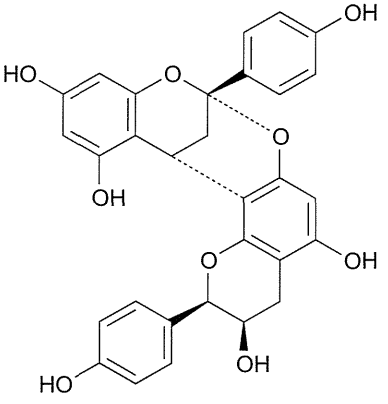
Compound 2



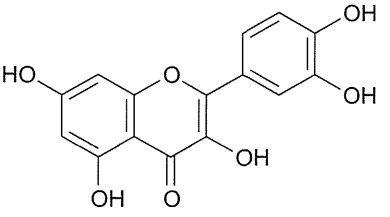
Compound 3



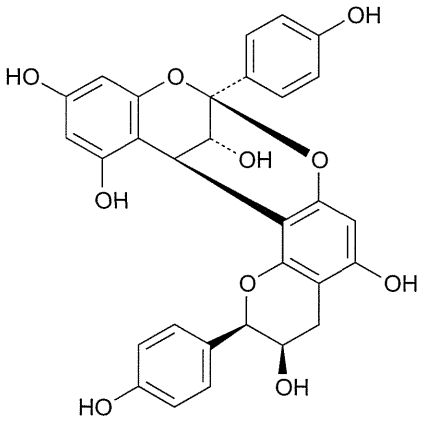
Compound 4



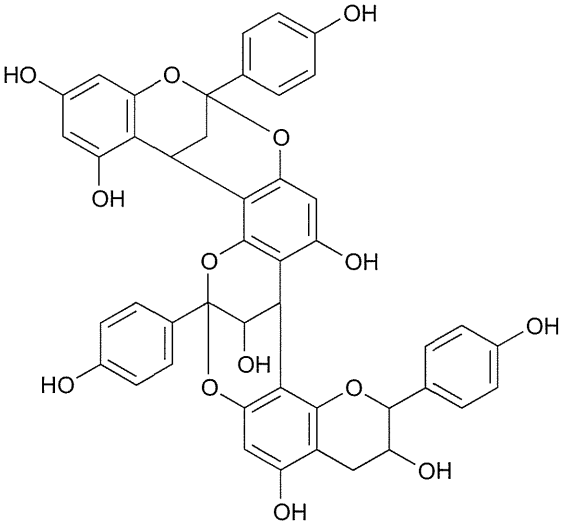
Compound 5



Compound 6



Compound 7



Compound 8

図７．化合物**1**～**8**の構造

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Anatomical, Chemical, and Molecular Genetic Studies of *Ephedra distachya*

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Ephedrae Herba (ma-huang in Chinese), the herbal stems of *Ephedra sinica* Stapf, which belongs to the Ephedraceae family, is an important crude drug in traditional Chinese medicine. Some researchers think that this species is identical to *E. distachya* L., which mainly grows in Europe. Thus, in this study, we carried out anatomical, chemical, and molecular genetic studies on *E. distachya* plants collected in Switzerland, France, and Turkey to consider whether *E. distachya* could be used to produce Ephedrae Herba. As a result, we found that the morphology of the subepidermal fiber bundles in cross sections of the herbal stems of *E. distachya* and the frequencies of ephedrine alkaloids in *E. distachya* were quite different from those observed in *E. sinica* and that the DNA sequence of the ITS1 region of *E. distachya* contains 11 base changes compared with that of *E. sinica*. Based on these facts, we concluded that *E. distachya* and *E. sinica* should be treated as different taxa and that, according to the prescriptions of the current Japanese and Chinese pharmacopoeias, *E. distachya* is not suitable for producing Ephedrae Herba.

Key words: alkaloid, anatomical study, *Ephedra distachya*, Ephedrae Herba, ITS1.

The genus *Ephedra*, which belongs to the Ephedraceae family, one of the three extant genera of the Gnetales, comprises about 50 species, which are native to arid and semiarid regions of Eurasia, northern Africa, western North America, and South America (Price 1996). Most species of this genus are erect or sprawling shrubs, except for a few species of vine-like climbing shrubs or small trees. Since ancient times, the aerial parts of *Ephedra* plants have been used for treating colds, coughs, bronchitis, etc. in traditional Japanese medicine (Kampo) and traditional Chinese medicine (TCM) (Tang 2002), under the name ma-huang (Ephedrae Herba). The ephedrine group alkaloids contained in Ephedrae Herba are considered to be active constituents and have been used as bronchodilators or decongestants against asthma or colds in Western medicine.

Ephedra sinica Stapf, one of three plant sources of Ephedrae Herba prescribed in both the Japanese and Chinese Pharmacopoeias, was

denominated in 1927 on the basis of a specimen collected in Hebei Province (Stapf 1927). This species is distributed from central China (Gansu province) and Mongolia, eastward up to the Gulf of Bohai (Hebei province) and the northeast of China (Jilin province) (Cheng 1978, Fu et al. 1999). Due to its relative abundance in nature, *E. sinica* dominates the crude drug market in China (Hong et al. 2011a). However, the export of Ephedrae Herba has been restricted in order to conserve natural ephedra resources and prevent desertification since 1999. Considering the continued use of Ephedrae Herba, substitutes for *E. sinica* should be sought.

Ephedra distachya L. was the first candidate that we considered might be a useful substitute for *E. sinica* because some researchers think that it is identical to *E. sinica*. *Ephedra distachya* was denominated in 1753 by Linnaeus and is widely distributed from Europe to west central Asia (Linnaeus 1753). *Ephedra distachya* subsp. *helvetica* (C. A. Mey.) Asch. & Graebn. was found in the dry Rhône valley in the Swiss Alps and was recorded as a variety of *E. distachya*, based on its slightly longer and twisted micropylar tubes. In morphology-based studies of plant taxonomy, it has been suggested that *E. sinica* and *E. distachya* subsp. *distachya* are conspecific as they both possess fleshy 2-seeded female cones and have rather short micropylar tubes (Kitagawa 1939, Yang 2002), whereas others have indicated that they can be distinguished by the size of the opening at the tips of their micropylar tubes and the shape of the free parts of their leaves (Cheng 1978, Fu et al. 1999). Recent molecular studies have shown that *E. distachya* subsp. *distachya* and *E. sinica* belong to the same clade but different subclades (Ickert-Bond and Wojciechowski 2004, Huang et al. 2005, Rydin and Korall 2009, Kakiuchi et al. 2011). However, because plants of the Genus *Ephedra* are polyphyletic, some species such as *E. przewalskii* Stapf and *E. intermedia* Schrenk & C. A. Mey., whose cone bracts and micropylar tubes are morphologically different from those of *E. sinica* and *E. distachya*, remained nested together with the latter species after molecular analysis (Ickert-Bond and Wojciechowski 2004, Rydin and Korall 2009, Kakiuchi et al. 2011). Moreover, *E. intermedia* can be easily distinguished by its long and spiral micropylar tube, but different accession samples of the species have been placed in different clades (Kakiuchi et al. 2011). Considering these conflicting results, it is not suitable to separate *E. sinica* from *E. distachya* (subsp. *distachya*) using molecular data alone.

