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ITS analysis of *Clematis* plants from East Asia and the botanical origin of Clematidis Radix sold in modern markets

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Abstract

Clematidis Radix (Wei Ling Xian in Chinese and Ireisen in Japanese) is a crude drug used in traditional Chinese medicine and is derived from the underground parts of *Clematis* plants, which belong to the family Ranunculaceae. The Clematidis Radix sold in modern markets is derived from a variety of botanical origins, whereas the Chinese and Japanese pharmacopoeias state that Clematidis Radix should be produced from *Clematis hexapetala, C. mandshurica,* or *C. chinensis*. To clarify the botanical origin of this crude drug, 9 closely related taxa of the genus *Clematis* growing in East Asia were subjected to molecular biological studies of their internal transcribed spacer (ITS) regions. We found that the ITS region nucleotide sequences of the 9 taxa had diverged. As a result, the 9 taxa could be successfully differentiated by comparing their whole ITS region sequences. Based on this result, 11 Clematidis Radix samples obtained from modern Japanese, Chinese, and Korean markets were genetically identified as follows: Of the samples from Japanese markets, 3 out of 4 were categorized as *C. mandshurica,* and one as *C. chinensis*. Among the samples from Chinese markets, 2 out of 4 were identified as *C. mandshurica,* and the other two as *C. brachyura.* Meanwhile, of the 3 samples from Korean markets, two were identified as *C. mandshurica,* and the other as *C. terniflora* var. *robusta.*

Key words Wei Ling Xian, Ireisen, Clematidis Radix, *Clematis*, ITS, botanical origin.

Abbreviations Ch, *C. hexapetala*; Cm, *C. mandshurica*; Ctr, *C. terniflora* var. *robusta*; Ctk, *C. terniflora* var. *koreana;* Cc, *C. chinensis*; Cb, *C. brachyura*; Ci, *C. ianthina*; Cp, *C. patens*; Cf, *C. florida*; and TP, type.

Introduction

The Chinese crude drug Clematidis Radix (Wei Ling Xian in Chinese, Ireisen in Japanese, and Wiryongseon in Korean) has long been used as a diuretic and antirheumatic remedy.[1] Recent studies showed that Clematidis Radix had anti-bacteria,[2] anti-inflammation, analgesia,[3,4] and anti-tumor[5] properties. Both the Chinese and Japanese pharmacopoeias state that *Clematis chinensis* Osbeck, *C. hexapetala* Pall. and *C. mandshurica* Rupr. of the family Ranunculaceae are the botanical sources of this crude drug.[6,7]

However, the Clematidis Radix sold in modern markets is derived from a variety of botanical origins. A previous study reported that these crude drugs were derived not only from the three official species but also from other species of the genus *Clematis*.[8] In addition, phytochemical studies have revealed that the roots and rhizomes of *C. chinensis* and *C. mandshurica* are rich in

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saponins,[9,10] while those of *C. hexapetala* are rich in flavonoids.[11] This observation means that Clematidis Radix derived from different *Clematis* species, even those derived from the three officially sanctioned *Clematis* species, will have different pharmacological effects. Therefore, it is important to precisely identify the origins of the Clematidis Radix sold in markets in order to ensure the correct use of this crude drug. However, it is difficult to identify the botanical origins of such crude drug samples macroscopically because the roots of *Clematis* species used as Clematidis Radix are morphologically similar and are sometimes cut into small pieces. Moreover, some of the *Clematis* species growing in East Asia display similar morphologies, whereas others are taxonomically disputable.

