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Analytical Methods

Genetic and chemical diversity of *Eleutherococcus senticosus* and molecular identification of Siberian ginseng by PCR-RFLP analysis based on chloroplast *trn*K intron sequence

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ABSTRACT

Siberian ginseng (SG), the rhizome and root of *Eleutherococcus senticosus*, has been used as a tonic and anti-fatigue agent in northeastern Asia from ancient time. In recent years, SG has been becoming fairly popular as dietary supplements and health foods worldwide. In order to establish a convenient and sensitive method for authentication, chloroplast *trn*K intron sequences of 6 *Eleutherococcus* species were determined and compared. Genetic polymorphism, representing by 14 types of *trn*K intron sequence, in *E. senticosus* was observed. However, characteristic nucleotide markers stable within this species enabled clear discrimination of it from other congeners. A PCR-RFLP method was further developed, which was demonstrated to be efficient for authentication of crude drugs as well as health foods. Quantitative evaluation of three main bioactive constituents indicated chemical diversity in *E. senticosus* collected from northeast China and the results suggested good producing areas of SG. The chemical data clearly revealed that *E. sessliflorus* was unsuitable to be used as SG.

1. Introduction

Siberian ginseng (SG), the rhizome and root of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim. (=*Acanthopanax senticosus* (Rupr. et Maxim.) Harms.) has been used as a tonic and anti-fatigue agent in northeastern Asia and eastern Russia from ancient time (Xiao, 2002). It has been called as "Ciwujia" in Chinese and "Shigoka" or "Ezo-ukogi" in Japanese. The common name "Siberian ginseng" seems to come from having ginseng-like adaptogenic effect and growing mainly in the far eastern areas, whereas, it is really different plant from *Panax ginseng* C. A. Meyer. In northeast China SG-liquor which has been made from soaking SG in alcohol, is a popular health supplement for weakness, rheumatism, impotence and haemorrhoids (Zhu, 1989). In recent years, SG has been becoming fairly popular as dietary supplement not only in Asia, but also in the United States and European countries. Many products which are in the form of capsule, powder, tea bag, etc., with the name of "Siberian ginseng" and "Eleuthero" are widely available in the health food markets. The aqueous and alcohol extracts of SG have been reported having anti-fatigue, anti-stress, immune-enhancing, anticancer effects, etc. (Davydov & Krikorian, 2000; Deyama, Nishibe, & Nakazawa, 2001; Kimura & Sumiyoshi, 2004). Moreover, the major constituents such as eleutheroside B (EB) had anti-fatigue and antioxidant effects, NGF-like effect in cultured PC12 cell (Davydov & Krikorian, 2000; Tasugi et al., 1985; Yamazaki, Hirota, Chiba, & Mohri, 1994), eleutheroside E (EE) possessed anti-stress and anti-fatigue effects, inhibition of gastric ulcer, and NGF-like effect in cultured PC12 cell (Davydov & Krikorian, 2000; Fujikawa, Yamaguchi, Morita, Takeda, & Nishibe, 1996; Nishibe, Inoshita, Takeda, & Okano, 1990; Yamazaki et al., 1994) and isofraxidin (IF) had antidepressant activity et al. (Chen & Liu, 1991; Davydov & Krikorian, 2000). Our previous study showed that the water and methanol extracts of SG had trophic and benefit effects on neurite outgrowth, synapse formation and neuronal protection in *in vitro* assay system for Alzheimer's disease, and eleutheroside B was one of the active constituents (Bai, Tohda, Zhu, Hattori, & Komatsu, 2011; Tohda et al., 2008). These evidences have corroborated the usefulness and benefits of SG.

"Shigoka" has been first listed in Japanese Pharmacopoeia from the 15th edition (2006), and is prescribed as rhizome and root of *E. senticosus* (Rupr. et Maxim.) Harms. In Chinese Pharmacopoeia (2005), stem is additionally included as medicinally used part besides the rhizome and root. In the main producing area of SG,