Besides molecular studies, attempts have been made to discriminate between *Ephedra* species using the anatomical characteristics of their stems (Chen 1989, Zhang 1989b, Fushimi 2008) or their ephedrine contents (Liu 1993, Hong 2011b) and have succeeded in identifying some *Ephedra* plants from China. However, no samples of *E. distachya* subsp. *distachya* from Europe have been subjected to studies of these two features. Thus, in the present study, anatomical, phytochemical, and molecular assessments were carried out on samples of *E. distachya* subsp. *distachya* and *E. distachya* subsp. *helvetica* collected from France, Switzerland, and Turkey, and the results were compared with those for *E. sinica*. Our intention is to clarify whether *E. distachya* and *E. sinica* are the same species and investigate the possibility of using *E. distachya* as a substitute for *E. sinica* in Chinese medicine.

Materials

Ephedra distachya subsp. *distachya* plant materials were collected in France in 2011 and in Turkey in 2009 and 2012, and *E. distachya* subsp. *helvetica* plant materials were collected in Switzerland in 2008. Each field study was performed in July or August, the seed-maturing season. All the plant specimens were collected by M. Mikage or M. Mikage & al., and were deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Kanazawa University (KANP), Japan.

1. *Ephedra distachya* subsp. *distachya*

France. Saint-Jean de Védas (Hérault), open hill slope, 9 km southwest of Montpellier, 43°33′03.3″ N, 3°49′24.1″ E, alt. 5 m (17 Sep. 2011, 1109171); Etang du Ponant, Le Boucanet, Le Grau-du-Roi (Gard), sandy shore of a lake, 20 km southeast of Montpellier, 43°33′31.4″ N, 4°07′17.9″ E, alt. 5 m (17 Sep. 2011, 1109172); Etang du Ponant, Le Boucanet, Le Grau-du-Roi (Gard), sandy shore of a lake, 20 km southeast of Montpellier, 43°33′42.4″ N, 4°07′04.8″ E, alt. 5 m (17 Sep. 2011, 1109173); Le Grand Travers, between Carnon and La Grande-Motte (Hérault), a sandy beach bordering the Mediterranean Sea, northern side of road D59, 13 km southeast of Montpellier, 43°33′20.3″ N, 4°01′03.2″ E, alt. 5 m (17 Sep. 2011, 1109174); Le Grand Travers, between Carnon and La Grande-Motte (Hérault), sandy beach bordering the Mediterranean Sea, northern side of road D59, 13 km southeast of Montpellier, 43°33′23.2″ N, 4°01′16.1″ E, alt. 5 m (17 Sep. 2011, 1109175); Les Aresquiers, Vic-la-Gardiole (Hérault), sandy shore, between Mediterranean and Etang de Pierre Blanche, 16 km southwest of Montpellier, 43°28′51.2″ N, 3°50′37.5″ E, alt. 5 m (17 Sep. 2011, 1109176); north of Etang de Pissevaches, Cabanes de Fleury, Fleury-d'Aude (Aude), west of a naturist resort, 1.2 km inland from the beach, 15 km south of Beziers, 43°12′29.1″ N, 3°12′41.3″ E, alt. 5 m (18 Sep. 2011, 1109181); Réserve Naturelle du Mas Larrieu, Argelès-sur-Mer (Pyrénées-Orientales), near the beach and south of the Le Tech river, 18 km southeast of Perpignan, 42°35′15.6″ N, 3°02′37.9″ E, alt. 5 m (19 Sep. 2011, 1109191); between Saint-Cyprien et Canet (Pyrénées-Orientales), sandy coastal land near the southern end of Lake Canet-St-Nazaire, 14 km southeast of Perpignan, 42°38′31.1″ N, 3°02′13.7″ E, alt. 5 m (19 Sep. 2011, 1109192); between Saint-Cyprien et Canet (Pyrénées-Orientales), near the baraques de pêcheurs, eastern side of Lake Canet-St-Nazaire, 13 km southeast of Perpignan, 42°39′37.7″ N, 3°02′07.3″ E, alt. 5 m (19 Sep. 2011, 1109193); Torreilles (Pyrénées-Orientales), sandy beach, south of Torreilles Plage, 15 km northeast of Perpignan, 42°45′26.9″ N, 3°02′26.5″ E, alt. 5 m (19 Sep. 2011, 1109194); Torreilles (Pyrénées-Orientales), sandy beach near the mouth of the River L'Agry, 16 km northeast of Perpignan, 42°46′40.0″ N, 3°02′27.7″ E, alt. 5 m (19 Sep. 2011, 1109195); Port Barcarès (Pyrénées-Orientales), sandy land along the Mediterranean coast, near Mas de l'Illa, 20 km northeast of Perpignan, 42°48′48.6″ N, 3°48′26.5″ E, alt. 5 m (19 Sep. 2011, 11091961; 11091962); Les Coussoules, La Franqui (Aude), sandy land near a camping ground, north of Leucate, 30 km north of Perpignan, 42°56′45.0″ N, 3°02′09.6″ E, alt. 5 m (19 Sep. 2011, 11091971; 11091972); Les Coussoules, La Franqui (Aude), edge of the tideland near a camping ground, north of Leucate, 30 km north of Perpignan, 42°56′57.2″ N, 3°20′09.6″ E, alt. 5 m (19 Sep. 2011, 1109198).

Turkey. B5 Kayseri, Yilanli Mountain, Sallibayir, Kulakli Baglari, alt. 1170 m (07 Jul. 2009, 09070701); Kayseri, 38°42′52″ N, 35°26′04″ E, alt. 1170 m (29 Jun. 2012, U62911).

2. *Ephedra distachya* subsp. *helvetica*

Switzerland. Sion. The slope around Sion castle, 46°13′40″ N, 7°21′41″ E, alt. 700 m (08 Aug. 2008, 0808S1; 0808S2; 0808S3); the back of Sion castle, 46°13′40″ N, 7°21′41″ E, alt. 700 m (08 Aug. 2008, 0808S5-1; 0808S5-2; 0808S6-1; 0808S6-2).

3. *Ephedra sinica*

China. Inner Mongolia. Chagannaoer, 60 km south of Sonid Zuoqi, grassland, 43°24′49.2″ N, 113°05′04.6″ E, alt. 1030 m (16 Aug. 2007, 7081605); 10 km from Bayachagan, Abagaqi, grassland, 43°59′30.4″ N, 115°06′57.0″ E, alt. 1200 m (17 Aug. 2007, 7081706); along Axi Highway marker 75, Dongwujimqin, grassland, 44°33′34.1″ N, 115°53′32.2″ E, alt. 1060 m (18 Aug. 2007, 7081803); along road 304 marker 510, 340km south of Tongliao, 43°19′53.7″ N, 122°13′33.2″ E, alt. 240 m (21 Aug. 2007, 7082102). **Liaoning Province**. Along road 304, Zhangwu County, beside the railway, 42°46′20.8″ N, 122°26′10.8″ E, alt. 255 m (21 Aug. 2007, 7082104). **Hebei Province**. Seashore, near the mouth of the Nandaihe river, 39°47′57.1″ N, 119°26′11.8″ E, alt. 2 m (22 Aug. 2007, 070822A01; 070822A02; 070822A03).

Methods

Anatomy

Transverse sections of internodes or herbal stems were examined using an optical microscope without treatment or after clarification with chloral hydrate solution. Three stems with one to six secondary xylem cell layers between their vascular bundles were chosen from each of the specimens to ensure that the experimental stems displayed uniform maturity. The following parameters were examined: the longitudinal and transverse lengths of the herbal stem and cambium ring; the presence of cuticular protuberances; and the numbers of subepidermal, cortical, and pith fibers. Moreover, the ratio of subepidermal fiber bundle length to cortex length was calculated for the 5 longest subepidermal fiber bundles in each stem. We also measured the angle between the two long edges of the subepidermal fiber bundle, as shown in Fig. 1A. This value was considered

to be negative if the lines extrapolated from the two long edges intersected on the epidermal side.

