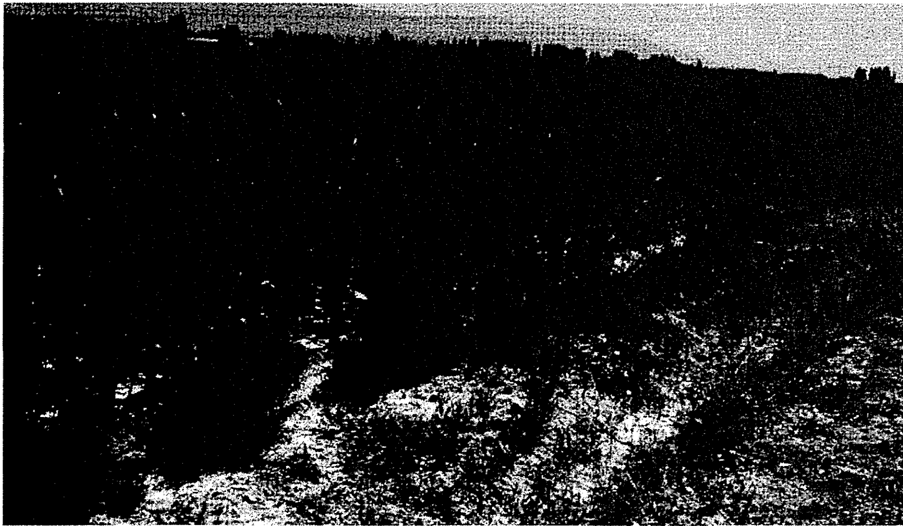

Fig.1 *Ephedra equisetina* and *E. sinica* cultivated in the same field in Pole, Xinjiang autonomous region, China. The stocks in this side (left, bigger) are *E. equisetina*, and in the other side are *E. sinica*.

ていたが，栽培生産物を換金できず，2013 年春に栽培を放棄し，トウモロコシに転作した．今回得られた試料は耕作機械が入らなかった畑の辺縁部に残存していた株である（Fig. 1）．

種の同定については，主に中国植物志[8]の記載に従って外部形態により行った．すなわち雌毬果が認められた３個体（130625A-1，２;*E. equisetina* 及び130625A-4；*E. sinica*）について，前者は１毬果あたり種子が１個，後者は１毬果あたり種子が２個，また，共に珠孔管が約１㎜で湾曲しないことにより同定した．雄株あるいは毬果の認められない株は前述の方法で同定可能であった株と木質茎の発達程度，草質茎の葉の鱗片の形状などを比較して判断した．それでも判定困難であった株については過去の報告[6]を参考に比較組織学的に検討した．

アルカロイドの定量

第16改正日本薬局方の定量法を参考にして以下の条件で測定した．試料溶液の調製：粉末約0.3 gを精密に量り，薄めたメタノール（1→2) 30 mLを加え，15 分間振り混ぜた後，遠心分離し上澄液をろ過し，試料溶液とした．

標準品：ephedrine-HCL，pseudoephedrine-HCl，methylephedrine-HCL, norephedrine-HCLは和光純薬㈱製のものを使用した．norpseudo-ephedrine-HCLはnorephedrine-HClからクラシエ製薬で合成したものを使用した．

HPLC測定条件：LC-20ADpump，SIL-20AC HT autosampler，CTO-20AC column oven，SPD-20A detector（Shimadzu），YMC-Pack ODS-A column (6.0mm I.D.×150mm), Column temperature:40°C, Flow rate: 1.0 mL/min, Detection wavelength: 210 nm, Mobile phase: 27 mM sodium lauryl sulfate (SDS) solution/MeCN/H_3PO_4 (640:360:1)

結果はstudent T-testにより統計処理をした．

結果・考察

１．アルカロイド含量測定結果をFig. 2，Table 1に示す．*Ephedra equisetina*の総アルカ

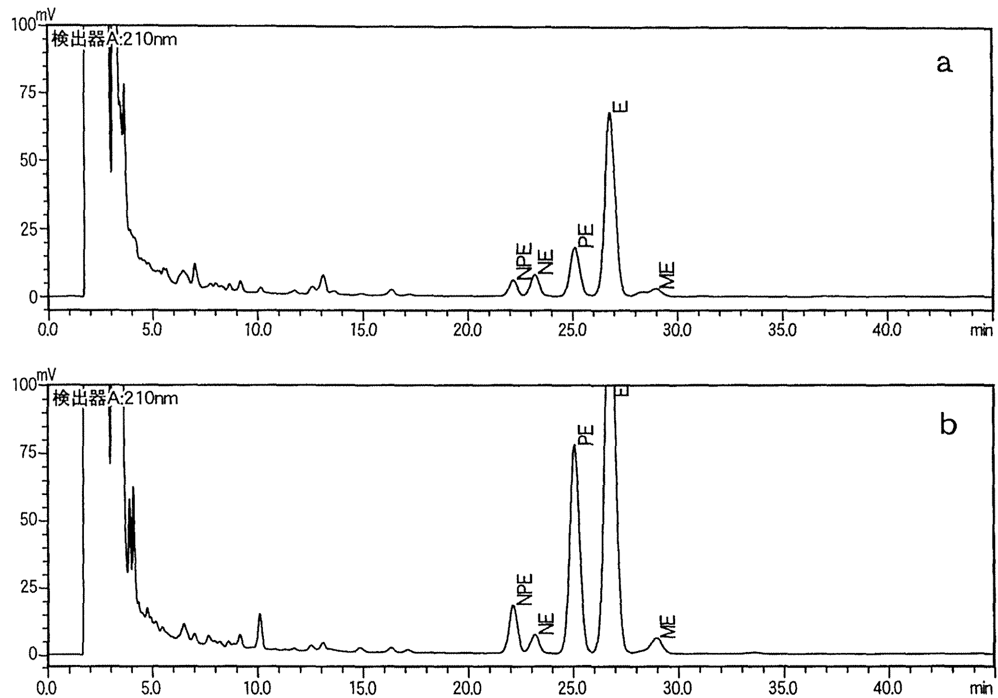

Fig.2 HPLC chromatograms of Ephedra plants samples.
a *Ephedra sinica* (130625A-3), b *Ephedra equisetina* (130625A-2)
NPE:norpseudoephedrine, NE:norephedrine, PE:pseudpephedrine, E:ephedrine, ME:methylephedrine