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northeast China, however, a congeneric species, *E. sessiliflorus* (Rupr. et Maxim.) Seem. is also distributed in the same areas and has also been locally used as SG (Xiao, 2002). Recently, Maruyama et al. (2008) demonstrated that SG available in Japanese market was derived from not only *E. senticosus* but also other relatives including *E. sessiliflorus* by analysis of rDNA ITS sequences from a number of commercial samples. The chemical constituents and contents of the two species are obviously different (Maruyama et al., 2008). Although the two plants can be easily differed by morphological features of inflorescence, leaflets, and prickles on stem, the medicinally used rhizome and root are extremely difficult to be morphologically discriminated from each other. As proved by many previous studies, sequence of chloroplast *trn*K gene with high rate of nucleotide variation provides useful information for assisting taxonomic classification and identifying the botanical origin of herbal drugs (Mizukami, Okabe, Kohda, & Hiraoka, 2000; Zhu, Fushimi, Cai, & Komatsu, 2003). In a preliminary experiment, intra-species polymorphism of *trn*K gene sequence in *E. senticosus* has been observed. Therefore, in the present study, we widely collected the plant specimens in northeast China and determined the nucleotide sequences of *trn*K gene, aimed to develop an objective and convenient method for identification of SG and its derived dietary supplements, as well as to investigate the intra-species polymorphism of *trn*K gene in *E. senticosus*. Furthermore, we quantitatively analysed contents of the three major bioactive compounds, EB, EE and IF to evaluate the quality of SG and to clarify chemical difference between *E. senticosus* and *E. sessiliflorus*, after clear identification of original sources by DNA sequence analysis.

2. Materials and methods

2.1. Materials

Ninety specimens of *E. senticosus* and 18 specimens of *E. sessiliflorus* collected in northeastern China (Heilongjiang, Jilin provs.), Japan and eastern Russia (Sakhalin), seven samples of other four congeneric species and 40 samples of crude drugs available in Japanese markets were used (*Tables* 1S and 2S, Supplementary data). The 40 commercial samples were identified by either analysing their *trn*K intron sequences or by PCR-restriction fragment length polymorphism (RFLP) assay. For most samples, two individuals cited as No.-A, B were analysed and their identities were provided separately. Samples used in chemical evaluation are shown by the code No. in *Table* 1S. Moreover, four health food products were purchased via internet with names of Siberian ginseng (capsule produced in USA, TMPW No. 22002), Eleuthero (capsule produced in USA, TMPW No. 22715), Shigoka tea (tea bag produced in Heilongjiang prov. China, TMPW No. 22716), Ukogi tea (tea bag produced in Japan, TMPW No. 22717), which were identified unambiguously by genetic analysis and were included in chemical evaluation. All specimens were stored in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW).

2.2. Isolation of total DNA and PCR amplification

Total DNA was extracted from dried leaf, rhizome or root of plant specimens and crude drug samples by using DNeasy™ plant Mini Kit (QIAGEN, Germany) with several modifications to the protocol provided by manufacturer (incubated sample with AP1 buffer at 65 °C for 4 h and then after adding AP2 buffer kept the mixture on ice for 1 h). The *trn*K gene region was amplified as two (in plant specimens) or four (in drug samples) fragments which were partially overlapped at both 5′- and 3′-ends (Fig. 1) via the polymerase chain reaction (PCR). The 30 μl reaction mixture was composed of 1× GoTaq® Flex buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM of each primer, 1.0 U of GoTaq® Polymerase (Promega, USA), and 10–100 ng total DNA as a template. Sequences of the used primers are as follows: trnK3914F, 5′-TGGGTTGCTAACTCA ATGG-3′; PT713R, 5′-TGATACATAGTGCGATACAG-3′; PT692F, 5′-GACTGTATCGCAC TATGTATC-3′; matK3F, 5′-CTCCAAATAAAGCCGG TTCTTC-3′; matK4R, 5′-CGGAGAAAGATGAAGATGG-3′; matK5F, 5′-GTAACG TATTGGGGCATCC-3′; matK2R, 5′-CAGAATCTGAT AAATCGGTCC-3′; trnK2R, 5′-AACTAGTCGGATGGAGTAG-3′. PCR amplifications were carried out in a Takara thermal cycler (Takara, Japan) by a cycling condition of hot start at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 52 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 10 min. Five microlitres of resulting PCR product was detected by 1.0% agarose gel electrophoresis and then the remained part was purified using Montage RCR column (Millipore, USA).

2.3. Sequencing analysis and phylogenetic analysis

The purified PCR products served as template, sequencing reaction were performed by using the Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, USA) with a set of primers shown in Fig. 1. The thermal cycling condition was 96 °C for 1 min, 25 cycles of 96 °C for 30 s, 50 °C for 5 s and 60 °C for 4 min. After removal of unincorporated fluorescent reagents from