Analysis of alkaloid content

Sample preparation for high-performance liquid chromatography (HPLC) analysis

First, 0.1 g powdered samples, which had been dried at 105°C for 15 h, were suspended in 5.0 mL of the mobile phase and then left at room temperature for 20 min, after which sonication extraction was performed for 20 min. After being centrifuged at 3000 r/min for 15 min, the supernatant solution was filtered through a membrane filter (pore size: 0.45 μm) into a HPLC vial, which was then capped.

HPLC conditions

The analysis was performed using a Hitachi Elite LaChrom HPLC system, consisting of an L-2130 pump, an L-2200 auto sampler, and an L-2400 UV detector. An ODS column was used as the analytical column (4.6 mm, 250 mm). The mobile phase consisted of 195 mL CH_3CN, 305 mL H_2O, 0.4 mL H_3PO_4, and 2.4 g sodium dodecyl sulfate. The flow rate was 1.0 mL/min, the sample injection volume was 10 μL, and the detector monitored the eluent at 210 nm.

DNA preparation, PCR amplification and Sequencing

Dried twigs were cut into pieces, frozen in liquid nitrogen, and ground into a powder. Using a DNeasy Plant Mini Kit (Qiagen), DNA was extracted according to the manufacturer's protocol. The primer sets for Eph-1F (GAC GTC GCG AGA AGT TCA TT) and 5.8S-R (CGG GAT TCT GCA ATT CAC AC) designed by Kakiuchi were used to amplify the ITS1 region. Standard PCR was carried out in a 25 μL reaction mixture containing 2.5 μL of 10× PCR buffer for KOD-Plus, 0.2 mM of each dNTP, 1 mM $MgSO_4$, 0.4 M of each primer, approximately 100 ng of the DNA sample, and 0.5 units of KOD-Plus DNA polymerase (Toyobo). The cycling parameters used for the PCR were as follows: 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 step at 68°C for 5 min. Three microliters of the PCR product were used for agarose gel electrophoresis, and the remaining product was purified using the QIA quick PCR Purification Kit (Qiagen).

The purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Result and Discussion

Microscopic characteristics of the herbal stems

1. *Ephedra distachya* subsp. *distachya*

Generally, the transverse sections were circular or elliptical in shape, and there were many ridges and furrows on their surfaces. Cuticular tubers were observed on the ridges, and stomata with guard cells were located within the furrows. The epidermal cells were arranged compactly and were covered with a thick cuticle. Below the surface, ridges composed of fiber bundles with rectangular shapes (Fig. 1B, C) were in contact with the epidermis. In the samples from France, the longest 5 of these subepidermal fiber bundles were 149.44 ± 22.87 μm in length and accounted for 42 ± 6% of the cortex, whereas those of the samples from Turkey measured 165.78 ± 26.28 μm in length and accounted for 50 ± 7% of the cortex (Table 1, Fig. 1B, C). The cortex mainly consisted of parenchyma cells, including radially elongated palisade cells in the outer cortex and circular cells in the inner cortex. Many fibers or small groups of fibers with irregular shapes were scattered throughout the cortex, and the samples from France contained many more of these fibers than the samples from Turkey (Table 1, Fig. 1B, C). The individual collateral vascular bundles were triangular and arranged in a ring, and their phloem bundles were usually capped by fibers. The pith consisted of large parenchyma cells, most of which were circular and were frequently

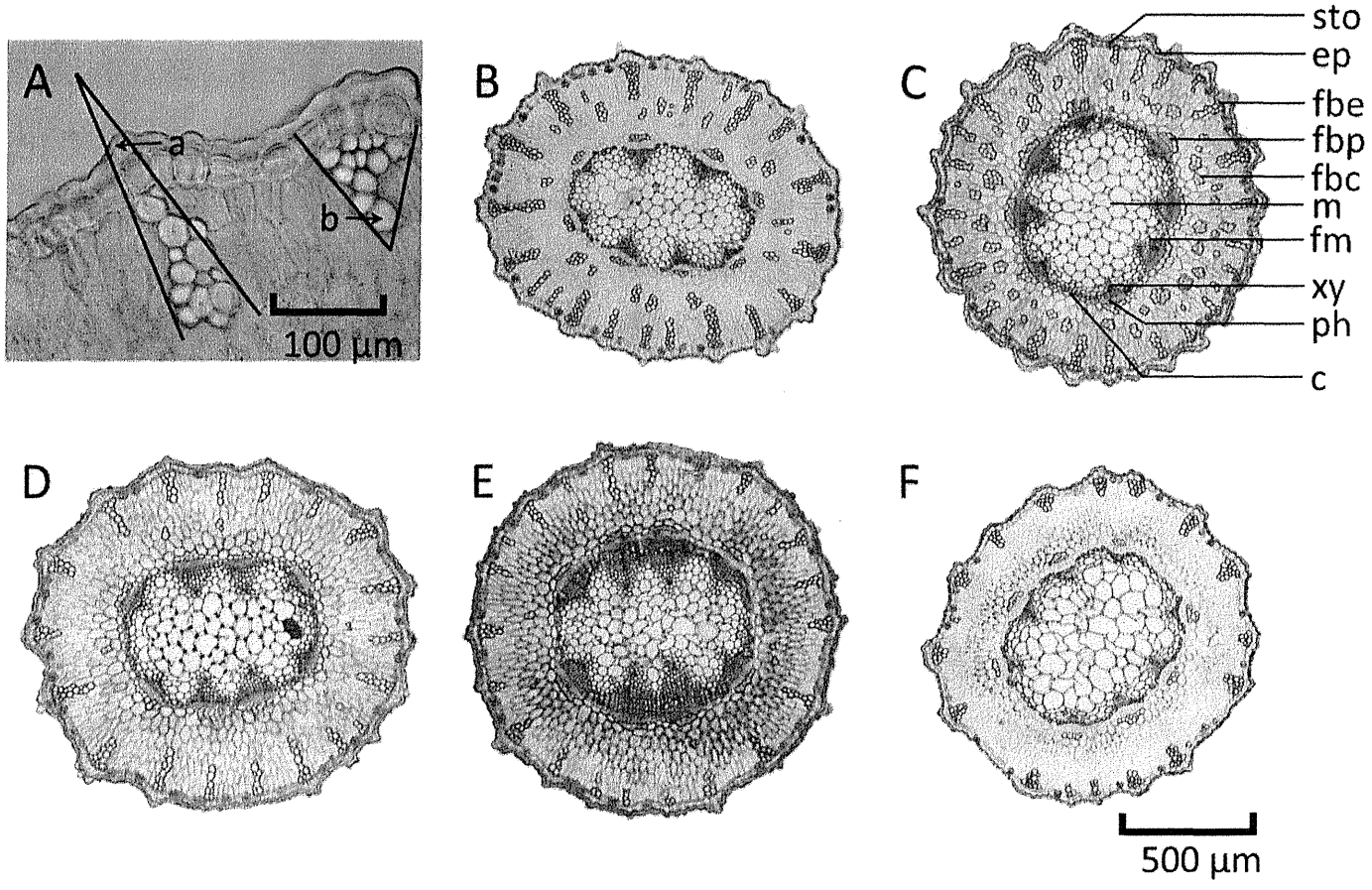

Fig. 1. Transverse section of herbal stems of *Ephedra distachya* and *E. sinica*. A. Angle of tip of subepidermal bundle fiber. a. Negative value. b. Positive value. B, C. *E. distachya* subsp. *distachya*. B. 1109173 (France). C. 09070701 (Turkey). D, E. *E. distachya* subsp. *helvetica*. D. No fiber existed in the pith (0808S5-2). E. Fibers existed in the pith (0808S6-2). F. *E. sinica* (7081706). Abbreviations: c, cambium; ep, epidermis; fbc, fiber bundle in the cortex; fbe, subepidermal fiber bundle; fbp, fiber bundle of the vascular bundle sheath; fm, fiber in pith; m, pith; ph, phloem; sto, stoma; xy, xylem.

