J. Trad. Med. **29**, 63－73, 2012

Medical and
Pharmaceutical Society
for WAKAN-YAKU

Quality evaluation of *Chotoko* - Local and specific variations in the alkaloid contents of *Uncaria* plants -

Wen Shi,[a] Fumiyo Kitaoka,[a] Misato Doui,[a] Katsunori Miyake,[a] Yohei Sasaki,[a] Nobuko Kakiuchi,[b] and Masayuki Mikage*[a]

[a]Herbal Medicine and Natural Resources, Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan. [b]Department of Pharmacognosy, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino-cho, Nobeoka, Miyazaki 882-8508, Japan.
(Received September 16, 2011. Accepted February 23, 2012.)

Abstract

The Japanese Pharmacopoeia states that the crude drug *Chotoko* is composed of the hooks of *Uncaria rhynchophylla* Miq., *U. sinensis* Havil., and *U. macrophylla* Wall., all of which belong to the Rubiaceae family. It has been reported that the indole and oxindole type alkaloids contained in *Chotoko* have different pharmacological effects, and *Chotoko* products derived from different species are composed of different alkaloids. However, there are no reports about the factors affecting the chemical compositions of *Uncaria* plants. In this study, we analyzed the alkaloid contents (the rhynchophylline, geissoschizine methyl ether, and hirsutine contents) of *Uncaria* samples collected from a broad range of sites by HPLC after identifying their species by DNA sequence analysis. As a result, we found that the hooks and small stems of *U. rhynchophylla* grown in habitats with lower annual precipitation levels tended to display higher alkaloid contents. We also found that the alkaloid compositions of cultivated *Uncaria* plants were different from those of wild plants, even those belonging to the same species, and crude *Chotoko* products displayed two types of alkaloid profile, the rhynchophylline-rich type and the geissoschizine methyl ether and hirsutine-rich type.

Moreover, in order to accurately identify the botanical origin of *Chotoko*, we established a method involving molecular genetics techniques; i.e., DNA sequence analysis and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Key words *Chotoko,* Uncaria hook, alkaloid, precipitation, cultivation, PCR-RFLP.
Abbreviations Um, *Uncaria macrophylla* Wall.; Ur, *U. rhynchophylla* Miq.; Usi, *U. sinensis* Havil.

Introduction

The Japanese Pharmacopoeia 16th edition (JP16) states that the crude *Kampo* drug *Chotoko* is composed of the hooks of *Uncaria rhynchophylla* Miq. (Ur), *U. sinensis* Havil. (Usi), and *U. macrophylla* Wall. (Um),[1] and in the Chinese Pharmacopoeia *Chotoko*, which is pronounced *GouTeng* in Chinese, is described as the hooks and small stems of these 3 species in addition to those of *U. hirsuta* Havil., and *U. sessilifructus* Roxb.[2]

Chotoko is the predominant component of the traditional *Kampo* formulas "yokukansan" and "chotosan". In recent clinical reports, these formulas were reported to relieve headaches and dizziness caused by hypertension and the symptoms of Alzheimer's disease.[3-6] In chemical studies,[7,8] indole and oxindole alkaloids; i.e., rhynchophylline, geissoschizine methyl ether, and

*To whom correspondence should be addressed.
e-mail : mikage@p.kanazawa-u.ac.jp

hirsutine, have been isolated from *Chotoko* as active compounds and have been shown to lower blood pressure, induce vasodilatation and sedation, and protect against ischemia-induced neuronal damage.[9-11] Furthermore, it was reported that the different types of alkaloid found in *Uncaria* plants have different pharmacological effects.[12]

In order to standardize the pharmacological effects of *Chotoko*, an investigation of its alkaloid composition is required. Differences in the plants it is produced from or the growing environments of these plants might be the main factors affecting the chemical components of the crude drug. As for the former, a previous report found that different *Uncaria* species contained different kinds of alkaloid; Usi mainly contains indole type alkaloids whereas Ur predominantly contains oxindole type alkaloids.[13] These results suggest that *Chotoko* derived from different plant species could have different pharmacological effects. However, there are no reports about the factors that affect the alkaloid compositions of *Uncaria* plants. Therefore, in this study, we collected Chinese and Japanese *Uncaria* plants that were grown in various habitats and analyzed their alkaloid contents (their rhynchophylline, geissoschizine methyl ether, and hirsutine contents) by HPLC after accurately identifying them by molecular genetics methods. We also found several environmental factors that affect the chemical composition of *Uncaria* plants. In addition, we evaluated the origins of commercially available *Chotoko* products.

Uncaria plants can be morphologically identified based on the characteristics of their flowers, stipules, and leaves. However, the crude drug *Chotoko* is usually composed of hooks with small stems, and so morphological identification is rather difficult.[14] Recently, molecular genetics methods have been used to identify crude drugs.[15-17] In this study, we genetically identified *Uncaria* plants using the method reported by Yamaji *et al*.[18]; i.e., we analyzed the DNA sequences of the internal transcribed spacer (ITS) 1 and 2 regions of nuclear ribosome DNA. In addition, we assessed the utility of another method; i.e., analyzing the DNA sequences of the genes of the ribosomal proteins L16 (*rpl*16) and S7 (*rps*7) (chloroplast DNA). Furthermore, we established a more convenient and economical method for identifying *Uncaria* species involving a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) procedure based on the DNA sequences of the ITS regions.

Materials and Methods

Plant and crude drug materials: Wild Usi, Ur, and Um plant materials were collected in China and Japan, and cultivated plant materials were collected from Guizhou in China. All the plant materials were collected when their leaves and hooks were green and dried naturally. The materials were preliminarily identified by the authors according to their morphological characteristics.[19]

Crude drugs materials were purchased from Chinese and Japanese markets. Information about these materials is shown in Table 1, and all of the materials were deposited in the Faculty of Pharmacy, Kanazawa University.