In the 1970s, Mikage[12-16] carried out a series of pharmacognostic studies on the Clematidis Radix sold in Japanese, Chinese, and Korean markets. However, the Clematidis Radix sold in these markets was thought to be changed since the 1970s. So, to build on these previous studies, we tried to develop a convenient method for identifying the botanical sources of Clematidis Radix. In recent years, nucleotide alignment analysis, especially of internal transcribed spacer (ITS) regions (ITS1-5.8S rDNA-ITS2) of nuclear ribosomal DNA (nrDNA), have been used as a practical way of identifying medicinal plants as well as crude drugs.[17,18] In this study, we conducted molecular genetic studies to identify the Clematidis Radix sold in Japanese, Chinese, and Korean markets by comparing their ITS sequences with those of *Clematis* plants growing in East Asia. Furthermore, we followed the classification systems proposed by Tamura.[19]

Materials and Methods

Plant and crude drug materials: Twenty-nine specimens of 9 *Clematis* taxa growing in East Asia, whose roots are similar to Clematidis Radix, were collected in Japan, China, Korea, and Russia, and were morphologically identified by Dr. M. Tamura of Osaka University and Dr. K. Yonekura of Tohoku University. The collected species are listed in Table 1 together with their taxonomic categories according to the classification systems proposed by Tamura[19] and Wang.[20]

In addition, 11 crude drug samples were purchased from Japanese, Chinese, and Korean markets. Detailed information about the plant and crude drug materials is shown in Table 2. All of the materials were deposited at the School of Pharmacy, Kanazawa University.

Table 1 Classification of the plant materials used in this study according to the systems developed by Tamura[19] and Wang[20]

	Tamura 1987				Wang 2005			
Species	**Subgenus**	**Section**	**Subsect.**	**Series**	**Subgenus**	**Section**	**Subsect.**	**Series**
***Clematis hexapetala* Pall.**	*Flammula*	*Flammula*	*Angustifolia*		*Clematis*	*Clematis*	*Angustifolia*	*Hexapetalae*
***C. mandshurica* Rupr.**	*Flammula*	*Flammula*	*Rectae*	*Rectae*	*Clematis*	*Clematis*	*Rectae*	*Rectae*
***C. chinensis* Osbeck**	*Flammula*	*Flammula*	*Rectae*	*Chinenses*	*Clematis*	*Clematis*	*Rectae*	*Rectae*
***C. terniflora* DC. var. *robusta* (Carr.) Tamura**	*Flammula*	*Flammula*	*Rectae*	*Rectae*	*Clematis*	*Clematis*	*Rectae*	*Rectae*
***C. terniflora* DC. var. *koreana* (Nakai) Tamura**	*Flammula*	*Flammula*	*Rectae*	*Rectae*	*Clematis*	*Clematis*	*Rectae*	*Rectae*
***C. brachyuran* Maxim.**	*Flammula*	*Pterocarpa*			*Clematis*	*Pterocarpa*		
***C. patens* C. Morren et Decne.**	*Flammula*	*Viticella*	*Patentes*		*Clematis*	*Viticella*	*Floridae*	*Patentes*
***C. florida* Thunb.**	*Flammula*	*Viticella*	*Floridae*		*Clematis*	*Viticella*	*Floridae*	*Floridae*
***C. ianthina* Koehne**	*Viorna*	*Viorna*	*Crispae*	*Fuscae*	*Viorna*	*Viorna*	*Crispae*	*Fuscae*