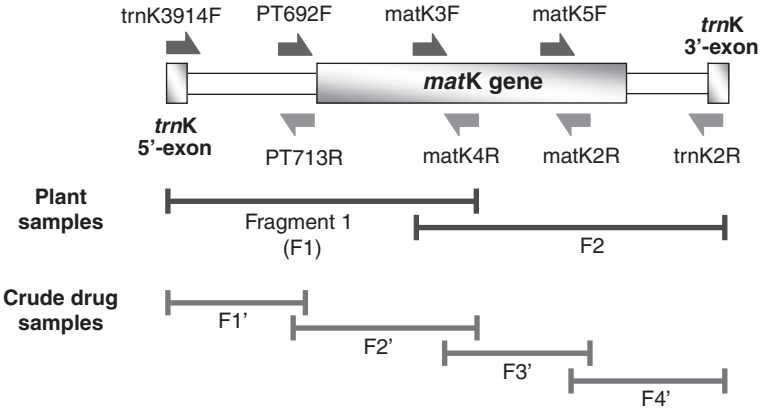

Fig. 1. Structure sketch of chloroplast *trn*K gene and strategy of PCR amplification. Exons of the *trn*K gene are divided into two parts by a long intron and *mat*K gene is embedded in the intron region. Arrows indicate location of primers which were used in PCR and sequencing reaction. The whole region is amplified as two fragments in case of plant specimens and four fragments in the crude drug samples.

Table 1
Comparison of *trn*K intron sequence among six *Eleutherococcus* species and Siberian ginseng.

	*mat*K gene (760th-2277th)																																										
	174	194	355	371	382	524	793	969	985	1001	1013	1094	1101 [b]	1102 [b]	1233	1334	1472	1540	1555	1614	1753	1787	1804	1813	1825	1955	1974	2034	2115	2136	2312	2352 - 2364	2443	2464	n=	Acces. No. in GenBank
E. senticosus																																											
Esn1	G	G	A	C	T	C	A	A	C	C	T	T	A	A	C	T	C	G	C	C	G	T	T	T	G	C	T	A	G	A	A	----------	A	G	22	AB571628
Esn2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	----------	*	*	11	AB571629
Esn3	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	6	AB571630
Esn4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	1	AB571631
Esn5	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	1	AB571632
Esn6	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	----------	*	*	8	AB571633
Esn7	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	4	AB571634
Esn8	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	----------	*	*	11	AB571635
Esn9	*	*	*	*	*	*	*	*	*	G	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	2	AB571636
Esn10	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	TAAATAATGAAGA	*	*	15	AB571637
Esn11	*	*	T	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	TAAATAATGAAGA	*	*	4	AB571638
Esn12	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	T	*	T	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	1	AB571639
Esn13	A	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	T	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	*	4	AB571640
Esn14 [a]	A	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	T	*	*	*	*	*	*	*	*	*	*	*	*	----------	*	T	5	AB571641
E. sessiliflorus																																										
Ess1	*	*	*	*	G	*	*	*	A	*	*	*	*	C	*	C	*	T	*	T	C	*	C	*	*	*	*	G	*	*	*	----------	T	*	6	AB571642
Ess2	*	*	*	*	G	*	*	*	A	*	*	*	*	C	*	C	*	T	*	T	C	*	C	*	*	*	*	G	*	T	*	----------	T	*	10	AB571643
E. sieboldianus	*	*	*	*	G	*	*	*	A	*	*	*	*	C	*	C	*	T	*	T	C	*	C	*	*	*	*	G	*	*	*	----------	T	*	1	AB571644
E. divaricatus	*	*	*	*	G	*	*	*	A	*	*	*	*	C	*	C	*	T	*	T	C	*	C	*	*	*	*	G	*	*	*	----------	T	*	4	AB571645
E. spinosus	*	*	*	*	*	*	*	G	*	*	*	*	C	*	*	*	*	*	*	*	*	C	C	G	T	*	*	G	T	*	C	----------	T	*	1	AB571646
E. trichodon	*	*	*	*	*	*	*	G	*	*	*	*	C	*	*	*	*	*	*	*	*	C	C	*	*	*	*	G	*	*	C	----------	*	*	2	AB571647

[a] This type of sequence has only been found in crude drug samples.
[b] The nucleotides at the 2 shade positions enable us to further develop PCR-RFLP method for identification of SG.

the produced mixture using AutoSeq™ G-50 columns (GE healthcare Biosciences, UK), sequencing products were analysed on an ABI Prism 3100-Avant Genetic Analyser (Applied Biosystems, USA). All the fragments were sequenced in both directions. The obtained sequences were edited and aligned by the AutoAssemble program (Version 1.3.0, Applied Biosystems, USA). A final consensus sequence was obtained by assembling the overlapped sequences in both directions. The boundary of the *mat*K gene region were investigated by using the computer program GENETYX-SV/SC (version 8.0.5, Japan) and were also confirmed by comparison with the sequence of tobacco (*Nicotiana tabacum*) and ginseng (*P. ginseng*) (Kim & Lee, 2004; Sugita, Shinozaki, & Sugiura, 1985). The determined nucleotide sequence data were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence database with the accession numbers shown in Table 1.

