Phylogenetic examination of crude drugs derived from Yunnanese Swertia plants

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NOTE

Phylogenetic examination of crude drugs derived from Yunnanese *Swertia* plants

Nobuko Kakiuchi · Naoko Iwaki · Masayuki Mikage · Huai Xiao · Zhigang Wang · Masao Hattori

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Abstract Aiming to examine whether the genetic background of the crude drugs derived from four Yunnanese *Swertia* plants and their chemical constituent profiles correlate, we analyzed the nucleotide sequences of their nuclear ribosomal DNA regions including ITS1, 5.8S ribosomal RNA gene, and ITS2, together with those of Japanese *S. japonica* and *S. pseudochinensis* from Hebei Province. The result that two of the Yunnanese *Swertia* plants, *S. binchuanensis* and *S. punicea*, were genetically similar may explain their similarity in chemical constituent profiles. On the other hand, in spite of differences in chemical profile, *S. decora* and *S. pseudochinensis* were genetically close. The other Yunnanese *Swertia* plants, *S. delavayi,* and *S. japonica*, stood at intermediate positions between these two genetically similar pairs. The result suggests that although genetic background would have an influence, environmental factors, e.g., soil and weather conditions, might be critical for their production of secondary metabolites.

Keywords *Swertia* · Yunnan · Ribosomal DNA · Phylogeny

Present Address:
N. Kakiuchi (✉)
School of Pharmaceutical Sciences, Kyusyu University of Health and Welfare, 1714-1 Yoshino-cho, Nobeoka, Miyazaki 882-8508, Japan
e-mail: kakiuchi@phoenix.ac.jp

N. Kakiuchi · N. Iwaki · M. Mikage
School of Pharmacy, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakumacho, Kanazawa 920-1192, Japan

H. Xiao
Department of Pharmacy, Dali University, Dali 671000, Yunnan Province, China

Z. Wang · M. Hattori
Division of Metabolic Engineering, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

Introduction

Swertia herb derived from various *Swertia* plants is widely used as a folk medicine in Asian countries including China, India, Korea, and Japan. The crude drug derived from *S. japonica* Makino, for example, is a notable Japanese folk medicine traditionally applied for various purposes such as a cure for stomach-ache and digestive disorders. Recent research has shown that *S. japonica* has a significant blood-sugar-lowering effect and a protective effect on liver cells in animal models [1, 2, 3]. These biological effects are attributable to the major and characteristic groups of the chemical constituents of *Swertia* plants (xanthones, iridoids and flavonoids). Recently, chemical constituent profiling of the crude drugs derived from four Yunnanese *Swertia* plants and Japanese *S. japonica* as well as *S. pseudochinensis* Hara from Hebei Province was conducted using LC/MS/MS [4]. The results showed the following similarities and differences in chemical profile of the major constituents of the 6 species: (1) *S. japonica* and *S. pseudochinensis* showed high swertiamarin content and universal occurrence of irridoids and flavonoids, (2) *S. decora* Franch and *S. delevayi* Franch showed high swertiamarin but very low xanthones and flavonoids content, (3) *S. binchuanensis* T. N. Ho & S. W. Liu and *S. punicea* Hensl had relatively high flavonoids content. Aiming to find out whether their genetic background and chemical constituent profile correlate, we analyzed the nucleotide sequences of nuclear ribosomal DNA

regions including ITS1, 5.8S ribosomal RNA gene, and ITS2 of these 6 *Swertia* plants.

Materials and methods

Plant and crude drug materials

Materials used in this study are summarized in Table 1; they were the same materials used in a previous study [4]. Cultivated *Swertia japonica* specimens were collected in Nagano Prefecture, Japan. Crude drug materials **2**, **3**, **4**, and **5** were obtained from Yunnan Province, China, and were identified as *Swertia decora* Franch, *Swertia binchuanensis* T. N. Ho & S. W. Liu, *Swertia punicea* Hensl, and *Swertia delevayi* Franch, respectively, by Prof. Tingnong He (Northwest Institute of Plateau Biology, Chinese Academy of Science). A crude drug material **6**, *Swertia pseudochinensis* Hara (collected in Hebei Province, China), was supplied by Alps Pharmaceutical Ind. Co. Ltd., Japan. The vouchers were deposited in the herbarium of the School of Pharmacy, Kanazawa University.

Isolation of total DNA

A single stem of each material (20–50 mg) was frozen in liquid nitrogen and ground into fine powder. Total DNA isolation from the powder was performed using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol with minor modifications.