Table 2 Materials used in this study

Plant materials

Species	Sample No.	Collection site/Source	Voucher No.	Collection date
***Clematis hexapetala* (Ch)**	h1	Side of Lake Khanka, Khankaysky Dist., Russia	H040811A	Aug. 2004
	h2		H040811B	
	h3		H040811C	
	h4		H040811D	
	h5	Unuer, Yakeshi County, Hulunbuir, Inner Mongolia Prov., China	H050712A	Jul. 2005
	h6		H050712B	
	h7	Nippon Shinyaku Co., Ltd. Japan	H110704	Jul. 2011
***C. mandshurica* (Cm)**	m1	Tonghua city, Liaoning Prov., China	M040918	Sep. 2004
	m2	Mt. Priozernaya, Khasanskii Dist., Primorsky Krai, Russia	M050628A	Jun. 2005
	m3		M050628B	
	m4		M050628C	
	m5	Samara State University, Russia	M110525	May 2011
***C. chinensis* (Cc)**	c1	Mianyang city, Sichuan Prov., China	C050925	Sep. 2005
	c2	Mt. Gusuku, Kunigami Gun, Okinawa Pref., Japan	C081030	Mar. 2007
***C. terniflora* var. *robusta* (Ctr)**	tr1	Haeundae-gu, Busan, Korea	R080907	Sep. 2008
	tr2	Medicinal herb garden of Kanazawa University, Japan	R090908	Sep. 2009
	tr3	Asahi-machi, Kanazawa city, Ishikawa Pref., Japan	R090909	Sep. 2009
	tr4	Kaimondake volcano, Kagoshima Pref., Japan	R090212	Feb. 2009
	tr5	Kakuma-machi, Kanazawa city, Ishikawa Pref., Japan	R100602	Jun. 2010
	tr6	Takaoka Gun, Kochi Pref., Japan	R110817A	Aug. 2011
	tr7		R110817B	
***C. terniflora* var. *koreana* (Ctk)**	tk1	Gwangneung, Gyeonggi-do, Korea	75112	Oct. 1975
	tk2	Anyang-si, Gyeonggi-do, Korea	78025	Jul. 1978
***C. brachyuran* (Cb)**	br1	Arboretum of the College of Agriculture, Seoul National University, Seoul, Korea	78009	Jul. 1978
	br2	Anyang-si, Gyeonggi-do, Korea	78021	Jul. 1978
***C. patens* (Cp)**	p1	Kochi Pref., Japan	P081111	Jun. 2007
	p2	Hyogo Pref., Japan	P110525	May 2011
***C. florida* (Cf)**	f1	Medicinal herb garden of Kanazawa University, Japan	F110526	Oct. 2011
***C. ianthina* (Ci)**	i1	Gwangneung, Gyeonggi-do, Korea	75110	Oct. 1975

The abbreviation used for each taxon is written in brackets ( ).

Crude drug materials

Sample No.	Purchase site/Source	Voucher No.	Production site	Collection date
w1	Tochimototenkaido Co., Ltd., Osaka, Japan	7535	Liaoning Prov., China	Apr. 2009
w2		7536	Anhui Prov., China	Apr. 2009
w3	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	7690	China	Oct. 2008
w4		7705	China	Apr. 2009
w5	Shanghai market, China	7709	Hebei Prov., China	Feb. 2010
w6		7708	Jiangxi Prov., China	Feb. 2010
w7	Ningde city market, Fujian, China	7950	Unknown	Feb. 2011
w8	Jingdezhen city market, Jiangxi, China	7952	Dongbei Dist., China	Feb. 2011
w9	Dong myong drug company, Seoul, Korea	7620	Korea	Dec. 2009
w10		7616	China	Dec. 2009
w11	Seoul market, Korea	7698	China	May 2010

Extraction of Total DNA: About 50-70 mg of dried leaves or crude drug pieces were frozen in liquid nitrogen and ground into powder. Total DNA was extracted from the powdered materials using the DNeasy Plant Mini Kit (QIAGEN, Germany), according to the manufacturer's protocol. After the extraction, the colored samples were treated with the Gene Clean II kit to remove their impurity (QBioGene, USA).

PCR amplification: The samples' ITS regions were amplified by PCR using 100-120 ng of the extracted total DNA as a template in a 25 μl 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 polymerase (TOYOBO, Japan), and 0.4 μM of each primer pair. Akebi-F (GCT CCT ACC GAT TGA ATG GT) and Akebi-26SR[17] (GTA AGT TTC TTC TCC TCC GC) were used as a standard PCR primer pair. Cle-1F (AAG TCG TAA CAA GGT TTC CG) and Cle-1R (TTA AAC TCA GCG GGT AGT CC) were also used as an inner pair of nested primers when nested PCR was necessary for the degraded DNA from old herbariums or crude drug samples.