Phylogenetic trees were reconstructed using the computer program PAUP* (Version 4.0 beta 10a, Sinauer Assoc. Inc., USA). Parsimony analysis was performed by Heuristic search with tree-bisection-reconnection (TBR) branch-swapping, MULPARS, a random addition sequence of 100 replicates. *P. ginseng* (AB087999) belonging to the same Araliaceae family was used as outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

2.4. PCR-RFLP method for identification of E. senticosus

Based on nucleotide differences among *E. senticosus* and other congeners at positions 1101st and 1102nd, a restriction enzyme *Ase* I which can recognize and cut a sequence unit AT^TAAT was used for PCR-RFLP analysis, enabling *E. senticosus* to be easily differed from others. A pair of primers, trnK891F (5′-GTT GGA AAA TGC GGG TTA TG-3′) and trnK1242R (5′-TTC CAG ATG GAC TGG GTA GG-3′) were newly designed to amplify a 352 bp small fragment. The location of primer was shown in Fig. 3. The resulting PCR products from all specimens were digested with a restriction enzyme, *Ase* I (BioLab, Inc., USA) at 37 °C for 4 h. The digested fragments were detected by 2.5% agarose gel electrophoresis and visualized by staining with GelRed™ Nucleic Acid Gel Stain (Wako Chemicals, Japan).

2.5. Standard compounds and reagents for HPLC analysis

All standards were isolated from a commercial SG sample (TMPW No. 24214), which was confirmed to be derived from *E. senticosus* by DNA analysis, in our laboratory. The compounds were identified by comparing the spectral data (Mass, ^{1}H and ^{13}C NMR) with those in the reported literatures. The purity was confirmed to be more than 98% by HPLC analysis. Reagents for HPLC analysis including acetonitrile and distilled water (both of HPLC grade) were purchased from Wako Pure Chem. Inc. (Japan). Potassium dihydrogenphosphate (analytical grade) was purchased from Wako Pure Chem. Inc. (Japan).

2.6. Apparatus and analytical condition

The HPLC system (SHIMADZU Co., Japan) is composed of a SCL-10AVP system controller, a LC-10AD VP pump, a DGU-14A degasser, a CTO-10-AS VP column oven, a SIL-10AD VP auto injector, and a SPD-M10A VP diode array detector (DAD). Analysis was carried out using a Waters XTerra phenyl (4.6 mm × 150 mm, 3.5 μm) column with column temperature at 40 °C. Mobile phase was a binary eluent of (A) acetonitrile and (B) 20 mM NaH_2PO_4 solution (pH 4.3) under gradient conditions: 0–5 min linear gradient from 8% to 13% A, 5–39 min from 13% to 30% A. Flow rate was 0.8 mL/min. Three wavelengths were used for detection, that is, 207 nm for eleutheroside E,