PCR amplification

The ITS region was amplified by polymerase chain reaction (PCR) using 30–100 ng of total DNA as a template in 25 μl of reaction mixture containing 2.5 μl of 10× PCR buffer for KOD-Plus, 0.2 mM of each dNTP, 1.0 mM of $MgSO_4$, 0.5 units KOD-Plus-polymerase (Toyobo), and 0.4 mM of primers Swer ITS1F (GAG GTC GCG AGA AGT CCA CT) and Swer ITS 1R (CTC AGC GGG TAA TCC CGC CT). Amplification was carried out under the following conditions: pre-heating at 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 2 min; with a final elongation at 68 °C for 5 min. One-tenth of the volume of the PCR product was analyzed by agarose gel electrophoresis and then the remaining part was purified using a QIAquick PCR Purification Kit (Qiagen).

Sequencing procedure

The purified PCR product was subjected to direct sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using ABI PRISM 310 (Applied Biosystems). The DNA sequences were aligned by 'DNASIS' version 3.0 (Hitachi).

Phylogenetic analysis

ITS1 nucleotide sequences were aligned by ClustalX software. Maximum parsimony phylogenetic analysis with 1000 replicates was performed by PAUP* 4.0.

Cloning of PCR products

The ITS region was amplified from total DNA using a tagged primer set of ER Swer 1F (AAA AGA ATT CGA GGT CGC GAG AAG TCC ACT) and BH Swer 1R (AAA ACC ATG **G**CT CAG CGG GTA ATC CCG CCT). After purification using a QIAquick PCR purification kit, the PCR products (100–200 ng) were digested with *Eco*RI and *Bam*HI (Takara Biotech) in a 10-μl reaction mixture containing 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM NaCl at 37 °C for 1 h, and the reaction mixture was heated at 60 °C for 15 min. Four microliters of the reaction mixtures were combined with 5 ng of plasmid Bluescript SK (−) digested with the same enzymes and 5 μl of DNA ligation solution Ver. 2.1 (Takara Biotech). The ligation reaction was performed at 16 °C overnight. Competent cells (Competent high DH5α, Toyobo) were transformed with the ligated product as recommended in the manufacturer's protocol. The transformed

Table 1 Materials used in this study

Sample	Species	Locality	Date	Status (ID)
1	*Swertia japonica*	Aokimura, Nagano, Japan	September 2007	Cultivated plant (071009)
2	*S. decora*	Dali, Yunnan, China	September 2001	Crude drug (KANP7581)
3	*S. binchuanensis*	Dali, Yunnan, Chian	October 2005	Crude drug (KANP7582)
4	*S. punicea*	Dali, Yunnan, China	September 2001	Crude drug (KANP7583)
5	*S. delavayi*	Dali, Yunnan, China	October 2005	Crude drug (KANP7584)
6	*S. pseudochinensis*	Hebei, China	2005	Crude drug (KANP7585)
7	*S. japonica*	Nagano, Japan	2005	Crude drug (KANP7586)

Table 2 Nucleotide differences in ITS1, 5.8S ribosomal RNA gene, and ITS2

Sample no.	Nucleotide no.[a]																				
	12	18	29	40	47–49	52	77	91–92	105	117	141	158–159	163	174	178–179	187	191	198	214	222	225
A: ITSI																					
1	C	G	A	C	GCG	C	C	CG	C	A	A	AA	G	C	TC	T	C	T	A	G	A
2[b, c]	S	*	W	Y	*YR	M	*	*S	Y	M	W	WG	K	R	CR	C	*	Y	G	R	G
3[b, d]	G	*	*	*	*T*	*	*	T*	T	*	*	*G	*	*	**	C	T	*	*	*	G
4[a]	G	*	*	*	*T*	*	T	T*	T	*	*	*G	A	*	**	C	*	*	*	*	G
5[d]	G	*	*	*	*T*	*	*	T*	T	*	*	*G	*	*	**	C	T	*	*	*	G
6[d]	*	T	*	*	C**	A	*	**	*	*	*	TG	*	*	CT	C	*	C	*	A	G
7[a]	*	*	*	*	***	*	*	**	*	*	*	**	*	*	**	*	*	*	*	*	*