Extraction of total DNA: Before the extraction of total DNA, about 50~70 mg samples of the dried leaves or crude drug were frozen in liquid nitrogen and ground into powder. The crude drug powders were then treated with cleaning buffer composed of 0.1M Tris-HCl (pH 0.8), 1% PVP, 0.05M ascorbic acid, and 5% 2-mercaptoethanol to remove any pigment and reduce their mucosity.

The DNA extraction was performed using a DNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's protocol.

PCR amplification: The ITS regions were amplified by the nested PCR method. One hundred~120 ng of total DNA as a template were mixed in 25 μl of reaction mixture containing 2.5 μl of 10× PCR buffer for KOD-Plus, 0.2 mM of dNTP, 1.0 mM of $MgSO_4$, 0.5 U of KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan), and 0.4 μM of each primer. Akebi-f (GCT CCT ACC GAT TGA ATG GT) and Akebi-26SR (GTA AGT TTC TTC TCC TCC GC) were used as the first pair of PCR primers, and Unc-2F (TCG AAT CCT GCG AAA CGC AC) and Unc-2R (TGC AAA CGA AAC GCG CAC TA) were used as the second pair of nested primers. The *rpl*16 gene was amplified using the same PCR solutions and the rpl16-F2 (GCG GAA CGA ACC GGA GAT CA) and rpl16-R2 (GGT TAT AGT TGA TGG TTC

Table 1 Materials used in this study

	Species	Samples No.	Collection site	Collection date	Voucher No.	Precipitation (mm/year)
Wild plant materials	*U. rhynchophylla* (Ur)	Rh-1	Tanegashima, Kagoshima Pref., Japan	2005/9/4	R050904A	2594
		Rh-2			R050904B	
		Rh-3		2005/9/6	R050906	
		Rh-4	Nobeoka city, Miyazaki Pref., Japan	2007/11/28	R071128	2430
		Rh-5	Miyazaki city, Miyazaki Pref., Japan	2009/2/14	R090214	2219
		Rh-6		2007/11/27	R071127	2465
		Rh-7	Munakata city, Fukuoka Pref., Japan	2011/8/10	R110810	1909
		Rh-8		2011/11/12	R111112A	
		Rh-9			R111112B	
		Rh-10	Miyajima, Hiroshima Pref., Japan	2011/7/25	R110725A	1625
		Rh-11			R110725B	
		Rh-12	Takaoka city, Kochi Pref., Japan	2011/8/15	R110815A	2592
		Rh-13			R110815B	
		Rh-14		2011/8/16	R110816	
		Rh-15	Mima city, Tokushima Pref., Japan	2005/9/11	R050911	1642
		Rh-16	Tairyujizan, Tokushima Pref., Japan	2009/9/26	R090926A	1978
		Rh-17			R090926B	
		Rh-18	Takamatsu city, Kagawa Pref., Japan	2010/5/4	R100504	1033
		Rh-19	Sumoto city, Hyogo Pref., Japan	2011/8/18	R110818A	2498
		Rh-20			R110818B	
		Rh-21	Kainan city, Wakayama Pref., Japan	2011/7/23	R110723	1751
		Rh-22	Tadokyo, Mie Pref., Japan	2011/7/5	R110705A	1738
		Rh-23			R110705B	
		Rh-24			R110705C	
		Rh-25			R110705D	
		Rh-26	Shinshiro city, Aichi Pref., Japan	2011/7/24	R110724A	2556
		Rh-27			R110724B	
		Rh-28	Yugawara city, Kanagawa Pref., Japan	2011/7/4	R110704A	2019
		Rh-29			R110704B	
		Rh-30			R110704C	
		Rh-31	Kamogawa city, Chiba Pref., Japan	2011/7/1	R110701A	1472
		Rh-32			R110701B	
		Rh-33			R110701C	
		Rh-34	Zhejiang Prov., China	2009/9/10	R090910	—
		Rh-35	Fujian Prov., China	1998/6/23	R980623	
	U. sinensis (Usi)	Si-1	Shaanxi Prov., China	2009/8/10	S090810	—
		Si-2		2010/7/26	S100726	
		Si-3	Sichuan Prov., China	2010/7/1	S100701	
	U. macrophylla (Um)	Ma-1	Guangxi Prov., China	2009/9/9	M090909	—
		Ma-2	Yunnan Prov., China	2010/10/6	M101006	
Cultivated plant materials	*U. rhynchophylla* (Ur)	Rh-36	Guizhou Prov., China	2011/8/22	R110822A	—
		Rh-37			R110822B	
		Rh-38		2011/10/20	R111020	
Crude drug materials		Ch-10	Chengdu market	2001/8/17	5797	—
		Ch-11	Hebei market	2010/2/28	7704	
		Ch-12		2010/7/29	7742	
		Ch-13	Shanghai market	2010/2/23	7740	
		Ch-14		2010/3/19	7746	
		Ch-15	Shaanxi market	2010/7/26	7735	
		Ch-16			7745	
		Ch-17			7747	
		Ch-18			7749	
		Ni-1	Japanese market	1995/11/16	3271	

The precipitation data were obtained from the Japan Meteorological Agency [21)]

TT) primers. The *rps*7 gene was amplified using the same PCR solutions and the rps7-F (GTA TAG ATC CTG TTG ATG GA) and rps7-R (TCA CGC TCA TGT CAC GTC GA) primers.

The PCR program for the ITS regions was as follows: 94℃ for 2 min; 40 cycles (for the first PCR) or 30 cycles (for the second PCR) of denaturation at 94℃ for 15 s, annealing at 55℃ for 30 s, and extension at 68℃ for 45 s; and a final extension step of 68 ℃ for 5 min. The PCR programs for the *rpl*16 and *rps*7 genes were as follows: 94℃ for 2 min; 30 cycles of denaturation at 94℃ for 15 s, annealing at 55℃ for 30 s, and extension at 68℃ for 45 s; and a final extension step of 68℃ for 5 min.