ロイド含量は，いずれも日局16の含量規格である0.7 %以上で，*E. sinica*は0.7 %未満のものが1検体認められ，総アルカロイド含量の平均値は*E. sinica*(0.80±0.24 %)よりも*E. equisetina*（2.32±0.39 %）の方が有意に高かった．中国での栽培者からの情報では，マオウ属植物は栽培当初はアルカロイド含量が低いが，播種後5～6年で安定するとされるため，本研究での実験材料のアルカロイド含量はすでに安定していると判断される．このことから，*E. equisetina*は生育環境によらず，*E. sinica*よりもアルカロイド含量が高い種であると判断された．すなわちアルカロイド含量は，生育環境だけではなく，種の違いにも影響を受けることが示唆され，局方3種のうち，*E. equisetina*の総アルカロイド含量が*E. sinica*よりも高いとする過去の複数の報告を支持する結果を得た．また，アルカロイド組成については，個体によってばらつきが認められた．*E. sinica*，*E. equisetina*ともにephedrine含量がpseudoephedrine含量より高い検体が多かったが，1検体ずつ比率が逆の検体が認められた．norephedrine 及び methylephedrine は ephedrine 及び pseudoephedrineと比較して含量が低かった．一方norpseudoephedrine 含量については個体差が大きく，*E. sinica*では最高0.37 %，*E. equisetina*では同じく0.41 %を含有する検体が認められた．前者では5種のアルカロイド総含量の約35 %となり，薬効に影響することも考えられる．

2．栽培株の地上部に関し，*E. equisetina*は基部が木質化し，一方の*E. sinica*は木質化して

Table1. Alkaloid contents in the herbals tems of *Ephedra* plants cultivated for8years fromseedlings in Pole, Xinjiang autonomous region, China.

			contents (%)								Eph	(Eph+P-Eph)	NP-Eph
	Voucher No.	sex	Eph	P-Eph	N-Eph	NP-Eph	M-Eph	E+PE	E+PE (mean±SD)	TAs	/P-Eph	/TAs	/TAs
Ephedra sinica	130625A-3	−	0.83	0.21	0.08	0.05	0.04	1.04	0.80±0.24	1.21	3.95	0.86	0.04
	130625A-4	♀	0.27	0.19	0.01	0.02	0.02	0.47		0.52	1.43	0.89	0.05
	130625A-5	−	0.84	0.08	0.06	0.02	0.05	0.92		1.05	10.78	0.88	0.02
	130625A-6	−	0.64	0.07	0.06	0.02	0.03	0.71		0.81	9.79	0.87	0.02
	130625A-7	−	0.50	0.65	0.02	0.28	0.02	1.15		1.47	0.76	0.78	0.19
	130625A-12	−	0.43	0.31	0.03	0.10	0.02	0.74		0.89	1.39	0.83	0.11
	130625A-13	−	0.36	0.23	0.08	0.37	0.00	0.59		1.04	1.53	0.57	0.35
E equisetina	130625A-1	♀	1.13	0.68	0.12	0.15	0.02	1.81	2.32±0.39*	2.09	1.65	0.86	0.07
	130625A-2	♀	1.94	0.92	0.07	0.15	0.09	2.86		3.17	2.12	0.90	0.05
	130625A-8	−	1.38	0.85	0.08	0.04	0.06	2.23		2.41	1.62	0.92	0.02
	130625A-9	−	1.25	0.88	0.06	0.41	0.03	2.13		2.64	1.41	0.81	0.16
	130625A-10	−	1.51	1.21	0.07	0.06	0.07	2.72		2.92	1.25	0.93	0.02
	130625A-11	−	0.89	1.28	0.03	0.07	0.05	2.18		2.32	0.70	0.94	0.03

Eph:ephedrine,P-Eph:psudoephedrine, N-Eph:norephedrine, NP-Eph:norpseudoephedrine, M-Eph:methylephedrine.
E+PE:ephedrine+pseudoephedrine, TAs:totalcontent of Eph, P-Eph, N-Eph, NP-Eph and M-Eph
*:stasistically significant (student T-test, $P<0.05$).

いなかったため，地上部の大きさについては，*E. equisetina* の方がやや大型であった．調査圃場においては地上部の特徴が同様の個体群は連続して植栽されており，特徴が異なるものとの混植はされていなかった（Fig. 1）．この事実は両種の種子が別の時期あるいは別系統として配布され，播種育苗後に順次定植された結果であると推定される．また政府機関から栽培が奨励されて配布されたマオウ属植物種子に関しては，これまでの複数の調査で情報収集し確認したものはすべて内蒙古で採取された *E. sinica* で，稀に *E. intermedia* と思われるものが混在していた[9]．*E. equisetina* の種子が配布されたことが確認されたのは今回が最初である．今回評価した *E. equisetina* の中に毬果が黄熟するものが認められたが，筆者らの調査ではこのような形態を示す *E. equisetina* は新疆ウイグル自治区北部において資源が豊富で，他の自生地ではほとんど認められなかったことから，配布された種子は新疆ウイグル自治区産の可能性が高いと考えられる．一方，*E. sinica* については新疆ウイグル自治区には自生がないため，内蒙古自治区など別の地方から移入された種子であると推測する．