The ITS region PCR was performed in the following conditions: 2 min at 94℃; 30 cycles of 15 s denaturation at 94℃, 30 s annealing at 55℃, and 45 s extension at 68℃; followed by a final extension step at 68℃ for 5 min.

Three microliters of the PCR products were analyzed in agarose gel electrophoresis, and the remaining products were purified with the QIA quick PCR Purification Kit (QIAGEN).

Sequencing procedure: The purified PCR products were subjected to sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. The Cle-1F, Cle-1R, Unc-5.8F (GCA TCG ATG ATG AAG AAC GTA GC), and Unc-5.8R[18] (GTT CAA AGA CTC GAT GGT TC) primers were used for sequencing reactions. The resultant DNA sequences were aligned using the DNASIS (version 3.0) software program (Hitachi, Japan), and a phylogenetic tree was constructed by the Neighbor-Joining (NJ) method in MEGA 5.10 with the Kimura 2-parameter model after aligning the sequence data with ClustalX v. 1.83.

ITS cloning procedure: Samples c1 and c2 of *C. chinensis* (Cc) were subjected to the cloning procedure. The ITS regions of the extracted total DNA were amplified by PCR with tagged primer sets; i.e., HD-Cle1F (TAA AAA GCT TAA GTC GTA ACA AGG TTT CCG) and ER-Cle1R (TTA TGA ATT CTT AAA CTC AGC GGG TAG TCC). After being purified, 100-200 ng of the PCR products were digested with *Hind* III and *Eco*R I (TaKaRa Bio. Inc., Japan) in a 10 μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, and 50 mM NaCl at 37℃

for 1h. Then, the reaction was terminated by heating the reaction mixture at 70℃ for 15 min. Four microliters of the reaction mixture and 5 ng of the pBluescript SK (-) plasmid, which was digested with *Hind* III and *Eco*R I, were mixed with 5 μl of the ligation solution from the DNA Ligation Kit Ver.2.1 (TaKaRa Bio. Inc.), then reacted overnight at 16℃. Competent high DH5α cells (TOYOBO) were then transformed with these ligated plasmids according to the manufacturer's protocol. The transformed cells were spread onto plates of lysogeny broth (LB) agar-Amp (1% yeast extract, 2% tryptone, 2% NaCl, 3% agar, and 100 μg/ml ampicillin) and incubated at 37℃ overnight. Each bacterial colony was collected for sub-culturing in LB-Amp liquid medium for 25 h. The resultant bacteria were collected after centrifugation, and the plasmids were obtained according to the standard protocol.

The plasmid DNA was isolated from the DH5α cells by alkaline lysis, treated with RNase A, and precipitation with polyethylene glycol. The sediments were then used as a template for amplifying the ITS region, and the resultant PCR products were purified and subjected to sequencing.

Results

Comparison of the ITS sequences of the *Clematis* plants

(1) Sequence length of the ITS region

Our results showed that the ITS regions of the 9 *Clematis* taxa varied in length (Table 3). *C. patens* (Cp) and *C. florida* (Cf) displayed the longest ITS regions among the 9 plant taxa (562 bp). The ITS regions of *C. hexapetala* (Ch) and *C. mandshurica* (Cm) were 551 bp long. The ITS sequences of *C. terniflora* var. *robusta* (Ctr), *C. terniflora* var. *koreana* (Ctk), and *C. brachyura* (Cb) were all of 547 bp while that of *C. ianthina* (Ci) were of 532 bp.

Samples of *C. chinensis* (Cc) were subjected to cloning because they showed ambiguous sequencing results involving overlapping sequences. As a result, 3 main types of ITS sequence containing different nucleotide deletions were found in Cc. All 3 sequence types were found in the 8 clones from sample c2, and two of them were found in the 16 clones from sample c1. The type 1 sequences were of 549 bp (2 clones from c2) or 548 bp (1 clone from c1), while the type 2 sequences were of 547 bp (2 clones from c2 and 9 clones from c1) or 546 bp (6 clones from c1), and the type 3 sequences were of 551 bp. Type 3 was found in c2 but not in c1.