Three μl of the PCR products were used for agarose gel electrophoresis, and the remaining PCR products were purified using a QIA quick PCR Purification Kit (QIAGEN).

Sequencing procedure: The purified PCR products were subjected to direct sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The Unc-2F, Unc-2R, Unc-5.8F (GCA TCG ATG ATG AAG AAC GTA GC), and Unc-5.8R primers (GTT CAA AGA CTC GAT GGT TC) were used to prime the sequencing reactions for the ITS regions. The rpl16-F2 and rpl16-R2 primers were used to prime the sequencing reactions for the *rpl*16 gene. The rps7-F and rps7-R primers were used to prime the sequencing reactions for the *rps*7 gene.

The DNA sequences were aligned using the DNASIS (version 3.0) software program (Hitachi, Tokyo, Japan).

PCR-RFLP: The purified PCR products (150~250 ng/μl) were digested for 120 min at 37 ℃ using the restriction enzymes *Stu*I and *Nae*I in a 12 μl reaction volume containing 3 μl of the purified PCR product, 1 μl of enzyme, and 1 μl of buffer. Agarose gel electrophoresis was performed after the reaction.

Sample preparation and HPLC conditions: The HPLC method was based on a method outlined in the JP16, as described by Mikage *et al*.[20] The recovery rates of rhynchophylline, geissoschizine methyl ether, and hirsutine, which were calculated using the standard addition method, were 98, 107, and 103%, respectively (mean of three experiments).

(1) Reagents

The standards for rhynchophylline (purity: 99.5%) and hirsutine (purity: > 98.0%) were purchased from Matsuura Yakugyo Co., Ltd and Wako Pure Chemical Industries, Ltd, respectively. The standard for geissoschizine methyl ether (the purity of the geissoschizine methyl ether standard was unknown because only a small amount was available. However, we considered that even if its purity was less than 100%, it would not affect our data as we did not discuss the precise amounts of this compound in the collected samples) was a gift from Prof. Hiromitsu Takayama of Chiba University.

(2) Sample preparation

The hooks and small stems of each sample were ground into powder. Each powdered sample (100 mg) was extracted with 5 ml of 70% methanol under ultrasonication for 10 min. After centrifugation at 3,000 rpm for 10 min, the samples were filtered through a 0.45 μm membrane filter. The resultant solutions were injected into the HPLC system. The extraction was repeated three times, and mean values were adopted.

(3) HPLC conditions

The apparatus comprised an L-2400 UV detector, an L-2130 pump, an L-2200 autosampler, a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan), a CTO-6A column oven (Shimadzu, Kyoto, Japan), and a Mightysil column (RP-18 GP; φ4.6 mm × 250 mm; 5 μm; Kanto Chemical, Tokyo, Japan).

The mobile phase was CH_3CN - H_2O - CH_3COOH - CH_3COONH_4 (26 ml: 73.3 ml: 0.7 ml: 0.3 g), the flow rate was 1.0 ml/min, and the column oven temperature was 40℃. The detection wavelength was set to 245 nm.

(4) Preparation of standard curves

Each standard curve was drawn using the peak areas of three different concentrations.

Results

Identification of *Uncaria* species by DNA analysis

(1) The sequence lengths of the ITS region, and the *rpl*16 and *rps*7 genes

In Ur, Usi, and Um, the ITS region was 594, 594, and 593 bp in length, respectively; and the *rpl*16 gene was

492, 489, and 492 bp in length, respectively. The *rps*7 gene was 435 bp in length in all three *Uncaria* plants.

(2) Wild plant materials

The deletions and substitutions found in the DNA sequences of each sample are shown in Tables 2 and 3. The ITS region sequences of the Usi samples were identical to those reported in GenBank (FJ980386), except for that of Ch-17, which displayed two nucleotide differences (at positions 10 and 89). The ITS2 region sequences of the Um samples were identical to that reported in GenBank (GQ434638), except for that of Ma-1, which had a nucleotide difference at position 413.

Compared with the ITS region sequence of Ur, that of Usi displayed 4 nucleotide differences, and that of Um demonstrated 17 differences. As for the *rpl*16 gene, we found that the *rpl*16 genes of Usi and Um had 8 and 7 nucleotide differences from that of Ur, respectively. Therefore, it was clarified that the three *Uncaria* species

Table 2 Nucleotide substitutions in the ITS regions of the three *Uncaria* species

Species	Sample type	Samples No.	Nucleotide number																						
			ITS 1												5.8S rRNA	ITS 2									
			10	37	43	78	85	89	94	169	177	209	211	215	365	412	413	415	450	461	532	536	554	559
Ur	WPM	Rh-1 ~ Rh-4, Rh-14, Rh-16 ~ Rh-18, Rh-24 ~ Rh-25, Rh-28 ~ Rh-30, Rh-34~Rh-35	G	A	T	G	T	G	A	C	T	T	T	T	T	T	A	C	A	G	T	T	G	T
		Rh-5 ~ Rh-13, Rh-19 ~ Rh-20, Rh-22~Rh-23, Rh-26 ~ Rh-27, Rh-31~Rh-33	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*
		Rh-21	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	R	*
		Rh-15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	C
	CPM	Rh-36 ~ Rh-38	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	CDM	Ch-10 ~ Ch-13, Ni-1-A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Usi		FJ980386	*	*	*	*	C	*	*	*	*	*	*	A	*	*	*	T	*	T	*	*	*	*
	WPM	Si-1 ~ Si-3	*	*	*	*	C	*	*	*	*	*	*	A	*	*	*	T	*	T	*	*	*	*
	CDM	Ch-14 ~ Ch-16, Ch-18, Ni-1-B	*	*	*	*	C	*	*	*	*	*	*	A	*	*	*	T	*	T	*	*	*	*
		Ch-17	T	*	*	*	C	R	*	*	*	*	*	A	*	*	*	T	*	T	*	*	*	*
Um		GQ434638	/	/	/	/	/	/	/	/	/	/	/	/	-	C	G	*	T	*	C	C	*	A
	WPM	Ma-1	A	G	C	A	C	*	C	T	C	C	C	G	-	C	S	*	T	*	C	C	*	A
		Ma-2	*	G	C	A	C	*	C	T	C	C	C	G	-	C	G	*	T	*	C	C	*	A