3．筆者らの寧夏回族自治区におけるこれ迄のマオウ栽培地の調査において，*E. equisetina* の栽培は困難であるとする情報を得ており[9]，実際，寧夏回族自治区だけではなく，内蒙古自治区や新疆ウイグル自治区の他の栽培地を含めても，*E. equisetina* の栽培は見られなかった．今回調査した栽培地において，定植後は管理のための灌水や除草作業を一度もしなかったとの情報を得たが，目立った欠株は見られず，生育状態も正常であった．このことから，この土地は *E. equisetina* を含めてマオウの栽培に適した環境であると判断された．すなわち降雨量が少なく，他の雑草がマオウの生長を阻害するほどに生長することができず，かつマオウの生育には影響を及ぼさない程度には降雨がある土地であると判断される．当地で栽培された *E. sinica* 株についてもアルカロイド含量が日局

16の規定を下回ったのは1株のみであり，降雨量の少ないことがアルカロイド含量を増加させている可能性が考えられる．

4．現在日本国内で消費される麻黄は，主に中国からの野生品に依存している[10]が，中国政府は資源保護や砂漠化防止を理由に輸出を制限している．我々はすでに日本での栽培研究に取り組み，地下茎による繁殖能力が高い *E. sinica* を中心に栽培方法の検討を行っているが，日本のような多雨多湿な環境下ではアルカロイド含量が全体的に低い傾向にあり，安定して日局16の含量規格に適合するものを生産するためには，今後種々の対策を検討する必要がある．本研究において，*E. equisetina* はアルカロイド含量が有意に高含量であることが認められたことから，今後日本で本種を栽培した場合のアルカロイド含量に興味が持たれる．しかし本種は根茎を伸長させる性質が弱く，栽培が困難な種であるとされる．根茎を伸長させる能力に優れて繁殖能力が高い *E. sinica* などに比して栽培が困難であり，今後は栽培方法の検討や育てやすい株（系統）の選抜の他，より栽培が容易な *E. sinica* と交配させるといった品種改良の取組みも必要であろう．

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The new finding about the classification of *Ephedra major* subsp. *procera* -Based on comparison of DNA and ephedrine alkaloid with *E. equisetina*-

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The classification of *Ephedra* plants has long been a matter of debate, as *Ephedra* plants have few morphological characteristics to aid classification. In particular, the distinction between *E. equisetina* and *E. major* ssp. *procera* is uncertain. Thus, in this study, an attempt was made to clarify their relationship by molecular analysis and content of ephedrine alkaloid. Molecular analysis revealed that *E. equisetina* and *E. major* ssp. *procera* have a close relationship with differing matrilineage, and chemical analysis showed that they have the same characteristic which varied alkaloid composition ratio by collection site. Thus, we suggest that on the basis of molecular phylogeny that *E. major* ssp. *procera* should be treated as a synonym or subspecies of *E. equisetina* instead of a subspecies of *E. major* ssp. *major*.

Key word: *Ephedra major* subsp. *procera*, *Ephedra equisetina*, ITS1, *trn* K, alkaloid

Introduction

The genus *Ephedra* is a taxon of approximately 50 species that are distributed in the Eurasian Continent, the northern part of the African Continent, and the arid area in the western American

continent (Price 1996). Fourteen species of them including *Ephedra sinica* Stapf and *E. intermedia* Schrenk et C.A. Meyer grow in China (Fu L. G. 1999). The classification of *Ephedra* plants has long been a matter of debate, because of the few morphological differences that exist between them. For example, regarding *E. sinica* in China and *E. distachya* L., which is distributed from Europe to Asia, some researchers think that *E. sinica* is identical to *E. distachya*. However, anatomical data and molecular analysis have revealed that these two species are different, although they are morphologically quite similar (Ni 2013). Moreover, many researchers have discussed the classification of *E. sinica* and *E. dahurica* Turcz., and it has been suggested that *E. sinica* should be reduced to a subspecies of *E. dahurica* (Kakiuchi 2011). Moreover, since the morphological characteristics of *E. equisetina* Bunge are similar to those of *E. major* Host ssp. *procera* (C.A.Mey.) Bornm., the distinction between *E. equisetina* and *E. major* ssp. *procera* is uncertain (Rydin 2010). It has been reported in Flora Reipublicae Popularis Sinicae that *E. equisetina* are morphologically similar to *E. procera* (= *E. major* ssp. *procera*) and could be treated as the same species (Cheng C. Y. 1978).

It is known that *E. equisetina* usually grow at dry and rocky places at high altitudes of about 800 to 3000 meters in China (Gansu, Hebei, Inner Mongol, Ningxia, Qinghai, Shanxi, Xinjiang), Afghanistan, Kazakhstan, Kyrgyzstan, Mongolia, Russia, Tajikistan, Turkmenistan, and Uzbekistan (Fu L. G. 1999). Plants in the genus *Ephedra* generally produce two seeds in each cone, but *E. equisetina* has only one (Fu L. G. 1999). Moreover, *E. equisetina* is a species that contains a high level of alkaloids (Cheng C. Y. 1978) and more ephedrine than pseudoephedrine (Zhang 1989). Some researchers have found diverse genotypes in *E. equisetina* by analyzing the DNA sequence of the non-coding region of nuclear ribosomal and chloroplast DNA. These genotypes have been registered in the DNA Data Bank of Japan (DDBJ).

Some subspecies have been classified under *E. major* including *E. major* ssp. *procera*. The distribution of *E. major* ssp. *major* is restricted to the western parts of the Mediterranean area. On the other hand, *E. major* ssp. *procera* has a wide distribution ranging from Western Europe and the Western Mediterranean area to Central Asia (Rydin 2010). In addition, *E. major* ssp. *procera* produces one seed in each cone and contains a high level of alkaloids, being not less than 2.5% of total alkaloids and more ephedrine than pseudoephedrine (Gokturk 2006). The classification of *E. equisetina* and *E. major* ssp. *procera* is complex as *E. equisetina* has various genotypes. Therefore,

we intended to study the phylogenic relationship of *E. equisetina* and related species by molecular analysis and the content of ephedrine alkaloid. In the molecular analysis, we investigated nuclear internal transcribed spacer (ITS) and chloroplast *trn*K genes used frequently for phylogenic analysis of closely related taxa of plants. In the analysis focusing on Ephedrine alkaloid content, we quantified ephedrine alkaloids (Ephedrine (E), Pseudoephedrine (PE), Norephedrine (NE), Norpseudoephedrine (NPE) and Methylephedrine (ME)) by high performance liquid chromatography (HPLC) analysis.