(2) Nucleotide differences in the ITS sequences of the *Clematis* plant

According to the results shown in Table 3, deletions around nucleotide position 60 were the main cause of the differences in length among the 9 *Clematis* taxa. Unlike Cp and Cf, seven taxa possessed deletions from positions 60 to 70 and positions 551 to 555, whereas deletions at positions 317, 544, 545, 560, and 561 were only found in Cp and Cf. Moreover, a deletion from positions 71 to 74 was found in Ctr, Ctk, Cb and the types 2 and 3 of Cc, whereas a 4 bp insertion from position 165 to 168 was observed only in the type 3 of Cc. Unlike the types 2 and 3 of Cc, the type 1 of Cc did not possess any deletions at positions 73 and 74. However, another deletion was found at position 485 in type 1 of Cc and in 6 clones from sample c1 belonging to type 2.

Both Ch and Cm possessed deletions from position 60 to 70, but no deletions from position 71 to 74. However, the ITS1 sequence of Cm exhibited nucleotides differences from that of Ch at positions 74, 79, 80, 143, 151, and 159 in all samples and at positions 33, 83, and 99 in some samples, while they shared common 5.8S rDNA and ITS2 sequences.

Ctr, Ctk, and Cb all possessed deletions at positions 60-74, and Ctk had 8 unique nucleotides at positions 83, 150, 155, 182, 265, 295, 344, and 373. Moreover, Cb was distinguished from Ctr by 8 nucleotide substitutions at positions 33, 57, 76, 77, 151, 159, 526, and 528.

Cp was differentiated from Cf by nucleotide variations at positions 138 (A and G) and 146 (G and A).

Thus, the 8 *Clematis* taxa mentioned above could be successfully discriminated according to their nucleotide substitutions.

The variable sequencing results of the clones from samples c1 and c2 demonstrated the genetic complexity of Cc. Three main types of ITS sequences were found in this species, as mentioned above. Among the 8 clones of sample c2, 2, 2, and 4 clones belonged to types 1, 2, and 3, respectively. Of the 16 clones of sample c1, one clone belonged to type 1, and the other 15 clones were classified as type 2. Moreover, among the same type, a few

nucleotide differences were noticed between samples c1 and c2 or even within c1 or c2. For example, the type 1 sequence observed in c2 differed from that in c1 at positions 150, 155, 159, 171, and 485. Furthermore, among the type 2 sequences in c1, nucleotide variations were found at 16 positions including positions 57 and 75. Interestingly, we obtained evidence that the ITS sequences of our Cc samples had undergone hybridization, and none of ITS sequences observed in clones from Cc were identical to the sequences of Cc registered in DDBJ. In addition, clone No. 2 from sample c1 possessed exactly the same sequence as Ctk throughout its whole ITS region, which showed characteristic nucleotides at positions 265, 295, 344, and 373.

Ci had a number of nucleotide differences from other 8 taxa in the ITS sequence. Although the sequence of Ci was not shown in Table 3, it was registered in DNA Data Bank of Japan (DDBJ) with the accession number AB773869.

A phylogenetic tree of the 9 *Clematis* taxa was constructed based on our ITS sequencing results (Fig 1). Ctk and clones from Cc except 3 clones with a same sequence (No. 5, 8 and 16) formed one subclade, which was closely related to another subclade composed of Cm, Ch, Ctr, Cb and the 3 clones from Cc. Both subclades formed a large clade, which was separate from another clade of Cf and Cp. Meanwhile, Ci was distantly related to all of 8 taxa.