The nucleotide numbers were adopted in accordance with the sequence of FJ980386. Asterisks (*) indicate the same nucleotides as the top sequence; hyphens (-) denote nucleotide gaps; slashes (/) denote no data for GQ434638; R indicates A or G, and S indicates G or C. Ni-1-A and Ni-1-B were subsamples derived from Ni-1. FJ980386 and GQ434638 were obtained from GenBank. Abbreviations: Ur, *Uncaria rhynchophylla*; Usi, *U. sinensis*; Um, *U. macrophylla*; WPM, wild plant materials; CPM, cultivated plant materials; CDM, crude drug materials.

Table 3 Nucleotide substitutions in the *rpl*16 genes of the three *Uncaria* species

Species	Samples No.	Nucleotide number in *rpl* 16 gene										
		71	74	79	163	164	303	311	348	355	380	381
Ur	Rh-5 (AB685338)	A	C	A	A	-	-	C	T	T	C	G
	Rh-6, Rh-11, Rh-15, Rh-17, Rh-21, Rh-22, Rh-23, Rh-28, Rh-31, Rh-34, Rh-36	*	*	*	*	*	*	*	*	*	*	*
Usi	Si-1 (AB685340)	G	-	-	-	*	*	T	*	A	T	T
	Si-2	G	-	-	-	*	*	T	*	A	T	T
Um	Ma-1	G	-	-	*	A	A	*	C	*	T	*

The nucleotide numbers were adopted in accordance with the sequence of AB685338. Asterisks (*) indicate the same nucleotides as the top sequence; hyphens (-) denote nucleotide gaps. The data for AB685338 and AB685340 are recorded in DNA Data Bank of Japan (DDJB). Abbreviations: Ur, *Uncaria rhynchophylla*; Usi, *U. sinensis*; Um, *U. macrophylla.*

can be distinguished from each other by comparing the DNA sequences of their ITS regions or *rpl*16 genes. On the contrary, we did not find any nucleotide differences among the *rps*7 genes of the three *Uncaria* species (data not shown; will be published in the DNA Data Bank of Japan (DDJB), accession No. AB690426.).

(3) Crude drug materials and cultivated plant materials

Based on our ITS region data, among the 10 crude drug samples, 4 were identified as Ur and 5 were identified as Usi. The samples bought in Chengdu (Ch-10) and Hebei (Ch-11, Ch-12) markets were identified as Ur, whereas those obtained at the Shaanxi (Ch-15 ~ Ch-18) market were identified as Usi (Table 2). Moreover, we found that the samples bought in the Shanghai market were derived from either Ur (Ch-13) or Usi (Ch-14). Meanwhile, both Ur (Ni-1-A) and Usi (Ni-1-B) were found in Ni-1, which was obtained from a Japanese market.

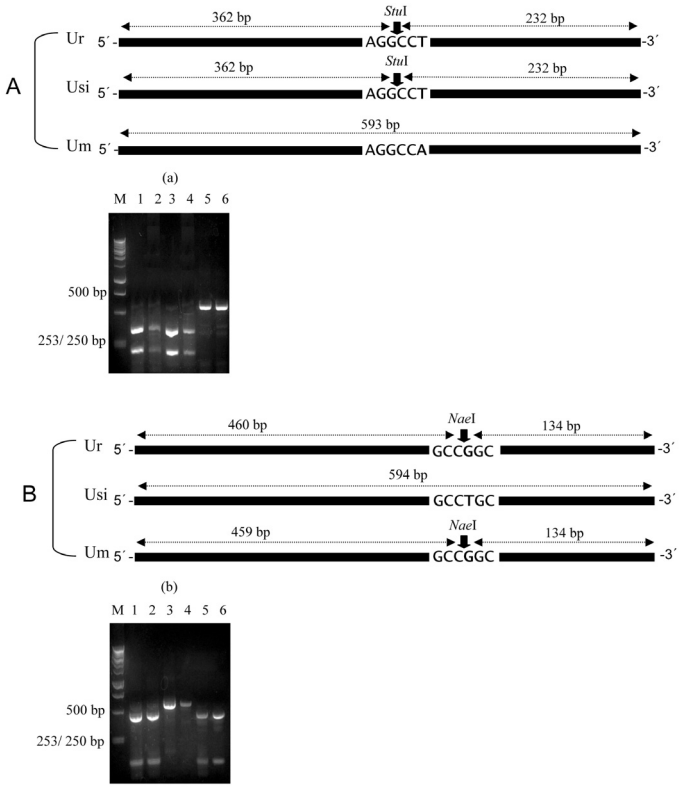

Fig. 1 *Stu*I (A) and *Nae*I (B) restriction sites on the ITS regions in the three *Uncaria* species, and agarose gel electrophoretogram of the PCR-RFLP products digested with *Stu*I (a) and *Nae*I (b). Arrows denote restriction sites of each enzyme.
M: 1 kb DNA ladder, 1: Rh-8, 2: Rh-11, 3: Si-1, 4: Si-3, 5: Ma-1, 6: Ma-2. Ur, *Uncaria rhynchophylla*; Usi, *U. sinensis*; Um, *U. macrophylla.*

The cultivated plant materials were all identified as Ur.