Table 1. Anatomical characteristics of transverse sections of herbal stems of *Ephedra sinica* and *E. distachya*

	E. distachya ssp. *distachya* (France)	*E. distachya* ssp. *distachya* (Turkey)	*E. distachya* ssp. *helvetica*	*E. sinica*
Shape of transverse sections	circular, elliptical	elliptical, triangular	circular, elliptical	elliptical, circular*[a)], triangular*
Longitudinal length of herbal stem (mm)	1.21–1.95 (1.53 ± 0.16)[b)]	1.32–1.73 (1.50 ± 0.16)	1.17–1.67 (1.41 ± 0.15)	1.27–1.74 (1.46 ± 0.14)
Transverse length of herbal stem (mm)	1.08–1.80 (1.44 ± 0.16)	1.14–1.55 (1.34 ± 0.15)	1.17–1.59 (1.33 ± 0.13)	1.14–1.53 (1.27 ± 0.11)
Longitudinal length of cambium ring (mm)	0.47–0.95 (0.68 ± 0.12)	0.58–0.91 (0.75 ± 0.12)	0.47–0.85 (0.67 ± 0.11)	0.68–1.10 (0.82 ± 0.11)
Transverse length of cambium ring (mm)	0.39–0.81 (0.55 ± 0.12)	0.45–0.70 (0.60 ± 0.09)	0.35–0.72 (0.54 ± 0.11)	0.50–0.77 (1.41 ± 0.08)
Existence of cuticular protuberances	present	present	present	present
Number of subepidermal fiber bundles	15–30 (20.23 ± 3.51)	19–31 (23.17 ± 4.79)	15–24 (20.37 ± 2.67)	14–28 (19.96 ± 3.22)
Number of fiber bundles in the cortex	10–92 (41.68 ± 13.79)	12–43 (27.50 ± 14.54)	1–19 (6.53 ± 5.54)	0–48 (9.50 ± 12.00)
Number of parenchyma cell layers in the cortex	4–7	5–7	4–5	5–6
Number of palisade cell layers	2–3	2–3	2–3	2–3
Number of fibers in the pith	8–137 (49.08 ± 30.47)	0	0–25 (8.53 ± 8.56)	0–9 (1.29 ± 2.77)
Mean length of subepidermal fiber bundles (longest 5 stems, μm)	92.06–243.68 (149.44 ± 22.87)	109.36–213.16 (165.78 ± 26.28)	61.52–188.77 (127.92 ± 29.39)	62.03–153.55 (95.90 ± 12.99)
Ratio of subepidermal fiber bundle length to cortex length (longest 5 stems)	0.25–0.65 (0.42 ± 0.06)	0.36–0.68 (0.50 ± 0.07)	0.21–0.63 (0.40 ± 0.07)	0.21–0.46 (0.34 ± 0.04)
Mean angle of the subepidermal fiber bundle tip (longest 5 stems, °)	−17.4–40.7 (11.3 ± 4.3)	5.4–30.3 (16.4 ± 2.4)	−1.4–50.8 (16.1 ± 6.0)	4.8–72.8 (33.0 ± 8.2)

[a)] *: unusual finding.

[b)] (MEAN ± S.D.).

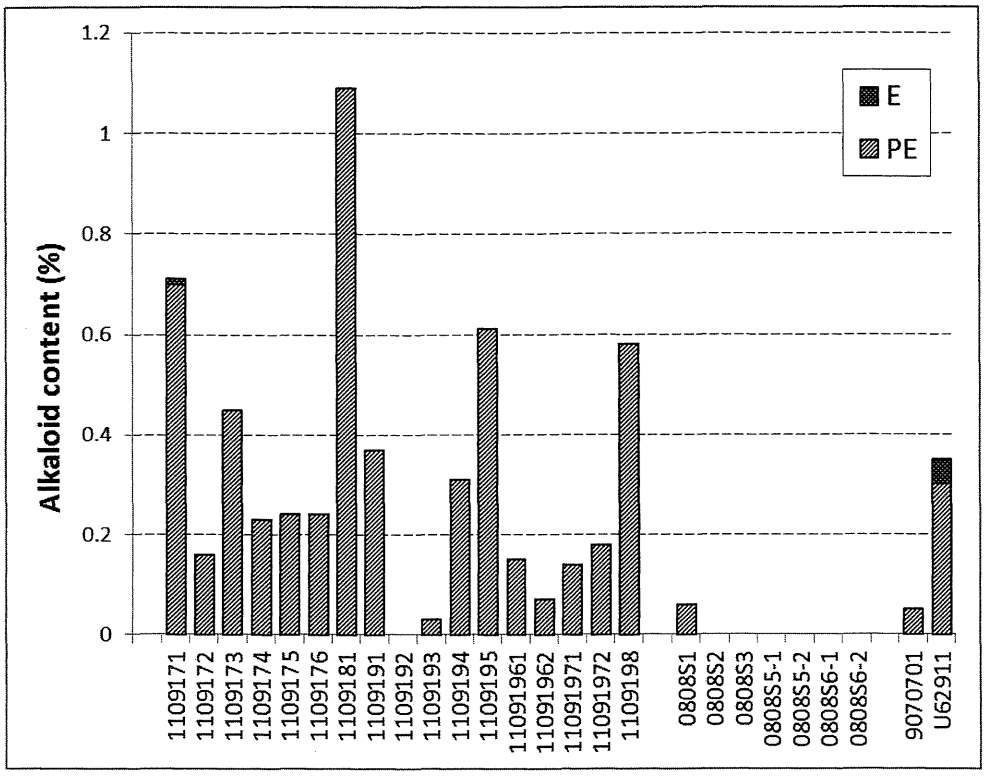

Fig. 2. Ephedrine and pseudoephedrine contents of *Ephedra distachya*. E. Ephedrine. PE. Pseudoephedrine.

filled with brown matter. Large numbers of fibers were observed in the pith of the samples from France, but no fibers were observed in the pith of the samples from Turkey (Table 1).

The anatomical characteristics of transverse sections of this species' herbal stems have been described previously using samples from China, but the results reported by these studies regarding the length and shape of the subepidermal fiber bundles were different from ours (Konoshima 1945b, Xu et al. 1992). Conversely, other studies have reported that *E. distachya* subsp. *distachya* samples collected from Inner Mongolia and Qinghai Province, China, belonged to the same species as *E. sinica* (Fushimi 2008), and another study could not confirm whether samples identified as *E. distachya* from Xinjiang Province, China, belonged to the same species as those from Europe (Fu et al. 1999).