Nucleotide no.[i]				
Sample no.	3–4	5	86	111	144–146
B: 5.8S ribosomal RNA					
1	AA	C	C	G	ACG
2[b, c]	–	Y	*	*	**R
3[b, d]	–	*	*	*	***
4[e]	–	–	*	C	CA*
5[f]	–	*	T	*	***
6[g]	–	*	*	*	***
7[h]	**	*	*	*	***

Sample no.	Nucleotide no.[i]																
	4	9	15–16	22	26	34	43	45–46	60	80	85	139–140	161	173	180–182	185	198
C: ITS2																	
1	C	C	CG	–	T	C	G	TG	G	C	A	GT	G	C	GG–	C	G
2[b, c]	*	*	*S	–	*	*	T	**	A	*	C	**	K	Y	K*–	*	*
3[b, d]	*	*	–*	–	*	Y	*	C*	A	T	C	R*	*	*	**–	*	*
4[e]	G	A	**	C	*	*	*	C*	A	T	C	**	*	*	**–	*	*
5[f]	*	*	AA	–	C	T	*	CA	*	*	C	*C	T	*	**C	T	A
6[g]	*	*	**	–	*	*	T	**	A	*	C	**	*	T	**–	*	*
7[h]	*	*	**	_	*	*	*	**	*	*	*	**	*	*	**_	*	*

S C + G, *W* A + T, *Y* C + T, *R* A + G, *M* A + C, *K* G + T

[a] Numbering is based on Hagen et al. (2001) [6]

[b] Sequence data deduced from those of clones

[c] GenBank Accession No. GQ848483

[d] GenBank Accession No. GQ848484

[e] GenBank Accession No. GQ848485

[f] GenBank Accession No. GQ848486

[g] GenBank Accession No. GQ848487

[h] GenBank Accession No.GQ848488

[i] Numbering is based on Liu et al. (2001) [7]

cells were spread on LB/Amp plates (2 % tryptone, 1 % yeast extract, 2 % NaCl, 1.5 % agar, and 100 μg/ml ampicillin) and incubated at 37 °C overnight. Bacterial colonies were picked up separately, and sub-cultured in liquid LB/Amp medium. After overnight incubation, the bacteria were collected by centrifugation and plasmids were obtained following the standard protocol [5].

Results and discussion

Direct sequencing of PCR products of the ITS region of samples **1**, **4**, **5**, **6,** and **7** was successful, while that of samples **2** and **3** gave ambiguous sequencing results caused by overlapping of divergent sequences of the respective samples. The PCR products of these 2 samples were

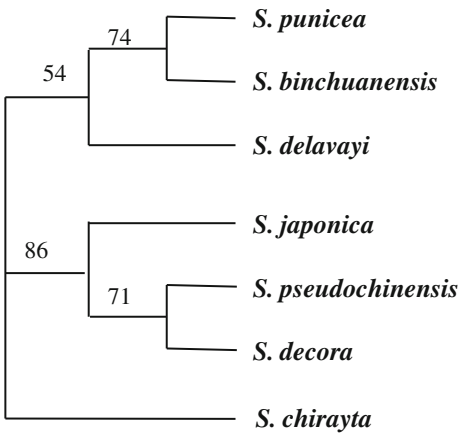

Fig. 1 Phylogenetic analysis of ITS1, 5.8S ribosomal RNA gene, and ITS2 of Yunnanese *Swertia* plants together with those of Japanese *S. japonica* and *S. pseudochinensis* from Hebei Province. The sequence data of *S. chirayta* (GenBank Accession No. AF255917) are used as an outgroup. The 50 % majority-rule consensus tree was based on maximum parsimony analysis. *Numbers above lines* are bootstrap values (%) with 1000 replicates