PCR-RFLP method: The restriction enzyme *Stu*I, which specifically recognizes the 5' AGGCCT 3' sequence in 5.8S rRNA, cleaved the PCR products of Ur and Usi, but not those of Um, into two fragments of 362 bp and 232 bp (Fig. 1-A). On the other hand, treating them with the *Nae*I enzyme (5' GCCGGC 3' in ITS2 region) resulted in the PCR products of Ur and Um, but not Usi, being cleaved into two fragments (Ur: 460 bp and 134 bp, Um: 459 bp and 134 bp) (Fig. 1-B). These results indicate that the PCR products of Ur can be digested by both *Nae*I and *Stu*I. Thus, by using the PCR-RFLP method we can identify the three *Uncaria* species (Fig. 1-(a) (b)), and this method can also be applied to identify the original species of *Chotoko* samples (Fig. 2). Accordingly, Ch-12 and Ch-13 were identified as Ur, and Ch-15 and Ch-16 were identified as Usi. The PCR products of Ch-12 and Ch-13 were digested into two fragments by *Stu*I and *Nae*I, suggesting that they were Ur, whereas those of Ch-15 and Ch-16 could not be digested by *Nae*I, suggesting that they were Usi. The results obtained with the PCR-RFLP method were the same as the results obtained by ITS sequence analysis.

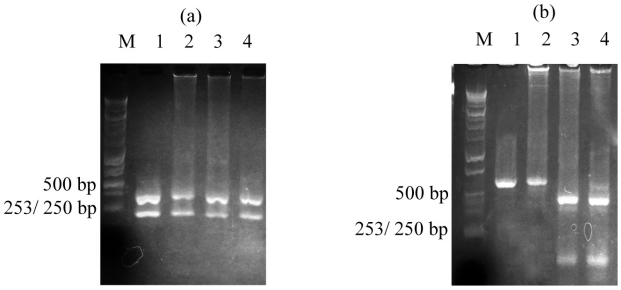

Fig. 2 Agarose gel electrophoretogram of the PCR-RFLP products of the ITS regions of the crude drug materials using *Stu*I (a) and *Nae*I (b).
M: 1 kb DNA ladder, 1: Ch-15, 2: Ch-16, 3: Ch-12, 4: Ch-13.

Alkaloid content analysis: The oxindole (rhynchophylline) and indole (geissoschizine methyl ether and hirsutine) alkaloid contents of the identified samples were analyzed using HPLC, and the results are shown in Fig. 3, Fig. 4, and Table 4.

(1) The relationship between the rhynchophylline content of *Uncaria* plants and the annual precipitation

Table 4 Mean value of three alkaloid contents of the samples collected in different locations in Japan.

Samples No.	Collection site	rhynchophylline (%)	geissoschizine methyl ether (%)	hirsutine (%)
Rh-7 ~ Rh-9	Munakata city, Fukuoka Pref.	0.349	N.D. **	N.D.
Rh-22 ~ Rh-25	Tadokyo, Mie Pref.	0.228	N.D.	N.D.
Rh-18	Takamatsu city, Kagawa Pref.	0.154	0.001	0.005
Rh-10 ~ Rh-11	Miyajima, Hiroshima Pref.	0.113	N.D.	N.D.
Rh-21	Kainan city, Wakayama Pref.	0.111	N.D.	N.D.
Rh-15	Mima city, Tokushima Pref.	0.108	0.017	N.D.
Rh-31 ~ Rh-33	Kamogawa city, Chiba Pref.	0.104	N.D.	N.D.
Rh-4	Nobeoka city, Miyazaki Pref.	0.099	N.D.	N.D.
Rh-28 ~ Rh-30	Yugawara city, Kanagawa Pref.	0.091	N.D.	N.D.
Rh-26 ~ Rh-27	Shinshiro city, Aichi Pref.	0.090	N.D.	N.D.
Rh-16 ~ Rh-17	Tairyujizan, Tokushima Pref.	0.090	N.D.	N.D.
Rh-12 ~ Rh-14	Takaoka city, Kochi Pref.	0.086	0.023	N.D.
Rh-6	Miyazaki city, Miyazaki Pref. (2007)*	0.075	N.D.	N.D.
Rh-5	Miyazaki city, Miyazaki Pref. (2009)*	0.057	N.D.	N.D.
Rh-1 ~ Rh-3	Tanegashima, Kagoshima Pref.	0.051	0.014	N.D.
Rh-19 ~ Rh-20	Sumoto city, Hyogo Pref.	0.050	0.009	N.D.

(*n* = 1-4)

* : Collection year
** : Not detected

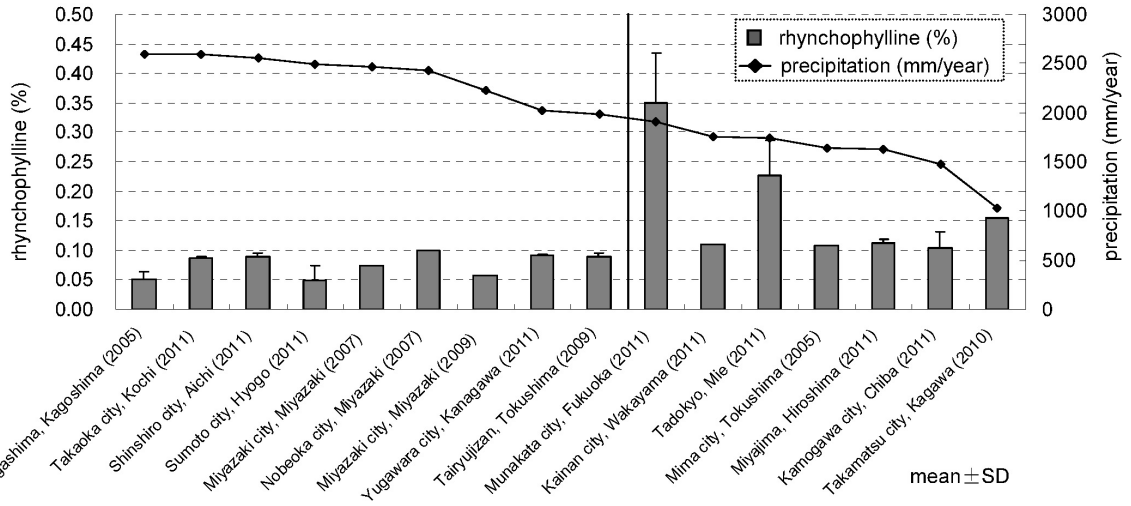

Fig. 3 Relationship between the mean rhynchophylline content and the annual precipitation level of the collection site (*n*=1-4).