2. *Ephedra distachya* subsp. *helvetica*

The anatomical characteristics of *E. distachya* subsp. *helvetica* were similar to those of *E. distachya* subsp. *distachya* (Fig. 1). However, 3 samples (0808S1, 0808S5-1, 2) contained less than 5 cortex fibers, and 2 of these samples (0808S1, 0808S5-1) contained no fibers in their pith (Fig. 1D). Other samples had more fibers in their cortex and pith (Fig. 1E). Thus, there was a great deal of variation in the findings for *E. distachya* subsp. *helvetica* (Table 1).

3. *Ephedra sinica*

The anatomical characteristics of *E. sinica* have been reported previously (Kimura 1930, Konoshima 1945a, Zhang 1989b, Fushimi 2008), and their results were in agreement with ours. Compared with *E. distachya* and *E. distachya* subsp. *helvetica*, *E. sinica* had shorter subepidermal fiber bundles (Table 1), which were almost trapezoidal in shape (Fig. 1F). Moreover, fewer fibers were observed in the

Table 2. Mutations in the ITS1 region of *Ephedra distachya* and its related species

	Nucleotide sequence of the ITS1 region														
	80	223	403	645	762	774	810	894	899	910	914	915	1023	1131	1134
E. distachya ssp. *distachya* (GU 065272)	C	G	—	C	T	G	A	C	T	C	A	G	C	Y	T
E. distachya ssp. *distachya* (France) 1109173, 1109176	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	*
E. distachya ssp. *distachya* (France) 1109191	S	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E. distachya ssp. *distachya* (France) 12 samples	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E. distachya ssp. *distachya* (Turkey) 2 samples	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
E. distachya ssp. *helvetica* (Switzerland) 6 samples	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
E. sinica (AY 394071)	*	*	A	A	C	T	C	*	C	A	G	T	A	C	C

*: same as the top sequence. —: gap., Y: C or T., S: C or G.

cortex and pith of *E. sinica* than in those of the *E. distachya* samples collected in France (Table 1, Fig. 1F).

Ephedrine alkaloid content (Fig. 2)

1. *Ephedra distachya* subsp. *distachya*

There were no significant differences in the total amount of ephedrine alkaloids (ephedrine and pseudoephedrine) between the *E. distachya* subsp. *distachya* samples from France (mean: 0.36 ± 0.29%) and Turkey (mean: 0.17 ± 0.17%). We also noticed that the ephedrine levels of all of the samples were so low that they could hardly be quantified. Moreover, only 3 of 21 samples met the requirements of the Japanese Pharmacopoeia (more than 0.7%), which suggests that *E. distachya* subsp. *distachya* does not have potential as a source of Ephedrae Herba.

However, two previous studies have reported that *E. distachya* subsp. *distachya* contained more ephedrine than pseudoephedrine (Moriyasu 1984, Kajimura 1994), which was contrary to our results. We noticed that the plant materials they used were cultivated in the Botanical Garden of Gifu College of Pharmacy and the Faculty of Pharmaceutical Sciences of Osaka University, respectively. However, they did not provide clear information about the provenance of their cultivated plants, so the differing results might have been due to the samples having different origins. It was reported that the alkaloid content of *Ephedra* plants was influenced by the area in which they grew (Zhang 1989a) and soil alkalinity (Kondo 1999), so we consider that the samples being grown in different environments is a reasonable explanation for the differences between their and our alkaloid content results, especially considering that Japan is not the natural habitat of *E. distachya* subsp. *distachya*.

2. *Ephedra distachya* subsp. *helvetica*

Only one of the *Ephedra distachya* subsp. *helvetica* seven samples had their ephedrine alkaloid contents quantified, so we were not able to reach any conclusion about the ephedrine/pseudoephedrine (E/PE) ratio. In addition, in the sample that was tested only pseudoephedrine was detected. Thus, like *E. distachya* subsp. *distachya*, *Ephedra distachya* subsp. *helvetica* could not be used as a source of Ephedrae Herba.

Nucleotide variations in the ITS1 regions of Ephedra distachya *and* E. sinica (Table 2)

Among 19 accession samples of *E. distachya* from France, two accession samples (1109173, 1109176) displayed substitutions at position 894, and 1 accession sample (1109191) had a substitution at position 80, whereas the other accession samples displayed identical ITS1 regions as the accession sample reported in a previous study (Kakiuchi et al. 2011). The specimens from Turkey displayed the same sequences as the *E. distachya* subsp. *helvetica* samples from Switzerland, whose ITS1 regions differed from those of the *E. distachya* subsp. *distachya* samples from France due to a substitution at position 223. In contrast to the accession samples of *E. distachya*, whose ITS1 region sequences were reasonably similar, *E. sinica* was found to display about 12 nucleotide differences compared with the *E. distachya* subsp. *distachya* samples from France, including a nucleotide insertion at position 403. Besides these 12 nucleotide differences, *E. sinica* differed from the *E. distachya* subsp. *distachya* samples from Turkey and *E. distachya* subsp. *helvetica* at position 223, whereas it displayed an identical sequence to the *E. distachya* samples from France.

Comparisons between Ephedra distachya *and* E. sinica

Microscopic characteristics

We first tried to discriminate between *E. distachya* and *E. sinica* according to the numbers of fibers in the cortex and pith because these features have often been used by researchers to identify the official origins of Ephedrae

Herba including *E. sinica* (Konoshima 1945a, 1945b, Zhang 1989b, Xu et al. 1992, Fushimi 2008). Although some values overlapped, the *E. distachya* subsp. *distachya* samples from France could be separated from *E. sinica* using these parameters ($P < 0.001$), as well as from the *E. distachya* subsp. *distachya* samples from Turkey ($P < 0.001$) and the *E. distachya* subsp. *helvetica* samples from Switzerland ($P < 0.001$). However, the other samples could not be distinguished from each other according to the morphology of the fibers in their cortex or pith.

On the other hand, we noticed that the subepidermal fibers of the *E. distachya* and *E. sinica* samples displayed different morphologies. Statistical analysis showed that the mean length of the longest 5 subepidermal fiber bundles in *E. distachya* was longer than that in *E. sinica* (*E. distachya* subsp. *distachya*: $P < 0.001$; *E. distachya* subsp. *helvetica*: $P < 0.001$). As subepidermal fiber bundles might be longer in places where the cortex is wider, we also calculated the ratio of the length of subepidermal fiber bundles to the length of the corresponding cortex. The results confirmed that *E. distachya* has longer subepidermal fiber bundles than *E. sinica* (*E. distachya* subsp. *distachya*: $P < 0.001$; *E. distachya* subsp. *helvetica*: $P < 0.001$). We examined the mean angle of the tip of the longest 5 subepidermal fiber bundles as another parameter of subepidermal fiber morphology and found that *E. distachya* displayed smaller angles than *E. sinica* (*E. distachya* subsp. *distachya*: $P < 0.001$; *E. distachya* subsp. *helvetica*: $P < 0.001$), and it was indicated that the subepidermal fiber bundles of these two species are rectangular. Thus, we conclude that *E. distachya* can be morphologically distinguished from *E. sinica* using this feature.

Ephedrine alkaloid content and DNA sequence of ITS1 region

In chemical analysis, we found that the alkaloid content of *E. distachya* was much lower than the values reported for *E. sinica* by us (Wang 2010) and other researchers (Hong 2011b), although the ranges of the two species overlapped a little. Moreover, *E. distachya* hardly contained any ephedrine. In contrast, we previously found that *E. sinica* normally contains more ephedrine than pseudoephedrine (Wang 2010), while a recent report also found that the E/PE ratio of *E. sinica* was greater than 0.7 (Hong 2011b). Therefore, we concluded that *E. distachya* is phytochemically different from *E. sinica*.