Materials

E. equisetina plant materials were collected in China and Mongolia in 2002, 2005, 2006, 2009, 2010 and 2012 (Table 1). *E. major* ssp. *procera* plant materials were collected in Turkey and France in 2012 (Table 1). The specimens were identified by Prof. M. Mikage and the curator of Musèum National d'Histoire Naturelle, and deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Kanazawa University (KANP), Japan. All the specimens identified as *E. major* were further distinguished as a ssp. *procera* based on their condensed branch and thin stem from ssp. *major*.

Methods

DNA preparation, PCR amplification and Sequencing

From the dried stem of the collected specimens, samples of about 50-100 mg were frozen in liquid nitrogen and ground into fine powder. Using a DNeasy Plant Mini Kit (Qiagen, Germany), DNA was extracted according to the manufacturer's protocol. The primer sets of Eph-1F2 (ACG TCG CGA GAA GTT CAT TG) and 5.8S-R (CGG GAT TCT GCA ATT CAC AC) were used to amplify the ITS1 region. 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 μM of each primer, approximately 100 ng of the DNA sample, and 0.5 units of KOD-Plus DNA polymerase (Toyobo, Japan). For the ITS region, the cycling conditions used for PCR were as follows. ITS1: 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 58 °C for 30 sec, and elongation at 68 °C for 45 sec, and a final elongation step at 68 °C for 5 min. While the primer sets and the cycling condition of *trn* K gene following previous study (Kitani 2009). Three microliters of the

PCR products were used for agarose gel electrophoresis, and the remained product was purified using the QIAquick PCR Purification Kit (Qiagen, Germany). The purified PCR products were used for cycle sequences using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, U.S.A.).

Analysis of ephedrine alkaloid content

We applied the optimized method from the quantification method described in JP16 (Society of Japanese Pharmacopoeia 2011).

Preparation of samples for injection: The mobile phase at 5.0 mL was added to each sample powder at 100 mg, and left at room temperature for 20 minutes. Subsequently, ultrasonic extraction was performed for 25 minutes. Each solution was centrifuged at 3,000 rpm for 15 minutes. The supernatant was filtered using a 0.45 μm membrane filter (Minisart RC25, Sartorius Stedim Biotech), and used as the sample solution. HPLC conditions: An L-2130 pump, L-2200 autosampler, L-2400 UV detector, D-2500 integrator (Hitachi), and Wakopak Handy ODS column (4.6 mm I.D. x 250 mm) (Wako Pure Chemical Industries, Co., Ltd.) were used. Column temperature: room temperature, Flow rate: 1.0 mL/min, Detection wavelength: 210 nm, Mobile phase: 27 mM sodium lauryl sulfate (SDS) solution/MeCN/H_3PO_4 (305:195:0.8)

Results

Nucleotide variations in the ITS1 regions of *E. equisetina* and *E. major*

We analyzed the ITS1 region of *E. equisetina* (57 specimens) from China and Mongolia and *E. major* ssp. *procera* (24 specimens) from Turkey and France (Table 1). The determined sequences were 1120 or 1121 bp in length for *E. equisetina* and 1121 bp in length for *E. major* ssp. *procera*. A schematic illustration of the different nucleotide positions of *E. equisetina* and *E. major* ssp. *procera,* based on the results of DNA sequence analysis of the ITS1 region, is shown in Fig. 1 and the phylogenetic tree constructed based on the ITS1 sequences using UPGMA method are shown in Fig. 2. The phylogenetic tree showed that *E. equisetina* (AY394073; registered in DDBJ) and the materials derived from *E. equisetina* (Table 1) formed the identical group, and *E. major* ssp. *procera* from Turkey and France formed the identical group also. However, *E. major* ssp. *major* was separated from *E. major* ssp. *procera* and *E. equisetina.*

Two types of overlapping nucleotide signal in ITS1 from two different alignments were observed in all specimens of *E. equisetina* (Fig. 3, Fig. 4). One type is the specimens 06c3094 (collected in China) and 20531051 (collected in Mongolia) which showed an overlapping signal from nucleotide position 770 (Fig. 3). From these complex ITS1 peaks, we divided the overlapping signal into two parts by signal intensity: the main peaks had high homology in *E. intermedia* and *E. sinica* and the 2nd peaks had high homology in *E. equisetina.* In another type of overlapping nucleotide signal in *E. equisetina* (except 06c3094 and 20531051), the nucleotide sequence from position 1 to 807 were identical to the nucleotide sequence of *E. equisetina* (GU968572) registered in DDBJ and all specimens of *E. major* ssp. *procera* used in this study. The overlapping nucleotide signal of those specimens were seen at a position starting at 808; two subtypes of overlapping peaks were found: subtype A and subtype B. Subtype A is a group of specimens in which main peaks were determined to have high homology with AY394073 (*E. equisetina*) and the 2nd peaks were determined to have high homology with GU968572 (*E. equisetina*) (Fig. 4-A), in contrast, subtype B is a group of specimens in which main peaks were determined to have homology with GU968572 and the 2nd peaks were determined to have high homology with AY394073 (Fig. 4-B). When we compared the selected nucleotide sequence of AY394073 and GU968572, it could be clearly understanding that the reason for the overlapping nucleotide signals were attributed to a lack of base cytosine at the 808th position. In this study, all of the specimens derived from *E. equisetina* except 06c3094 and 20531051 were classified into type A or B based on the main peaks and 2nd peaks in the nucleotide sequence starting from the 808th position.