levels of their habitats

Geissoschizine methyl ether and hirsutine could not be detected in most samples collected from Japan. Then, we compared the rhynchophylline contents of Ur samples collected from different habitats in Japan and found that they differed. The Ur samples collected from Munakata city in Fukuoka prefecture had the highest rhynchophylline content; i.e., 0.349%, while the samples collected from Sumoto city in Hyogo prefecture had the lowest rhynchophylline content (0.050%) (Table 4).

Then, we investigated the environmental factors that affect the alkaloid contents of Ur. By investigating the annual precipitation level[21] of collection year of each site (Fig. 3), we found that the samples collected from areas with precipitation levels of less than 1950 mm/year had higher rhynchophylline contents (more than 0.1%).

(2) Differences in alkaloid composition between wild and cultivated *Uncaria* plants and an analysis of crude *Chotoko* samples

We found that all of the wild plant materials identified as Ur, Usi, or Um had higher rhynchophylline contents and lower geissoschizine methyl ether and

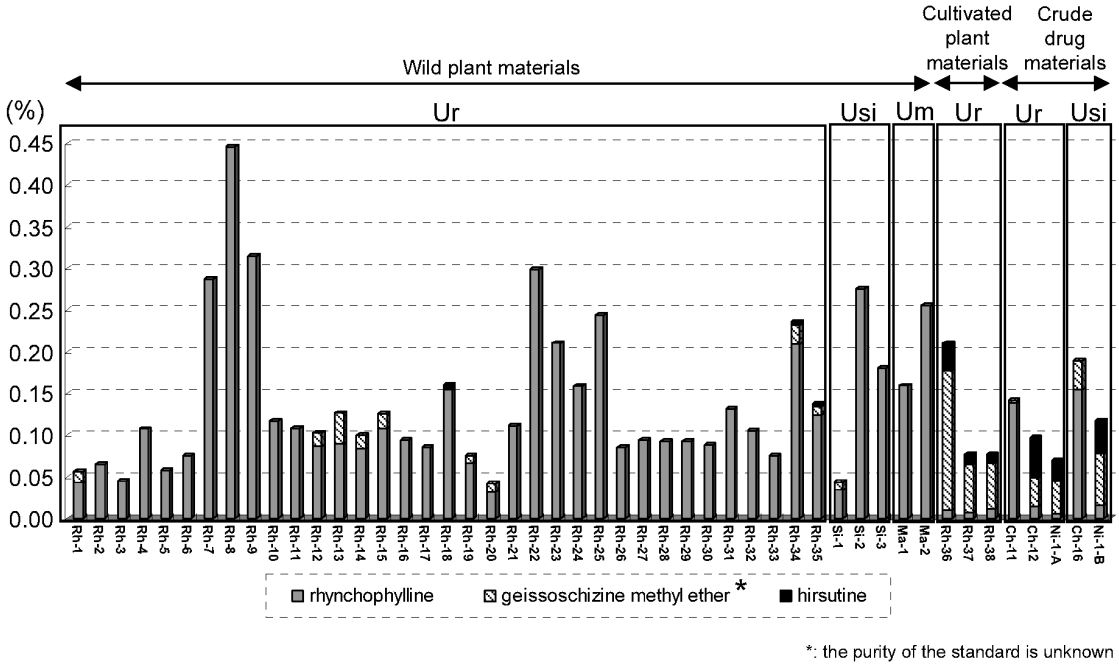

Fig. 4 Mean alkaloid contents of the wild plant, cultivated plant, and crude drug materials (n= 3: each sample was independently tested 3 times)
Ur, *Uncaria rhynchophylla*; Usi, *U. sinensis*; Um, *U. macrophylla.*

hirsutine contents. This type of alkaloid profile is defined as the R type (Fig. 4). While, the cultivated plant materials identified as Ur had higher geissoschizine methyl ether and hirsutine contents, and lower rhynchophylline contents (the GH type).

As for the crude drug materials, although Ch-11 and Ch-12 were identified as Ur, they displayed different alkaloid profile types; i.e., Ch-11 belonged to the R type while Ch-12 was defined as the GH type, and Ch-16, which was identified as Usi, displayed an R type alkaloid profile. In addition, the two crude drug samples derived from Ni-1, Ni-1-A (Ur) and Ni-1-B (Usi), both displayed GH type profiles.

Discussion

Identification of *Uncaria* species by DNA analysis

(1) We analyzed a region of nuclear ribosomal DNA (the ITS region) and two chloroplast DNA (*rpl*16 and *rps*7) genes in the three plant species that the JP16 states are used to produce *Chotoko*; i.e., *Uncaria rhynchophylla, U. sinensis*, and *U. macrophylla.* Although it was difficult to extract total DNA from crude *Chotoko* due to the lower amount of DNA present in the hooks and stems, we were able to improve our method by using cleaning buffer before extracting the total DNA and then used a nested PCR program to amplify the ITS region. As a result, we were able to identify the 3 *Uncaria* species using the DNA sequences of their ITS regions, as was previously reported by Yamaji *et al.*[18] In addition, we found that the *rpl*16 gene displayed nucleotide differences among the three species, and these methods were successful in identifying the botanical origins of *Chotoko* products sold in Chinese and Japanese markets, most of which were found to be derived from Ur or Usi.