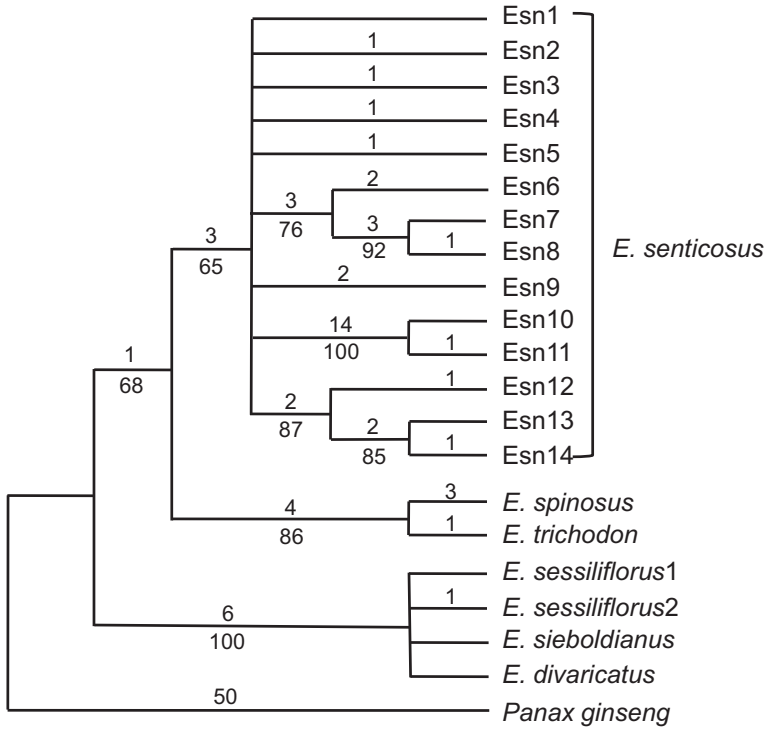

Fig. 2. Strict consensus tree of two most parsimonious trees reconstructed on the basis of *trn*K intron sequence from *Eleutherococcus* species. Gaps were treated as "fifth status". Tree length = 105, CI = 0.9328, RI = 0.8987, RC = 0.8303. Number above line is branch length, and number below line is the bootstrap value with 1000 replicates. Type Esn14 was observed only in crude drug samples.

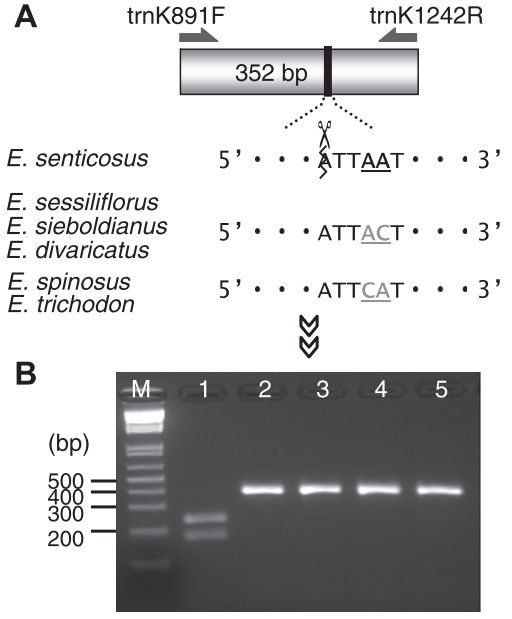

Fig. 3. PCR-RFLP analysis using restriction enzyme *Ase* I on partial *trn*K intron region. (A) *Ase* I restriction site in *E. senticosus* and the sequences of other five *Eleutherococcus* species. Nucleotides with bold face indicate the defined marker nucleotides at positions 1101 and 1102. Arrows indicate location of primers which were new designed for amplification of small fragment (352 bp) in PCR-RFLP analysis; (B) electrophoretogram of digested PCR product of each species which was analysed by 2.5% agarose gel and stained by gel-red. Lane 1: *E. senticosus;* Lane 2: *E. sessiliflorus;* Lane 3: *E. sieboldianus;* Lane 4: *E. spinosus*; Lane 5: *E. trichodon*; M: 1 kb plus DNA ladder.

264 nm for eleutheroside B, 340 nm for isofraxidin. The chromatographic data were collected and processed by LC solution Software (Ver. 1.03, SHIMADZU Co., Japan).

2.7. Preparation of calibration curve and procedure of sample preparation

The individual stock solutions of the standard compounds, eleutheroside B, eleutheroside E and isofraxidin were separately prepared at a concentration of 1.0 mg/mL in methanol. The calibration curves were prepared at five different concentration levels. The calibration curve was obtained by plotting the peak area (y) of each compound in HPLC chromatogram against the injected amount of corresponding compound (x) and then performing a linear regression.

Five hundred milligram of rhizome or stem samples of *E. senticosus* and *E. sessiliflorus* were extracted by using 10 mL 50% MeOH/H_2O (v/v) in a 15 min continuous shaking condition for two times and each time followed by a centrifugation for 5 min. The extracted solutions were combined together into a 20 ml volumetric flask and finally an exact 20 ml solution was obtained. Aliquot 10 μl of each sample was injected into the HPLC system after being filtered through a 0.2 μm Millipore filter (Advantec. Tokyo, Japan).

3. Results and discussion

3.1. trnK intron sequences of E. senticosus and five related species

Total 116 specimens belonged to six *Eleutherococcus* species were analysed and the sequences were compared. As shown in Fig. 1, the determined sequence included *trn*K intron region and the embedded *mat*K gene region. Length of the determined sequences was of 2506 or 2519 bp in *E. senticosus* due to a 13-bp indel was observed in several samples; whereas, the other five species were of 2506 bp in length. The inside *mat*K gene region was found to be 1518 bp in all species (Table 1).