In the part of the ITS1 sequence of *E. major* ssp. *procera*, the specimens of *E. major* ssp. *procera* had almost identical GU968555 (*E. major* ssp. *procera*) in the DDBJ except one base indel at nucleotide position 35th and identical with the main peaks of subtype A after the 808th position observed in *E. equisetina* (Fig. 1 and Fig. 5). On the other hand, 71 or 73 bp of different bases in total were found when we compared *E. major* ssp. *procera* with *E. major* ssp. *major* (GU968557) as standard subspecies (Table 2). As a result, *E. major* ssp. *procera* and *E. equisetina* were sharing the quite identical sequence with the exception of one cytosine at the 808th position.

Nucleotide variations in the *trn* K regions of *E. equisetina* and *E. major*

In the resulting DNA sequence analysis of a part of the *trn* K gene of *E. equisetina* (27 specimens) from China and *E. major* ssp. *procera* (24 specimens) from Turkey and France. The

length of a part of the *trn* K gene of *E. equisetina* and *E. major* ssp. *procera* was 2307 bp, and 2307 bp or 2316 bp respectively (Table 3). Upon comparison of the nucleotide sequence of *E. equisetina* (AB453795) registered in DDBJ with the specimens derived from *E. equisetina*, it was clear that they were identical each other except two base-substitutions at the 1602th and 1864th positions. Besides, the two genotypes (type M-1, type M-2) were observed also in the *trn* K gene on the specimens of *E. major* ssp. *procera* which had the identical sequence of ITS1 region. The sequences of type M-1 and M-2 were different by 1 or 2 substitutions (1630th, 2014th) and 9 indels (TTT TCA ATG) from the 186th position to the 194th position. For the *E. major* ssp. *procera* from Turkey and France, five specimens (Ankara (n=2), Kirikkale (n=3)), were grouped into type M-1, whereas there were 18 specimens from Turkey: Karadiken (n=1), Kaiseri (n=4), Cappadocia (n=10), Kirikkale (n=3), and France: Paris (n=1) were divided into type M-2. Among the type M-2 collected from different countries (Turkey and France), their sequencing results showed one different base at the 1630th position. About the differences of sequence in *E. equisetina* and *E. major* ssp. *procera* (type M-1 and M-2), our results showed that there were 10 base variations between *E. equisetina* and type M-1, and there were 11 or 12 base variations and 9 indels between *E. equisetina* and type M-2.

Ephedrine alkaloid content

The *Ephedra* specimens were analyzed for their ephedrine alkaloid contents, as shown in Fig. 6. In Fig. 6-A, 57 specimens of *E. equisetina* from six sites showed the average of total alkaloid content (E+PE) 1.72%, which was more than two times the value prescribed in JP16. Moreover, the average total alkaloid content at each collection site was more than 0.7%, according to JP16. The specimens that had the highest alkaloid content were *E. equisetina* from Qinghai (2.64%). We showed five types of alkaloid composition ratio: E, PE, NE, NPE and ME in Fig. 7-A. Nonetheless, E and PE were the most prominent compounds. The composition ratio of *E. equisetina* from Xinjiang was E: 64%, PE: 20%, Hebei was E: 64%, PE: 22%, Qinghai was E: 13%, PE: 80%, Gansu was E: 12%, PE: 73%, Inner Mongolia was E: 7%, PE: 84%, Mongolia was E: 9%, PE: 82%.

The ephedrine alkaloid analysis of *E. major* ssp. *procera* (23 specimens except the French specimens) from five sites was shown in Fig. 6-B. Their average of total alkaloid content (E+PE)

was 0.70% which reached the value prescribed by JP16. Among most of the *E. major* ssp. *procera*, the specimens from Ankara had the highest alkaloid content (1.17%). The composition ratio of ephedrine in *E. major* ssp. *procera* specimens was analyzed according to the different locations (Fig. 7-B): Karadiken was E: 0%, PE: 93%, Kaiseri was E: 25%, PE: 65%, Kirikkale was E: 0%, PE: 97%, Cappadocia was E: 31%, PE: 62%, Ankara was E: 1%, PE: 95%. All of the specimens collected from Karadiken and Kirikkale contained an undetectable level of ephedrine, furthermore one specimen from Ankara had a low level of ephedrine.

Discussion and conclusions

(1) The relationship between *E. equisetina* and *E. major* ssp. *procera* has long been a topic of discussion.

There have been widely different opinions about the similarity of morphological characteristics in both groups. In addition, the histology and other characteristics of *E. equisetina* are quite variable and the distinction between *E. equisetina* and *E. major* ssp. *procera* is uncertain (Rydin 2010). The common morphological characteristics of *E. major* ssp. *major* are upright branchlets that are relatively thick (2-3 mm) and rougher than those of *E. major* ssp. *procera*. *E. major* ssp. *procera* has numerous, smooth, and thin (1 mm) branchlets (Rydin 2010, Freitag 2007). Based on morphological characteristics, it is assumed that *E. major* ssp. *procera* and *E. equisetina* are more similar to each other than to *E. major* ssp. *major*.

(2) In the DNA analysis of the ITS1 region, it could be clear that all of the specimens derived from *E. equisetina* had overlapping sequences. We found two types of sequences in *E. equisetina* used in this study (57 specimens). One is the overlapping sequence starting at the 770th position, another is the overlapping sequence starting at the 808th position. The classification of *E. equisetina* and *E. monosperma*, *E. equisetina* and *E. gerardiana* was difficult in the past, because of the high level of homology on ITS region (Kitani 2010). Here, we could clearly distinguish *E. equisetina* from *E. monosperma* and *E. gerardiana* by considering the existence of the overlapping sequence, because *E. monosperma* and *E. gerardiana* did not have overlapping sequence. The sequence of *E. equisetina* started at the 808th position, where the peaks overlap, is species-specific, and as such we are able to identify *E. equisetina*. It suggested that these sequences with overlapping

peaks derived from a cross-fertilization. The possibility of a hybrid on *E. equisetina* could be explained the morphological diversity of *E. equisetina*.