(2) We also established a PCR-RFLP method based on the DNA sequence of the ITS region for identifying the species origins of *Chotoko* products. Direct analysis of the DNA sequence of a crude drug is helpful for identifying the species it is derived from, but it requires a long time, expensive reagents, and special equipment. Conversely, applying the PCR-RFLP technique is more convenient and cheap.

Environmental factors that affect alkaloid content

(3) Plant samples were collected from a broad range of areas from Kyushu to Kanto (areas of Japan). All of them were identified as Ur by DNA analysis, but they displayed different rhynchophylline contents. In

addition, we found that the samples grown in areas with lower precipitation levels tended to display higher rhynchophylline contents. As for the materials collected from areas that received less than 1950 mm precipitation per year, they displayed rhynchophylline contents of more than 0.1%, with the highest value being 0.349%. Considering the low correlation coefficient for the relationship between the rhynchophylline content and the precipitation level of the collection site (r= -0.411) for all samples, we supposed that alkaloid content might be influenced by other factors such as humidity or the collection season, as well as precipitation. It is interesting that this phenomenon; i.e., higher alkaloid contents being detected in plants from lower precipitation areas, coincides with the results reported by Wang, L.L. *et al*.[22] and Wang, Z.Y. *et al*.[23]

(4) Wakan-sansai-zue,[24] which was written in the Edo era in Japan, stated that of the *Chotoko* collected in Bunshu-Nakatsu, which is now called Houshu, those from Fukuoka, Oita, and Geishu-Hiroshima were of high quality. In the present study, the samples collected from Fukuoka prefecture displayed high alkaloid contents; however, those collected from Hiroshima prefecture did not. Therefore, *Uncaria* plants grown in Fukuoka in northern Kyushu might be of higher quality in terms of their alkaloid content.

Environmental factors affecting alkaloid composition

(5) Sakakibara *et al*.[13] reported that the alkaloid composition of Uncaria hooks is related to their species: Usi mainly contains indole type alkaloids (GH type) whereas Ur predominantly contains oxindole alkaloids (R type). In our study, all the wild Ur, Usi, and Um plant materials displayed R type alkaloid profiles. Morphologically, the Si-1, Si-2, and Si-3 samples had no hair on their leaves, stems, or hooks; their stipules were entire; and their DNA sequences were identical to those reported for Usi in GenBank, so we identified them as Usi; however, they displayed an R type alkaloid profile, which was different from the findings of a previous report.[13] Therefore, we consider that species differences might not be the decisive factor affecting the alkaloid compositions of *Uncaria* plants.

(6) Mikage *et al*.[20] reported that hirsutine was not present in the hooks of wild Japanese Ur, and the same result was obtained in this study. In other words, we could not detect a high hirsutine content in any of the wild plant materials. However, the cultivated plant materials collected from Guizhou displayed a high hirsutine content. We consider that the chemical composition of *U. rhynchophylla* might be affected by the cultivation conditions. Further studies are necessary to investigate the effects of cultivation on the alkaloid compositions of *Uncaria* plants.

Alkaloid composition of the crude drug *Chotoko*

(7) The crude drug samples that we collected were all derived from Ur or Usi; however, both the R and GH type alkaloid profiles were seen in samples derived from both species. Thus, we considered that processing might affect the chemical composition of *Chotoko*; therefore, we analyzed the alkaloid contents of the *Chotoko* samples after steaming the wild hooks or keeping them in acidic fluid; however, neither of these treatments had any effect on the alkaloid contents of the hooks (data not shown). In recent years, *Uncaria* plants have begun to be cultivated, so commercially available crude drug materials can be derived from either cultivated or wild plants, and we consider that the alkaloid composition of *Chotoko* is related to the source of the crude drug. Actually, the crude drug sample obtained from the Japanese market (Ni-1) was mainly derived from Ur and partly derived from Usi, and the predominant alkaloid types of the two Ni-1 subsamples were the same (GH type), indicating that some of the crude drug was derived from cultivated Ur. Assuming that the alkaloid components of *Chotoko* are responsible for its clinical efficacy, analyses of the alkaloids within *Chotoko* samples should be given priority over the identification of their origins.

Acknowledgements

We are grateful to Professor Akiyo Sakushima of Kyushu University of Health and Welfare; Professor Hironori Deguchi and Associate Professor Hiromi Tsubota of the Faculty of Science, Hiroshima University; Mr. Teruo Katsuyama of Kanagawa Prefectural Museum of Natural History; Professor Hiroshi Kohda of Yasuda Women's University; Miyajima Natural Botanical

Garden and Professor Xiaojun Ma of Guangxi Botanical Garden of Medicinal Plants for their help with the *Uncaria* plant collection. In addition, we are grateful to Professor Hiromitsu Takayama of the Graduate School of Pharmaceutical Sciences, Chiba University, for donating the standard sample of geissoschizine methyl ether.