A preliminary experiment clearly indicated intra-species variation of the *trn*K intron sequences in *E. senticosus*, which led us to

widely collect samples of this species. Herein, 90 specimens widely collected from northeastern China, Japan and Russia were investigated and their *trn*K sequences were compared (Table 1). The nucleotide position was indicated as the aligned position, starting from the 1st nucleotide of *trn*K intron region. Thirteen types of *trn*K intron sequences (Esn1–13) were detected, among which 15 sites of nucleotide differences and a site of 13-bp indel were observed. Types Esn10 and Esn11 had a 13-bp insertion at positions 2352–2364, resulting in these two types with longer length than others. Among 90 specimens, four types of sequences appeared in higher frequency, that is, 22 specimens collected from wide areas possessed type Esn1 sequence; 15 specimens had type Esn10 and both of 11 specimens had types Esn2 and Esn8 sequences, respectively. Comparing with the type Esn1, sequences of types Esn2–5, Esn7 and Esn10 differed at only 1 nucleotide site; types Esn6, Esn8–9 and Esn11 differed at two nucleotide sites; types Esn12 and Esn13 differed at three or four sites, respectively. Sequences of types Esn7–9 shared a common nucleotide guanine (G) at position 1001 and types Esn9–11 shared a common G at position 1013. Types Esn12 and Esn13 were characterized by having Tyrosine (T) at positions 1472 and 1555.

Plotting the genotype of each sample on the collection map, certain relationship was implied from the *trn*K intron sequence types and distribution regions of many samples collected in Heilongjiang province. As shown in *Fig.* 1S (Supplementary data), types Esn7 and Esn8 were inclined to distribute in northwestern area, type Esn10 appeared in the northern part, types Esn12 and Esn13 in the eastern part, respectively. The specimens collected from Heilongjiang prov. of China were of 12 genotypes, showing a notable genetic polymorphism.

Two types of *trn*K intron sequence were found in *E. sessiliflorus* and only a nucleotide at position 2136 was different between them. Comparing the sequences of *E. senticosus* with those of *E. sessiliflorus*, nucleotides at positions 382, 985, 1102, 1334, 1540, 1614, 1753, 1804, 2034 and 2443 were found to be common within each species, but different between the two (Table 1). Therefore, those 10 sites of nucleotides enabled the two species to be discriminated clearly.

E. sieboldianus and *E. divaricatus* collected in Japan showed the same sequence as one type of *E. sessiliflorus*. Other 2 species, *E. spinosus* and *E. trichodon* possessed their specific sequences, which differed from type Esn1 by 10 or 6 sites of nucleotides, respectively. The above results showed that even the significant polymorphism of *trn*K intron sequence in *E. senticosus* were observed, *E. senticosus* and the five other species could be distinguished clearly by 6–11 sites of nucleotide differences.

3.2. Phylogenetic relationship of E. senticosus and five related species

Including all types of *trn*K intron sequences obtained from six *Eleutherococcus* species and using *P. ginseng* as an outgroup, a phylogenetic tree was constructed by maximum parsimony analysis (Fig. 2). The tree revealed three clades which were supported by high bootstrap value. As mentioned above significant polymorphism in *trn*K intron sequence was observed in *E. senticosus*, however, all types of sequences from this species were clustered into the same clade. *E. spinosus* and *E. trichodon* formed one clade which showed close relationship with *E. senticosus*-clade. *E. sessiliflorus, E. sieboldianus* and *E. divaricatus* formed a clade located at a basic position of the tree.

3.3. Development of PCR-RFLP method for identification of E. senticosus

Based on the sequence of *trn*K intron region, we tried to develop a convenient PCR-restriction fragment length polymorphism (RFLP) method which would enable rapid and accurate authentication to be performed in field site of drug control administrations. A restriction enzyme *Ase* I which is able to recognize 6-bp sequence unit (ATT**AA**T) specific to *E. senticosus* was found. In the other species, however, different sequence units of ATT**AC**T or ATT**CA**T were observed at the same positions, which would not be recognized by *Ase* I. For applying the PCR-RFLP assay, a new primer set (trnK-891F and trnK-1242R) was designed to amplify a short fragment of 352-bp in length, because the DNA extracted from the crude drug sample was generally degraded and therefore difficult to amplify a long PCR fragment. As a result, the PCR product from each sample was easily amplified with new primer set, and subsequently the PCR products were digested with *Ase* I. As shown in Fig. 3, only the PCR products from *A. senticosus* were cleaved into two fragments of 209 bp and 143 bp (lane 1), while those from the other 5 species kept the intact size of 352 bp (lanes 2–5).