We found the differences between *E. major* ssp. *major* (GU968557, Algeria) and *E. major* ssp. *procera* in 71 or 73 bp of different bases on the nucleotide sequence. On the other hand, when we compared *E. equisetina* with *E. major* ssp. *procera*, the difference of both species was the existence of the overlapping sequence from the 808th position; one deletion of cytosine at the 808th position were found either main peaks or 2nd peaks. As a result, from our phylogenetic comparison of the ITS1 region, we found that *E. major* ssp. *procera* has a closer relationship with *E. equisetina* than with *E. major* ssp. *major*. The phylogenetic trees showed that *E. major* ssp. *procera* was located between the materials derived from *E. equisetina* (Table 1) and *E. equisetina* (GU968572; registered in DDBJ), so, *E. major* ssp. *procera* contained in the diversity of *E. equisetina*.

(3) Analyzing the *trn* K gene, we found two genotypes of *E. major* ssp. *procera* containing 10-11 bp of base-variations: type M-1 and type M-2. Type M-1 are specimens from Kirikkale and Ankara while type M-2 are from Karadiken, Kaiseri, Kirikkale, Cappadocia and France. The differences between *E. equisetina* and type M-1 were 10 bp of base-variations, while *E. equisetina* and type M-2 were 20-21 bp of base-variations. In addition, we found that *E. major* ssp. *procera* from France belong to the type M-2 group. Thus we showed that *E. major* ssp. *procera* consists of at least two types of matrilineage, because the chloroplast DNA generally is obtained through maternal inheritance. Moreover, it was assumed that type M-2 was a cluster of *E. major* ssp. *procera* produced by seed dispersal around the Mediterranean area. According to the analysis results of the *trn* K gene, we found two types of specimens on different matrilineage morphologically identified as *E. major* ssp. *procera*.

(4) Two specimens (06c3094 from Xinjiang and 20531051 from Mongolia) thought to be hybrids were confirmed in specimens identified as *E. equisetina*. It was assumed that one specimen from Xinjiang was hybrid from *E. equisetina* and *E. intermedia*, because *E. sinica* doesn't grow wild in Xinjiang (Yang C. Y. 1992).

(5) We found that the alkaloid composition ratio varied by collection site. For example, the composition ratio of ephedrine of *E. equisetina* from Xinjiang and Hebei was 64%, but specimens from Chinghai, Gansu, Inner Mongolia and Mongolia were 7~13%. A previous study has reported that *E. sinica*, *E. intermedia* and *E. equisetina* can be classified chemically by alkaloid composition

ratio (Hong 2011), which was contrary to our results. The composition ratio of ephedrine of *E. equisetina* varied by collection site in this study. We noticed that *E. equisetina* used in the above Hong's study (2011) were exclusively Xinjiang. Therefore, our results with specimens from Xinjiang are in accordance with the previous study. Similarly, the composition ratio of ephedrine in *E. major* ssp. *procera* from Kaiseri and Cappadocia was 25-31%, but in specimens from Karadiken, Kirikkale and Ankara it was 0-1%. Thus, we showed that the composition ratio of E and PE depends on the collection site for *E. equisetina* and *E. major* ssp. *procera*. It is likely that *E. equisetina* and *E. major* ssp. *procera* make a cluster differing in genetic background based on collection site, because the alkaloid composition ratio was influenced by genetic factors (Matsumoto 2014). Besides, the alkaloid composition ratio and genotype were not related, according to analysis of ITS1 and *trn* K sequence.

(6) We concluded that *E. major* ssp. *procera* had a closer relationship with *E. equisetina* in morphology and molecular phylogeny than *E. major* ssp. *major*. We think that these classifications have been difficult because the taxa had a close relationship with differing matrilineage and a different genetic background. Thus, we suggest that *E. major* ssp. *procera* should be treated as a synonym or subspecies of *E. equisetina* instead of a subspecies of *E. major*, based on molecular phylogeny. *E. equisetina* and *E. major* ssp. *procera* contained no less than 0.7% of total alkaloids. In general, *E. equisetina* is a medicinal species prescribed in JP16. However, *E. major* ssp. *procera* is not used as medicine in Japan, though it is used as a medicinal plant in Turkey (Fakir 2009, Altundag 2011). It is necessary that we discuss the usage of *E. major* ssp. *procera* as a medicinal plant (Ephedrae Herba) in Japan.

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和文要旨

Ephedra 属植物は外部形態的な分類形質が少なく，種分類が困難な一群であるため，種の位置づけが常に議論されている．特に，形態学的特徴が類似している *E. equisetina* と *E. major* ssp. *procera* の分類は不確かで混乱している．そこで本研究では，DNA 配列とアルカロイド含量組成をもとに両種の近縁関係を調べることを目的とした．その結果，ITS1 領域及び *trn* K 遺伝子の解析結果では，母系の異なる近縁関係にある事が示された．また，含有成分の分析結果では，両種ともに産地によって全く異なるアルカロイドの組成を持つことが明らかになった．以上の結果から，*E. major* ssp. *procera* は *E. major* ssp. *major* の亜種とするより，形態学的に類似し，分子系統学的にも近縁関係にある *E. equisetina* のシノニム又は亜種とする事が適切であると提案した．