References

1) The Japanese Pharmacopoeia Sixteenth Edition 2011, Hirokawa Shoten, Tokyo, pp. D559-562, 2011.
2) The Chinese Pharmacopoeia (Chinese Edition), Chemical Industry Press, Beijing, p.240, 2010.
3) Mimaki, Y., Toshimizu, N., Yamada, K. and Sashida, Y.: Anti-convulsion effects of Choto-san and Chotoko (Uncariae Uncis cum Ramulus) in mice, and identification of the active principles. *J. Pharm. Soc. Jpn.,* **117**, 1011-1021, 1997.
4) Watanabe, H., Zhao, Q., Matsumoto, K., Tohda, M., Murakami, Y., Zhang, S.H., Kang, T.H., Mahakunakorn, P., Maruyama, Y., Sakakibara, I., Aimi, N. and Takayama, H.: Pharmacological evidence for antidementia effect of Choto-san (Gouteng-san), a traditional Kampo medicine. *Pharmacol. Biochem. Behav.,* **75**, 635-643, 2003.
5) Zhao, Q., Murakami, Y., Tohda, M., Watanabe, H. and Matsumoto, K.: Preventive effect of Chotosan, a Kampo Medicine, on transient ischemia-induced learning deficit is mediated by stimulation of muscarinic M_1 but not nicotinic receptor. *Biol. Pharm. Bull.,* **28**, 1873-1878, 2005.
6) Kawakami, Z., Kanno, H., Ikarashi, Y. and Kase, Y.: Yokukansan, a Kampo medicine, protects against glutamate cytotoxicity due to oxidative stress in PC12 cells. *J. Ethnopharmacol.,* **134**, 74-81, 2011.
7) Heitzman, M.E., Neto, C.C., Winiarz, E., Vaisberg, A.J. and Hammond, G.B.: Ethnobotany, phytochemistry and pharmacology of *Uncaria* (Rubiaceae). *Phytochemistry,* **66**, 5-29, 2005.
8) Laus, G.: Advances in chemistry and bioactivity of the genus *Uncaria. Phytother. Res.,* **18**, 259-274, 2004.
9) Kang, T.H., Murakami, Y., Takayama, H., Kitajima, M., Aimi, N., Watanabe, H. and Matsumoto, K.: Protective effect of rhynchophylline and isorhynchophylline on in *vitro* ischemia-induced neuronal damage in the hippocampus: putative neurotransmitter receptors involved in their action. *Life Sci.,* **76**, 331-343, 2004.
10) Yuzurihara, M., Ikarashi, Y., Goto, K., Sakakibara, I., Hayakawa, T. and Sasaki, H.: Geissoschizine methyl ether, an indole alkaloid extracted from Uncariae Ramulus et Uncis, is a potent vasorelaxant of isolated rat aorta. *Eur. J. Pharmacol.,* **444**, 183-189, 2002.
11) Yuan, D., Ma, B., Wu, C., Yang, J., Zhang, L., Liu, S., Wu, L. and Kano, Y.: Alkaloids from the leaves of *Uncaria rhynchophylla* and their inhibitory activity on NO production in lipopolysaccharide-activated microglia. *J. Nat. Prod.,* **71**, 1271-1274, 2008.
12) Sakakibara, I., Takahashi, H., Yuzuhara, M., Yatoh, T., Kubo, M., Hayashi, K., Ishige, A., Amagaya, A., Okada, M. and Maruno, M.: Pharmacognostical and pharmacological evaluations of *Uncaria sinensis* (Rubiaceae). *Nat. Med.,* **51**, 79-83, 1997.
13) Sakakibara, I. and Takahashi, H.: Chemical and pharmacological evaluations of Chinese crude drug "Gou-teng". *Nat. Med.,* **52**, 353- 359, 1998.
14) Mikage, M., Kiuchi, F., Sakai, S. and Tsuda, Y.: Morphological study and alkaloidal analysis of *Uncaria scandens* (Rubiaceae) from Nepal. *Nat. Med.,* **48**, 155-160, 1994.
15) Kitaoka, F., Kakiuchi, N., Long, C.F., Itoga, M., Yoshimatsu, H., Mitsue, A., Atsumi, T., Mouri, C. and Mikage, M.: Difference of ITS sequences of *Akebia* plants growing in various parts of Japan. *J. Nat. Med.,* **63**, 368-374, 2009.
16) Yang, D.Y., Fushimi, H., Cai, S.Q. and Komatsu, K.: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system (ARMS) analyses of medicinally used *Rheum* species and their application for identification of Rhei rhizoma. *Biol. Pharm. Bull.,* **27**, 661-669, 2004.
17) Ding, X., Xu, L., Wang, Z., Zhou, K., Xu, H. and Wang, Y.: Authentication of stems of *Dendrobium officinale* by rDNA ITS region sequences. *Planta. Med.,* **68**, 191-192, 2002.
18) Yamaji, H.: Method of identifying the plant species of the genus *Uncaria*. Japan Patent Kokai WO2009014269 (2009.1.29), < http://patent.astamuse.com/ja/published/JP/No/WO2009014269>.
19) Sakakibara, I., Takahashi, H., Terabayashi, S., Kubo, M., Higuchi, M., Okada, M., Cheng, B.Q., Hao, X.J., Shu, G.M. and Huang, H.: Discrimination of nine species of

Uncaria (Rubiaceae), original plants of Chinese natural medicine, Diao-teng-gou, based on stem anatomy and HPLC analysis. *J. Jpn. Bot.,* **74**, 42-52, 1999.

20) Mikage, M., Endo, H., Katsuki, S. and Kakiuchi, N.: Historical studies about medicinal part of Chinese crude drug "Uncaria Hook" (Part 2) -alkaloid contents in different part used-. *Kampo Med.,* **59**, 279-285, 2008.

21) Japan Meteorological Agency: weather and climate information in Japan. <http://www.data.jma.go.jp/obd/stats/etrn/index.php/>.

22) Wang, L.L., Kakiuchi, N. and Mikage, M.: Studies of *Ephedra* plants in Asia. Part 6: Geographical changes of anatomical features and alkaloids content of *Ephedra sinica. J. Nat. Med.,* **64**, 63-69, 2010.

23) Wang, Z.Y.: Study on active component from the fruit of *Lycium barbarum* in different regions. *Bull. Bot. Res.,* **23**, 337-339, 2003.

24) Terashima, R.: Wakan-sansai-zue (1713), reprinted edition, Tokyo-Bijutu, Tokyo, p.1382, 1970.