3.4. Identification of crude drug samples by the established genetic methods

By using the established genetic methods, 40 samples of commercial SG drugs available in Japanese markets were identified. Twenty-five samples were identified by analysing the *trn*K intron sequences and 15 samples were identified by the developed PCR-RFLP method (Details in *Table* 2S). Within the 25 samples, two samples presented a new type of sequence (Esn14 in Table 1) which differed from type Esn13 sequence by only 1 nucleotide at position 2464th. Although the type Esn14 sequence was not detected in plant specimens, we hold that these two samples were of *E. senticosus*-source because of high similarity of the sequence to type Esn13 and obvious polymorphism of the *trn*K intron sequences in *E. senticosus*. In the phylogenetic tree, this type was unambiguously fitted into the subclade of *E. senticosus*. Among 40 commercial drug samples, 12 samples, accounting for 30% of the total samples, were derived from other species than *E. senticosus*. This finding was consistent with the results reported by Maruyama et al. (2008). It is noteworthy that two samples were of mixed sources by testing two individual pieces in the same lot. High frequency of adulterant appearing must hazard effective use of SG, therefore, strict regulation is necessary for preventing this situation. Our developed PCR-RFLP method enabled *E. senticosus* derived SG in the markets to be identified simply and rapidly, which must be powerful and helpful for this purpose.

3.5. Contents of three main bioactive compounds in the rhizome and stem of E. senticosus and E. sessiliflorus

In order to clarify chemical difference between *E. senticosus* and *E. sessiliflorus* as well as between different used parts and producing areas, 35 samples of *E. senticosus* and five samples of *E. sessiliflorus* collected from Heilongjiang and Jilin provinces of China, whose botanical sources were identified clearly by genetic analysis, were quantitatively analysed by HPLC-DAD method. The rhizome and the stem parts of each sample were analysed separately. Three main active constituents, EB, EE and IF were detected by different wavelengths for sensitive monitoring of every compound: EB (R_t 8.5 min) was detected at 264 nm, while EE (R_t 15.9 min) at 207 nm and IF (R_t 18.0 min) at 340 nm. As shown in *Table* 3S (Supplementary data), the contents of the three compounds varied significantly among different samples as well as different parts of the same sample. From Fig. 4, it is easy to find out the difference between *E. senticosus* and *E. sessiliflorus*: (1) only one compound, EE was found in the rhizome of *E. sessiliflorus*, ranging from 0.010% to 0.073%, while, all the three compounds or at least EE and EB were detected in that of *E. senticosus*; (2) the stem part of *E. sesilliflorus* contained none of these three compounds.

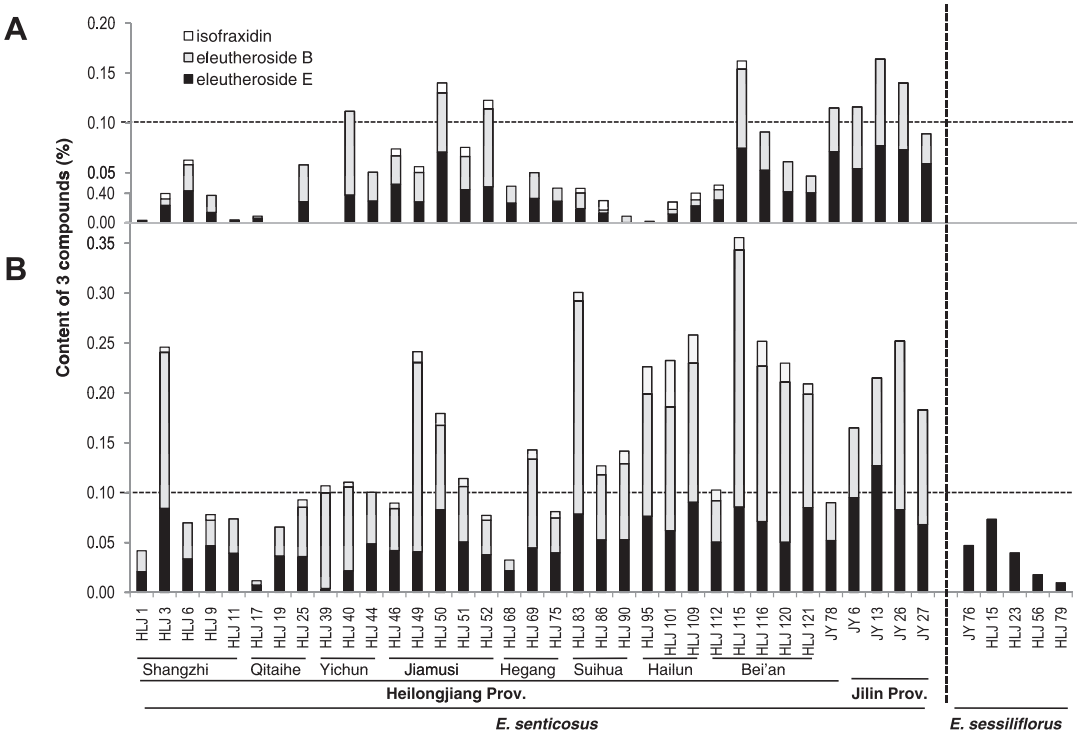

Fig. 4. Contents of three main bioactive components, eleutheroside B, eleutheroside E, and isofraxidin in the stem (A) and rhizome (B) of *E. senticosus and E. sessiliflorus*. Black, grey, and open boxes indicate the contents of eleutheroside E, eleutheroside B, and isofraxidin, respectively.