Species	Locality of voucher	Date of collection	Voucher No.
E. equisetina	Qinggil, Xinjiang, China	2012.7.10	71031, 71032, 71033, 71034, 71037, 71038
	Fuyun, Xinjiang, China	2012.7.11	71121, 71122, 71123, 71124
		2012.7.12	71201, 71202, 71203, 71204
		2012.7.13	71301, 71302
	Kumul, Xinjiang, China	2006.6.27	06C3024, 06C3025
	Qinggil, Xinjiang, China	2006.6.30	06C3046
	Fuyun, Xinjiang, China	2006.6.30	06C3047, 06C3048, 06C3049
	Altay, Xinjiang, China	2006.6.30	06C3051, 06C3056, 06C3057
	Jeminay, Xinjiang, China	2006.7.1	06C3062, 06C3063
	Ili Kazakh, Xinjiang, China	2006.7.4	06C3091, 06C3092
	Xinyuan, Xinjiang, China	2006.7.5	06C3094, 06C3095
	Fukang, Xinjiang, China	2006.7.14	06C3138
	Zhangjiakou, Hebei, China	2002.6.8	02136
	Zhangjiakou, Hebei, China	2002.7.27	02612-1, 02612-2, 02612-3
	Xunhua, Qinghai, China	2002.7.30	02303-1, 02303-2, 02304, 02305
	Xining, Qinghai, China	2002.8.1	02314
	Shandan, Gansu, China	2002.8.11	02356
	Gulang, Gansu, China	2002.8.12	02359
	Bayan Nur, Inner Mongolia, China	2009.8.15	90815102, 90815103
	Bayan Nur, Inner Mongolia, China	2009.8.15	90815104, 90815105, 90815106
		2010.7.22	1007221
	Alxa Zuoqi, Inner Mongolia, China	2010.7.22	1007222, 1007224, 1007226, 1007227, 1007228
	Bayanhongor District, Mongolia	2005.7.31	20531022, 20531023
	Dundgovi District, Mongolia	2005.8.4	20531051
E. major ssp. procera	Karadiken, Turkey	2012.3.30	U120330
	Kaiseri, Turkey	2012.6.20	U120620
		2012.6.29	U62921, U62922, U62923
	Kirikkale, Turkey	2012.6.29	U62901, U62902, U62903, U62904, U62905, U62906
	Cappadocia, Turkey	2012.6.30	U63001, U63002, U63003, U63004, U63005, U63006, U63007, U63008, U63009, U63010
	Ankara, Turkey	2012.7.1	U70101, U70102
	France	2012.9.3	U201209031

Table 1 Plant materials used in this study (Chinese, Mongolian, Turkish and French origin)