subjected to cloning analysis using an *E. coli* system, and the clones were sequenced. The sequence data of samples **2** and **3** were deduced from their 10 or 6 clones, respectively. The data obtained from direct sequencing as well as that deduced from clones are shown in Table 2, where the ITS1, 5.8S ribosomal RNA gene, and ITS2 sequences are aligned with those of *S. japonica* [6] or *S. chirayta* [7]. Maximum parsimony analysis using PAUP* 4.0 ver. 10 (Altivec, Sinauer Assoc. Inc.) was performed on the combined sequence of ITS1, 5.8S ribosomal RNA gene, and ITS2 together with that of *S. chirayta* as an outgroup (Figs. 1, 2). The phylogenetic tree shows that two of the Yunnanese *Swertia* plants, *S. binchuanensis* and *S. punicea*, were genetically similar, and so were *S. decora* and *S. pseudochinensis*. The other Yunnanese *Swertia* plants, *S. delavayi*, and *S. japonica*, stood at intermediate positions between these two genetically similar pairs. The similarity in chemical constituent profiles of *S. binchuanensis* and *S. punicea* was correlated with their genetic background. On the other hand, although *S. decora* and *S. pseudochinensis* showed differences in chemical constituent profile and had relatively distant habitats, their genetic structure seemed similar. To survey the phylogenetic structure of *Swertia* plants growing in a wider range, further phylogenetic analysis was performed on the ITS1 data obtained from the present study and the database of *Swertia* plants distributed in Asia. Similar to the previous finding obtained from combined ITS1, 5.8S ribosomal RNA gene, and ITS2 sequences, *S. decora* and *S. pseudochinensis* were positioned in the same clade, but not in the clade of the other plants from Yunnan, *S. binchuanensis*, *S. delavayi*, and *S. punicea*. In addition, the position of *S. decora* and

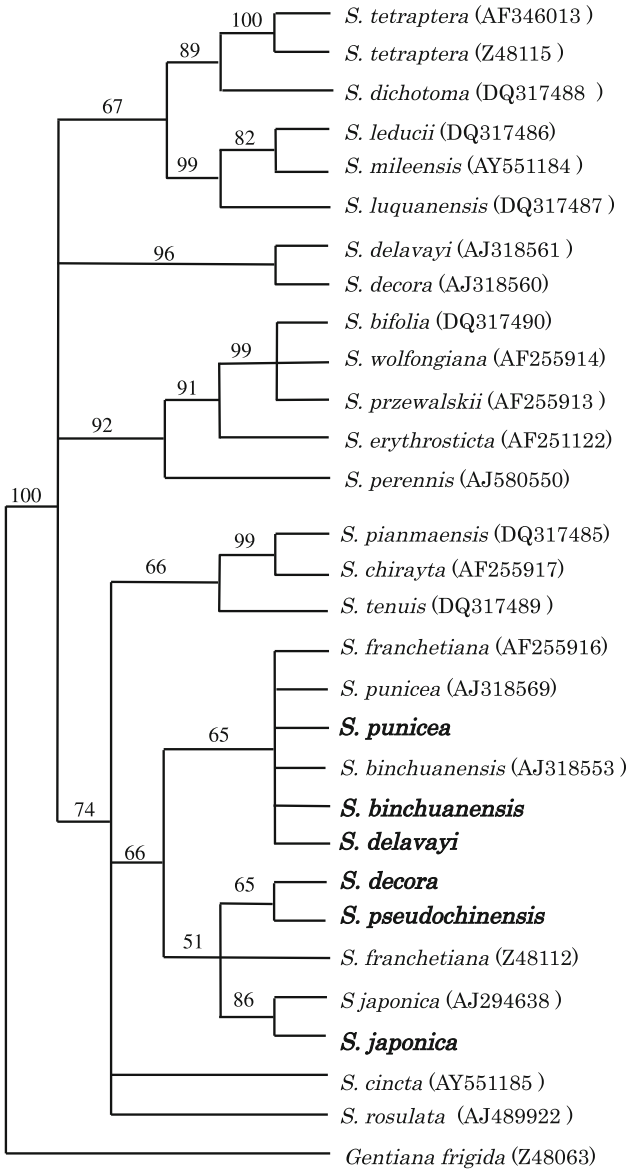

Fig. 2 Phylogenetic analysis of ITS1 sequences of *Swertia* plants. The sequence data of *Gentiana frigida* (GenBank Accession No. Z48063) are used as an outgroup. The 50 % majority-rule consensus tree was based on maximum parsimony analysis. *Numbers above lines* are bootstrap values (%) with 1000 replicates. Species shown in *bold* are the results of this study. Data from GenBank are shown with accession numbers in *parentheses*

S. delavayi in this study was inconsistent with that reported by Chassot et al. [8]. Re-examination of both of the materials might be necessary. However, *Swertia* plants from Yunnan, Hebei, and Japan analyzed in this study were phylogenetically close in comparison with *Swertia* plants obtained from Qinghai and Tibet (*S. bifolia*, *S. przewalskii*, and *S. erythrosticta*) [9] and from South Asia including India and Nepal (*S. chirayta*) [8]. The result of our study suggests that although genetic background would have an influence, environmental factors, e.g., soil and weather

conditions, might be critical for their production of secondary metabolites.

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