At the same time, both our molecular phylogenetic results and those reported previously (Ickert-Bond and Wojciechowski 2004, Huang et al. 2005, Rydin and Korall 2009, Kakiuchi et al. 2011) showed that *E. distachya* possesses a different ITS1 region from *E. sinica*.

Comparisons between Ephedra distachya *subsp.* distachya *and* E. distachya *subsp.* helvetica

Microscopic characteristics

The *Ephedra distachya* subsp. *distachya* samples from France could be easily distinguished from *E. distachya* subsp. *helvetica* according to the morphology of the fiber bundles in their cortex ($P < 0.001$) and pith ($P < 0.001$). The length ($P < 0.001$) and angle ($P < 0.01$) of the subepidermal fiber bundles of these two species were also found to differ. It was difficult to determine the botanical characteristics of the *E. distachya* subsp. *distachya* samples from Turkey because only two samples were studied, but generally they did not display any significant difference from *E. distachya* subsp. *helvetica* with regard to the morphology of the fiber bundles in their cortex or pith; however, they did have significantly longer subepidermal fibers than the *E. distachya* subsp. *distachya* ($P < 0.001$) samples from France and *E. distachya* subsp. *helvetica* ($P < 0.001$). Thus, we concluded that these longer subepidermal fiber bundles can be used to differentiate between *E. distachya* subsp. *distachya* and *E. distachya* subsp. *helvetica*. It was unclear why the *E. distachya* subsp. *distachya* samples from France had many

more fiber bundles in their cortex and pith than those from Turkey, although we consider that environmental differences might have played a role as the plants from France grew on a sandy seashore while those from Turkey grew in the clay soil at the foot of a mountain.

Ephedrine alkaloid content and the DNA sequence of the ITS1 region

Chemical analysis showed that *E. distachya* subsp. *helvetica* hardly contained any alkaloids, which was different from *E. distachya* subsp. *distachya*. In addition, a molecular phylogenetic study showed that these two species possessed no more than two nucleotide variations in their ITS1 regions.

Conclusion

We concluded that *Ephedra distachya* and *E. sinica* differ to some extent, based on the differences in the shapes of their subepidermal fiber bundles, their E/PE ratios, and their ITS1 region sequences. Thus, we suggest that these two species represent different taxa although they are morphologically similar.

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倪　斯然[a]，松本昌士[a]，下山祐依[b]，N. Allain[c]，M. Coşkun[d]，T. Yilmaz[d]，御影雅幸[a]：**Ephedra distachya の組織学的，化学的，分子遺伝学的研究**

マオウ科の *Ephedra sinica* Stapf の草質茎は，中国伝統医学で麻黄の名称で薬用にされる重要生薬である．本種はヨーロッパを中心に自生する *E. distachya* L. と同種であるとする説がある．そこで本研究では，*E. distachya* を麻黄として利用可能か否かを検討するため，スイス，フランス及びトルコで採集した株について，内部形態，含有アルカロイド，並びに DNA 塩基配列を検討した．その結果，本種は *E. sinica* とは，草質茎の横断面では表皮下繊維群の形，化学的にはエフェドリン類アルカロイドの組成比が大きく異なり，また ITS1 領域の DNA 配列は約 11 塩基が異なっていた．以上の観点から，両種は別の分類群であると判断され，現在の日・中の薬局方に照らし合わせると，*E. distachya* は麻黄として利用できないと結論した．

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マオウ属植物の栽培研究（第２報）[1)]
海水がシナマオウの生長およびアルカロイド含量に及ぼす影響

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Studies of Cultivation of Ephedra Plants (part 2).
Effect of sea water on the growth and alkaloid content of *Ephedra sinica* Stapf

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Summary

Ephedra plants have salt tolerance to some degree. We reported the salt tolerance of ephedra in germination stage in the previous paper. In this study, we report the salt tolerance of ephedra in growing stage, comparing with some wild plants, and the effect of salt water on alkaloid content of ephedra. The result showed that the salt tolerance of *Ephedra sinica* Stapf was superior than *Amphicarpaea edgeworthii* Benth. and *Artemisia indica* Willd. var. *maximowiczii* H.Hara (= *Altemisia princeps* Pamp.), same as *Imperata cylindrica* Raeusch., and less than *Chenopodium album* L. Moreover, the alkaloid content of herbal stem of ephedra intentionally increased by giving the artificial sea waters thinned to 1/16 once a week.

要　旨

マオウ属植物はある程度の耐塩性を有している．前報ではマオウ種子の発芽期における耐塩性を検討した．本研究では生長株の耐塩性を他の野生植物と比較検討するとともに，アルカロイド含量への影響を調査した．その結果，シナマオウの耐塩性はヤブマメ，ヨモギに勝り，チガヤと同程度であり，シロザに劣っていた．また，16分の1希釈した人工海水を週１回間灌水することにより，アルカロイド含量が有意に増加することが明らかになった．

緒　言

著者らの中国における*Ephedra* 属植物の自生地における現地調査の結果，日局「麻黄」の１基原植物である *Ephedra sinica* Stapf (=*E. dahurica* Turcz.) シナマオウは，野生地では土質を選ばず生育し，塩性地や海岸などにも生育する一方，背が高くなる雑草との生存競争に弱いことが明らかになっている[2]．中国では麻黄の栽培が行なわれているが，マオウ属植物は多年草で，同一場所で継続栽培されることから，他の一年生の農作物の場合には毎年耕作時に除草可能であるのに対して，除草に手間がかかる欠点がある．そこで，塩性地での栽培は除草の手間が少なくなる可能性があると考え，前報[1]ではシナマオウの発芽期の耐塩性について検討し，アカミタンポポやレンゲソウなどの一般の植物や海岸の砂地で自生するカワラヨモギよりも高く，日本の海岸砂地に一般的に見られるハマダイコンなどと同等の耐塩性があることを報告した．また，塩性地で生育した*Ephedra* 属植物は，通常よりアルカロイド含量が高いという報告がある[3]．そこで，本報では，発芽後の生育を他の一般植物と比較するとともに，塩分がアルカロイド含量に及ぼす影響について検討した結果を報告する．

【実験方法】

実験材料：発芽後４年目のシナマオウ及び金沢大学医薬保健学域薬学類・創薬科学類附属薬用植物園（以下，薬草園）内に自生する４種の植物（キク科のヨモギ，アカザ科のシロザ，イネ科のチガヤ及びマメ科のヤブマメ）を用いた．これらの４種の植物は，中国における麻黄栽培畑地において多く見られた有害雑草と同科あるいは同属植物種である[4]．

人工海水の調製：塩化ナトリウム434.0g，硫酸マグネシウム七水和物103.9g，塩化マグネシウム六水和物78.6g，塩化カルシウム二水和物22.4g及び塩化カリウム11.1gを薬草園の地下水に溶解して15 Lにし，人工海水とした．人工海水１/２，１/４，1/8及び１/16希釈液は上記電解質の量をそれぞれ１/２，１/４，１/８及び１/16にし，薬草園の地下水に溶解して15 Lとして調製した．