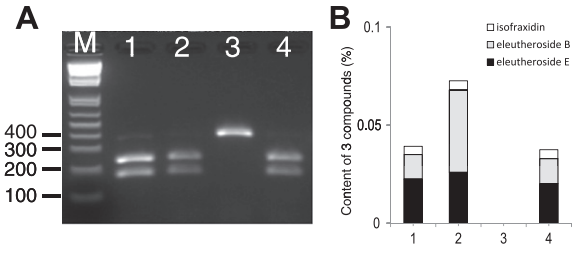

Fig. 5. Electrophoretogram of PCR-RFLP analysis (A) and contents of three main bioactive components, eleutheroside B, eleutheroside E, and isofraxidin (B) in the four health food products (1–4). 1: Siberian ginseng (capsule, TMPW No. 22002); 2: Eleuthero (capsule, TMPW No. 22715); 3: Shigoka tea (tea bag, TMPW No. 22716); 4: Ukogi tea (tea bag, TMPW No. 22717). (A) M: 1 kb plus DNA ladder. (B) Black, grey, and open boxes indicate the contents of eleutheroside E, eleutheroside B, and isofraxidin, respectively.

These data obviously indicated that *E. sesilliflorus* was unsuitable to be used as alternative source of SG.

Compared the contents in different used parts in *E. senticosus*, we found that the rhizome had much higher contents of the three bioactive compounds than those in the stem. Some samples even showed more than 10 folds difference in the total contents. In the rhizomes of *E. senticosus* (Fig. 4B), contents of the three compounds were generally in an order of EB > EE > IF; EB ranged from 0.004% to 0.258%, EE was from 0.004% to 0.127%, and IF was only obtained either in a small amount (trace to 0.047%) or even not detected. EB and IF have been reported as the two marker components for *E. senticosus* (Maruyama et al., 2008), however, our results revealed that only EB was beneficial for this purpose. Among various producing area of SG in China, samples from northwestern Heilongjiang province (Bei'an, Hailun) and eastern Jilin province showed relatively high contents of the three components, suggesting SG with high quality could be obtained from these regions.

3.6. Genetic identification and chemical evaluation of four health foods

Four samples of health foods available in market were also identified by PCR-RFLP method. The results showed that three of them were made from *E. senticosus*, but one product (Shigoka tea, TMPW No. 22716) was not (Fig. 5A). By further determining the sequence amplified by the primer set (trnK891F and trnK1242R) and then doing homology search in GenBank, we found that the partial *trn*K intron sequence of this Shigoka tea sample was completely same as that of *P. ginseng*. It indicated that this health food product was made from *P. ginseng*, but not *E. senticosus*. Quantitative analysis revealed that the three products derived from *E. senticosus* contained all the three compounds (EB, EE and IF) but with low contents, and one sample (TMPW No. 22716) contained none of the three compounds (Fig. 5B). The results of genetic and chemical analyses were completely consistent with each other.

4. Conclusion

The present study demonstrated that *E. senticosus* could be clearly distinguished from *E. sessiliflorus* as well as other four relative species by *trn*K intron sequence. *Trn*K intron sequence of *E. senticosus* showed a significant polymorphism and further investigation on the 13 genotypes and their geographic distribution revealed that genetic information might be useful in supposing of their producing area. A convenient PCR-RFLP method was developed for identification of SG based on the *trn*K intron sequence, which was demonstrated to be effective to identify crude drugs as well as health food products. From the viewpoint of bioactive constituent contents, *E. sessiliflorus* was unsuitable to be used as SG. Of the *E. senticosus* derived crude drug, the rhizome had higher contents of the three main compounds than that in stems, suggesting that the rhizome was the proper use part than the stem. Moreover, *E. senticosus*, which grow in eastern Jilin province and northwestern area of Heilongjiang province, China possessed

higher contents of the three bioactive constituents, therefore, SG produced in those areas might be with high quality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.05.128.

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