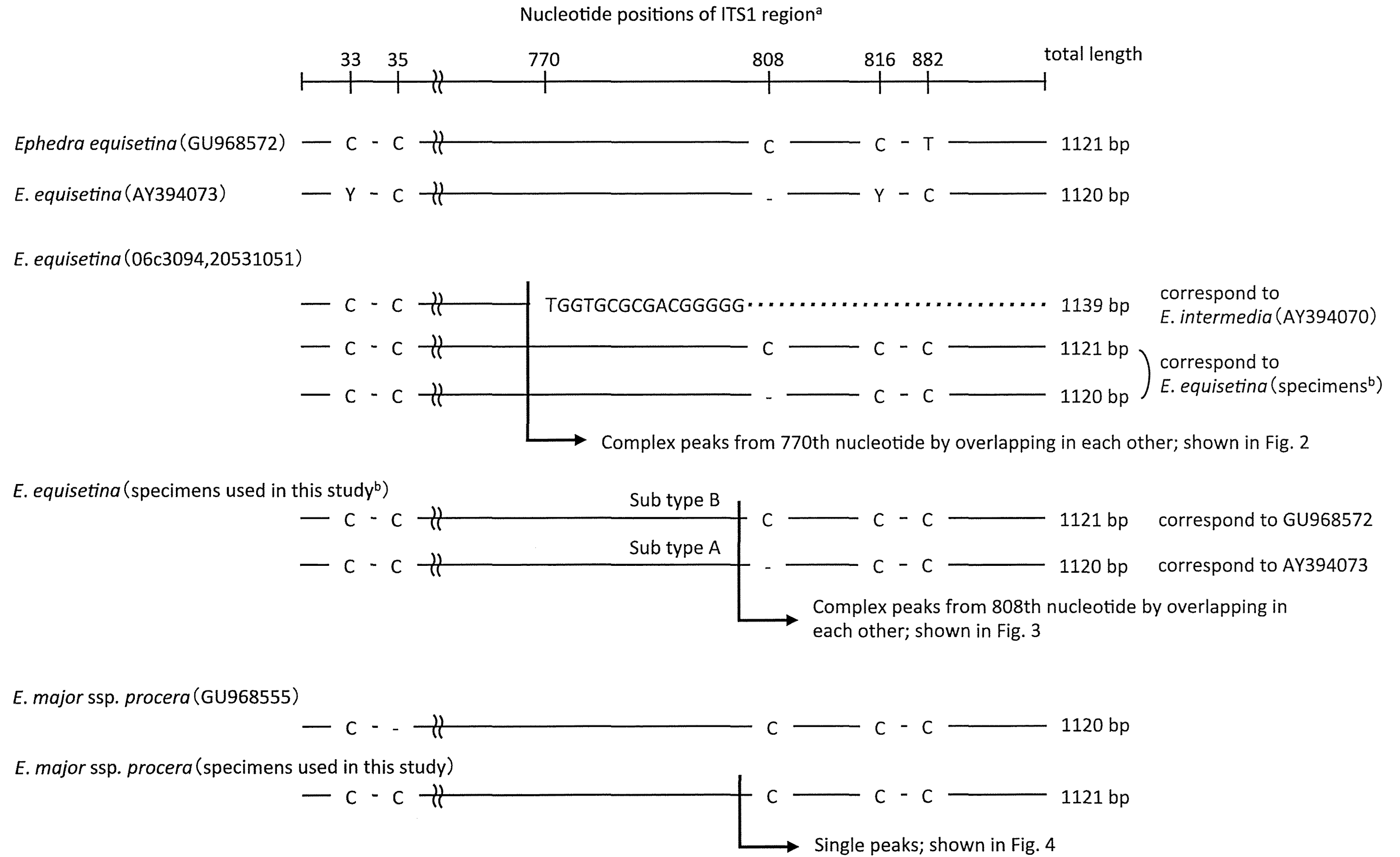


Fig. 1 Schematic illustration of ITS1 region

[a] Nucleotide positions are counted from 5'- terminal of AY394073

[b] All of the specimens except 06c3094 and 20531051

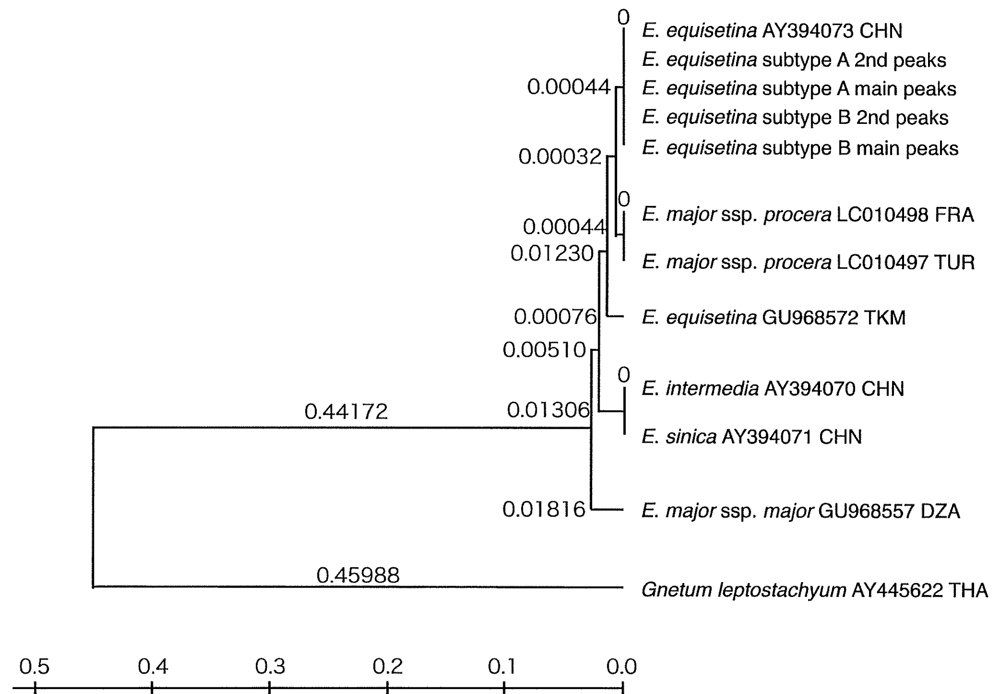
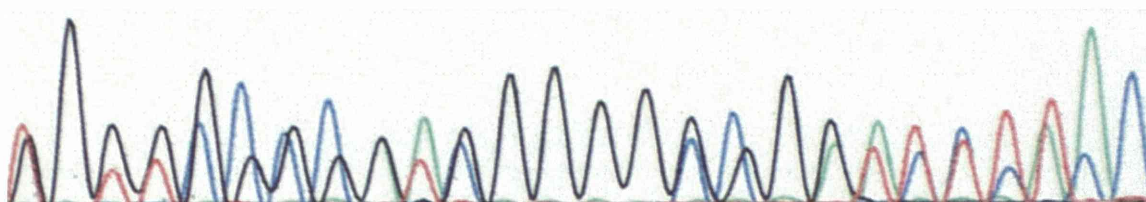


Fig. 2 The phylogenetic trees using the UPGMA method for *Ephedra* plants obtained from ITS1 sequence

The trees were outgroup-rooted using the sequence data of *Gnetum leptostachyum*. Branch lengths were calculated by Kimura's two-parameter method. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

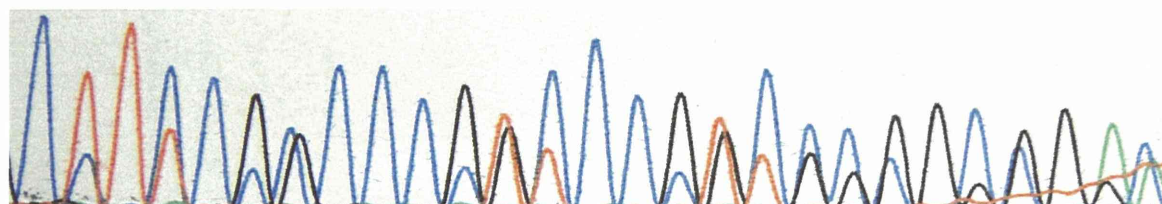


	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795
Main peaks	T	G	G	T	G	C	G	C	G	A	C	G	G	G	G	G	C	G	G	A	T	C	T	T	A	C
2nd peaks	G	G	T	G	C	G	C	G	A	T	G	G	G	G	G	C	G	G	A	T	C	T	C	A	C	C
06c3094, 20531051	K	G	K	K	S	S	S	S	R	W	S	G	G	G	G	S	S	G	R	W	Y	Y	Y	W	M	C

Fig. 3 Example of direct sequencing Electropherogram of *E. equisetina* from China and Mongolia.

Y: C and T S: C and G K: G and T R: A and G M: A and C W: A and T

Overlapping peaks were analyzed on ITS1 about 06c3094 and 20531051 from the nucleotides position 770th. The main and 2nd peaks were elucidated by comparing with the corresponding regions of *E. equisetina* and *E. intermedia* respectively.



	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833
Main peaks	C	T	T	C	C	G	C	C	C	C	G	T	C	C	C	G	T	C	G	C	G	G	C	G	G	A	C
2nd peaks	C	C	T	T	C	C	G	C	C	C	C	G	T	C	C	C	G	T	C	G	C	G	G	C	G	G	A
Subtype A	C	Y	T	Y	C	S	S	C	C	C	S	K	Y	C	C	S	K	Y	S	S	S	G	S	S	G	R	M

Fig. 4-A Example of direct sequencing Electropherogram of *E. equisetina* from China and Mongolia.

Y: C and T S: C and G K: G and T R: A and G M: A and C W: A and T

Subtype A is complex ITS1 peaks from 808th position by overlapping of peaks from two different bases. The main peaks determined to have high homology with AY394073 (*E. equisetina*), the 2nd peaks determined to have high homology with GU968572 (*E. equisetina*).