栽培容器と土壌：容器として1/2000aのワグネルポット，栽培土壌として川砂を用い，土壌下層に元肥として化成肥料［普通化成８号（フジカワエッグ，N:P:K = 8:8:8)］25 g/potを混合し，実験植物を１ポットあたり３株定植した．定植直後に置肥としてプロミック遅効きタイプ中粒（ハイポネックスジャパン，N:P:K = 8:8:8）６錠/potを与えた．2007年7月11日に同内容の置肥を追加した．

定植と管理：定植日はシナマオウが実験第一年目（2007年）の４月８日～９日，ヨモギ及びチガヤが４月24日，シロザが５月２日，ヤブマメが５月21日．シナマオウは54株，他の植物は各18株定植した．実験は雨の当たらないビニールハウス内で行なった．

実験群と灌水方法：上記の方法で定植した実験材料の中から生長度が揃った株を選択し，１群あたりシナマオウ９株，他の植物３株とし，各６群ずつを準備した．毎週月曜日に各群にそれぞれ無希釈の人工海水，１/２，１/４，１/８，１/16希釈の人工海水，及びブランクとして薬草園の地下水を灌水した．土壌中塩分の濃縮を防ぐため，毎週金曜日にすべての群に地下水を灌水した．１回の灌水量は２L/potとした．上記の灌水を実験一年目は2007年６月４日～2007年11月12日に行なった．

実験二年目（2008年）はシナマオウについてのみ，一年目に枯死しなかった株を３月31日～４月１日にすべて１/5000aのワグネルポットに１株ずつ新たな川砂で再定植し，施肥は元肥を10g/pot，置肥を２錠/potとした．実験材料は定植直後からビニールハウス内にて管理した．一年目に人工海水１/８，１/16希釈液及び地下水を与えた群は引き続き同濃度の希釈人工海水及び地下水を与えて栽培し，人工海水１/２及び１/４希釈液を与えて栽培した群の株には人工海水１/16希釈液を与えた．毎週金曜日に希釈人工海水を灌水し，毎週火曜日に地下水を灌水した．１回の灌水量は400 mL/potとした．上記の灌水を4月11日～11月７日に行なった．

生長の評価：シナマオウについては草質茎の総長（全ての茎の長さの総和），ヨモギ及びシロザについては草丈及び葉の枚数，ヤブマメについては草丈及び小葉の枚数，チガヤについては葉の総長をそれぞれ生長評価の指標とした．一年目の生長評価は，シナマオウは５月９日～11日，６月１日，７月３日～４日，７月30日～31日，９月11日～12日，11月8日～13日に行なった．ヨモギ，シロザ，ヤブマメ，チガヤについては６月から10月まで，各月の初旬に行なった．シナマオウの二年目の生長評価は４月８日～12日，６月30日～７月４日，９月８日～９月11日に行なった．

アルカロイドの定量：実験一年目の11月13日に，分析に十分量の草質茎を有する36株のシナマオウから３～５本の草質茎を基部から採取し，それらの全量を乾燥粉末化し，アルカロイド[ephedrine(E)及びpseudoephedrine (PE)]を定量した．〈装置：日立製〉ポンプ: L-2130，オートインジェクター：L-2200，紫外部検出器：L-2400，クロマトデータ処理及びシステムコントロールソフト：D-2500．〈HPLC条件〉カラム:ODS(4.6mm×250mm)，カラム温度：室温，流速：1.0mL/min，検出波長：210nm，注入量：10μL，移動相：$C_{12}H_{25}OSO_3Na$溶液（１→１２７)/MeCN/H_3PO_4 (305:195:0.8)．

二年目は9月11日に同様に36株から草質茎を採取し，測定した．また，前年のデータと比較するため，11月14日に上記36株のうち３株から再び草質茎を採取し測定した．

【結果】

灌水液中塩分濃度が実験植物の生長に及ぼす影響

実験一年目における灌水液の塩分濃度別の平均草質茎長をFig.１に示す．無希釈の人工海水を灌水した群では実験終了までにすべての株が枯死した．人工海水1/2希釈液を灌水した群では２株が枯死し，生存していた株も著しく生長が抑制され，最終生長評価時における平均草質茎長はブランクの半分程度であった．人工海水1/4，1/8及び1/16希釈液を灌水した群ではブランクと比較してわずかに生長が抑制されたものの，ほとんどの株がほぼ正常に生長した．

ヨモギについては，無希釈の人工海水および1/2希釈人工海水の群では全株が年内に枯死した．1/4液希釈液群ではブランクと比較して草丈では約90％，葉の枚数では55％程度であった．1/8希釈以下では顕著な生長不良は認められなかった．

シロザについては，無希釈の人工海水群で３株のうち２株が枯死し，残った１株の草丈も地下水群の70％程度であった．また，1/2希釈液群では枯死株はなかったが，明らかな生長阻害が見られ，1/4希釈以下では顕著な生

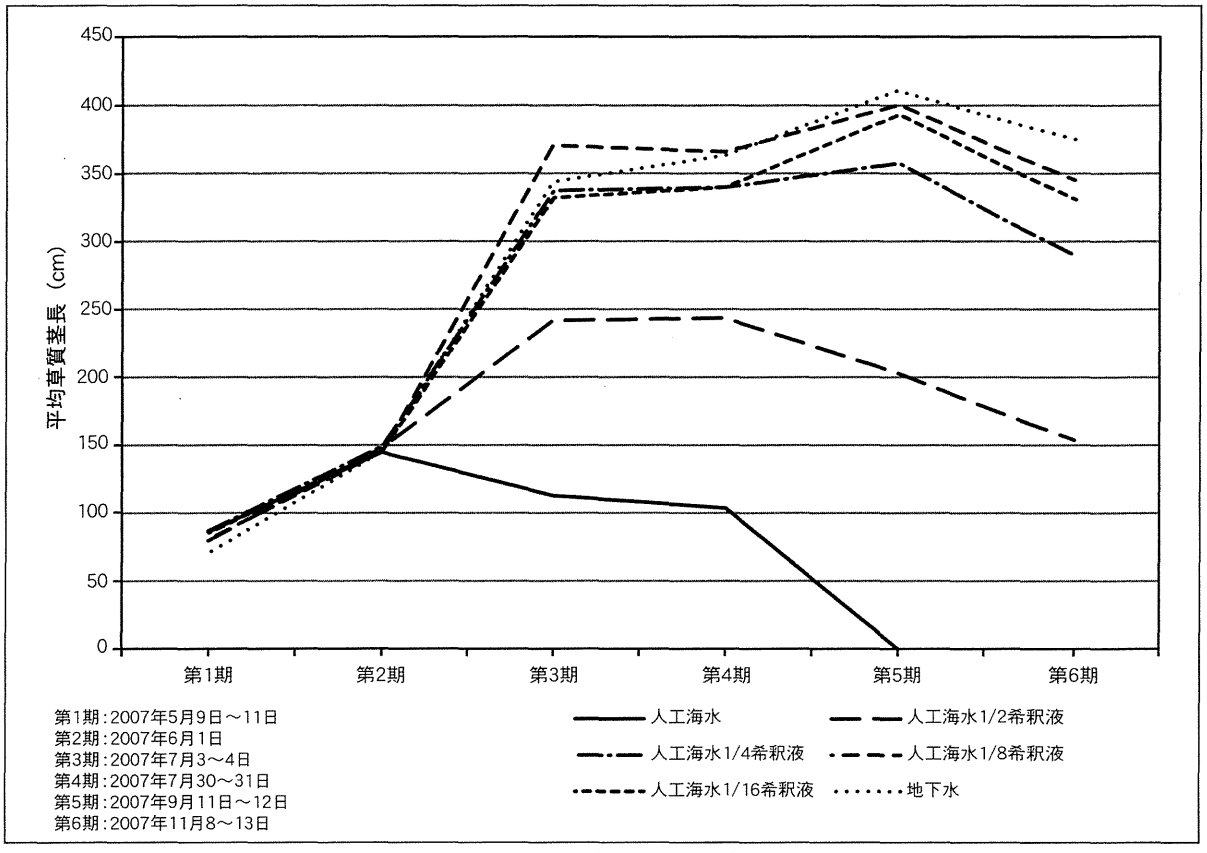

Fig. 1　灌水液の塩分濃度とシナマオウの平均草質茎長（実験一年目）

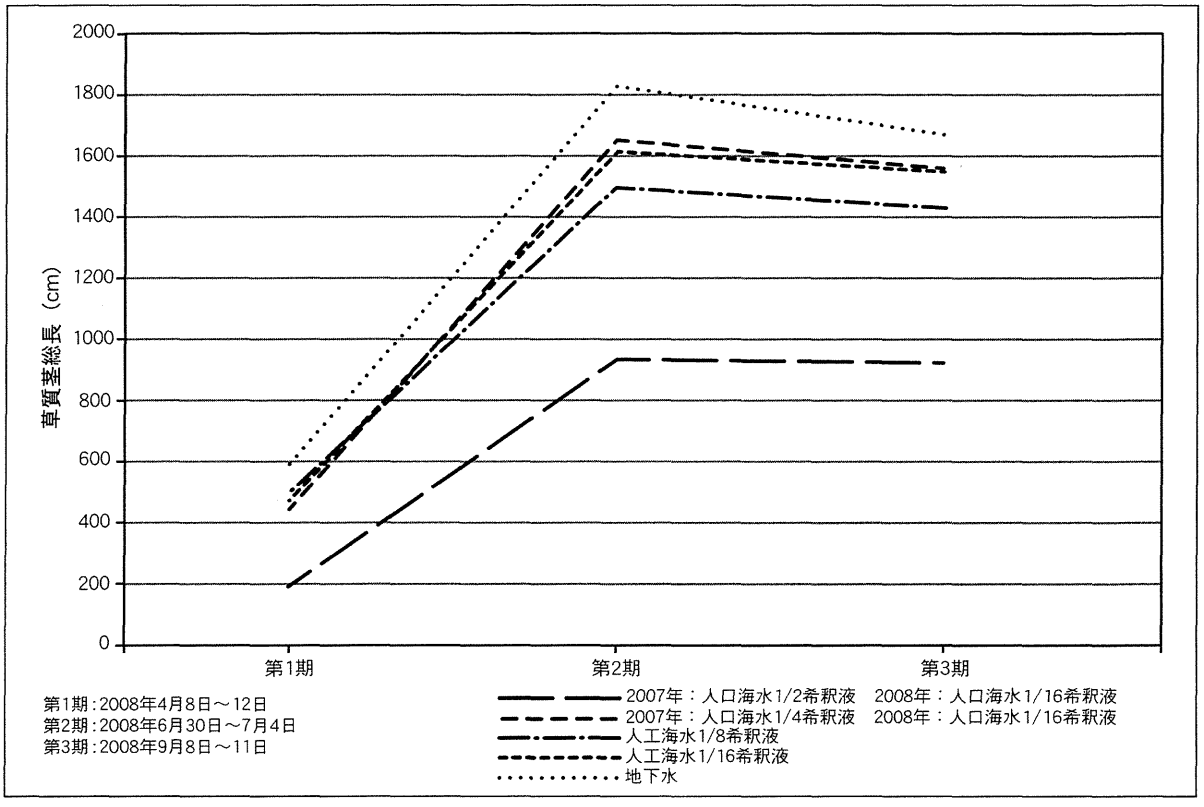

Fig. 2：灌水液の塩分濃度とシナマオウの平均草質茎長（実験二年目）

長不良は認められなかった.

チガヤについては，無希釈の人工海水を灌水した群ではすべての株が枯死した．1/2希釈群では明らかな生長阻害が見られ，葉の総長は地下水群の30％程度であった．1/4希釈群以下では有意な阻害は認められなかった.

ヤブマメについては，1/16希釈以上の人工海水濃度では全て第１期には生存していたが，実験終了までには全株が枯死した.

以上，ヤブマメは最も耐塩性が低く,ヨモギはヤブマメに次いで耐塩性が低く,チガヤは耐塩性が比較的高く，シロザが最も耐塩性が高いことが明らかになった.

実験二年目のシナマオウの灌水液の塩分濃度別の平均草質茎長をFig.２に示す．一年目に人工海水１/２希釈液を，二年目に人工海水１/16希釈液を灌水した群の平均草質茎総長はブランクの半分程度であったが，それ以外の群の平均草質茎長はブランクとほぼ同等であった．ブランクの平均草質茎総長は７月に最大となり，その値は約1800cmであった.

塩分濃度がシナマオウのアルカロイド含量に及ぼす影響

異なる塩分濃度の人工海水を灌水した実験一年目のマオウ草質茎の平均アルカロイド含量をFig.３に示す．人工海水１/16希釈液を灌水した群の平均アルカロイド含量は約0.70%であり，ブランクの約1.7倍であった．人工海水１/２，１/４及び１/８希釈液を灌水した群の平均アルカロイド含量はブランクよりも低く，いずれも0.3%前後であった.

実験二年目の９月11日に採取した草質茎のアルカロイド含量をTable １ に示す．人工海水１/16希釈液を灌水した群の平均アルカロイド含量は0.845±0.35%であり，ブランクの約1.2倍であった．人工海水１/８希釈液を灌水した群の平均アルカロイド含量はブランクより低く，約0.48%であった．2007年に人工海水１/２及び１/４希釈液を，2008年に人工海水１/16希釈液を灌水した群の平均アルカロイド含量はそれぞれ約0.54及び0.58%と，ブランクには劣るものの，人工海水１/８希釈液を灌水した群よりも高かった.

結論および考察

１．シナマオウは人工海水１/４希釈液以下の塩濃度においてほぼ正常に生育できることが明らかになった．また，シナマオウの耐塩性はヤブマメ，ヨモギに勝り，チガヤと同程度であり，シロザに劣っていた．以上，人工海水１/４希釈液を灌水して栽培を行なえば，シナマオウを正常に生長させ，ヤブマメやヨモギの生長を抑え，除草の省力化が期待できると判断できる．しかし，この条件ではチガヤとシロザの生長を抑えることはできなかった．とくにチガヤは地下に根茎を蔓延して繁殖するため，除草作業により根絶させることは困難である．中国の麻黄栽培地においても根茎を引いて増殖するイネ科植物の除草に苦労しており[4]，有害雑草はできる限り早期に除草する必要があろう．なお，人工海水１/４希釈液を灌水した群の土壌中塩分濃度を塩分計(Salt Tester 11, EUTECH INSTRUMENTS) を用いて測定した結果，表面において0.80%，深部において0.08%を超えることはなかったことから，この土壌中塩分濃度がシナマオウが正常に生長できる限界の濃度であると判断された（測定法，データ等省略).

２．灌水液中の塩分濃度がシナマオウのアルカロイド含量に及ぼす影響に関しては，人工海水1/16希釈液を灌水した群の平均アルカロイド含量が最も高く，一年目がブランクの約1.7倍，二年目が約1.2倍であった。これま