Table 3 Variations in the ITS regions of the plant materials

Species	Sample No.	Clone No.	DDBJ Accession No.	Nucleotide No. ITS1																						Length of ITS1	TYPE
				12	33	57	59	60-80	83	99	127, 128	138	143, 144	146	150, 151	155, 156	159	162	165-168	171, 172	182	185					
			GU732597	C	A	A	C	----------TTAGCGGGGA	T	C	TA	A	GA	G	GT	AG	G	A	----	AA	C	C					
Clematis hexapetala (Ch)	h1, h2, h3, h4		AB775144	*	*	*	*	----------**********	*	*	**	*	**	*	**	**	*	*	----	**	*	*				172	
	h5		AB775145	*	*	*	*	----------**********	*	*	**	*	**	*	**	**	*	*	----	**	*	*					
	h6, h7		AB775146	*	*	*	*	----------**********	*	*	**	*	**	*	**	**	*	*	----	**	*	*					
			JF778872	*	T	*	*	----------***A****TC	*	*	**	*	A*	*	*C	**	A	*	----	**	*	*					
	m1		AB775147	*	*	*	*	----------***R****TC	Y	*	**	*	A*	*	*C	**	A	*	----	**	*	*					
C. mandshurica (Cm)	m2		AB775148	*	*	*	*	----------***R****KM	*	*	**	*	R*	*	*Y	**	R	*	----	**	*	*				172	
	m3		AB775149	*	*	*	*	----------***R****KM	Y	Y	**	*	R*	*	*Y	**	R	*	----	**	*	*					
	m4		AB775150	*	W	*	*	----------***A****TC	Y	*	**	*	A*	*	*C	**	A	*	----	**	*	*					
	m5		AB775151	*	*	*	*	----------***A****TC	*	*	**	*	A*	*	*C	**	A	*	----	**	*	*					
			GU732584	T	*	*	*	----------*CC*TC	C	*	**	*	A*	*	**	**	A	*	----	**	*	*				168	
		15	AB775161	*	*	*	*	----------TTG*T*TC	C	*	**	*	A*	*	TC	G*	A	*	----	**	G	*				170	TP1
		7, 10, 12	AB775162	*	*	*	*	----------TT**TC	C	*	**	*	A*	*	TC	**	A	*	----	**	G	*					
		2	AB775163	*	*	*	*	----------TT**TC	C	*	**	*	A*	*	TC	G*	A	*	----	**	G	*					
		1, 6, 14	AB775164	*	*	*	*	----------*C**TC	C	*	**	*	A*	*	TC	G*	A	*	----	**	G	*					
	c1	13	AB775165	*	*	*	*	----------*C**TC	C	*	**	*	**	*	TC	**	A	*	----	**	G	*					
		4	AB775166	*	*	*	*	----------*C**TC	C	*	**	*	**	*	TC	**	A	*	----	**	G	*				168	TP2
C. chinensis (Cc)		9	AB775167	*	*	*	*	----------*C**TC	C	*	**	*	A*	*	TC	G*	A	*	----	**	G	*					
		3	AB775168	*	*	*	*	----------*C**TC	C	*	**	*	**	*	TC	**	A	*	----	**	G	*					
		11	AB775169	*	*	*	*	----------*C**TC	C	*	**	*	**	*	TC	**	A	*	----	**	G	*					
		5, 8, 16	AB775170	*	*	G	*	----------*T**TC	*	*	**	*	A*	*	*C	**	A	*	----	**	G	*					
		4, 8	AB775171	*	*	*	*	----------TTG*T*TC	C	*	**	*	A*	*	*C	**	*	*	----	G*	G	*				170	TP1
	c2	1	AB775172	*	*	*	*	----------*T**TC	C	*	**	*	A*	*	TC	**	A	*	----	**	G	*				168	TP2
		6	AB775173	*	*	*	*	----------*T**TC	C	*	**	*	A*	*	TC	**	A	*	----	**	G	*					
		2, 3, 5, 7	AB775174	*	*	*	*	----------*T**TC	C	*	**	*	A*	*	TC	GA	A	*	GACC	**	G	*				172	TP3
			AB120183	*	T	*	*	----------*CC*TC	*	*	**	*	A*	*	**	**	*	*	----	**	*	*					
	tr1		AB775152	*	W	*	*	----------*CS*TC	*	*	**	*	A*	*	*Y	**	R	*	----	R*	*	*					
	tr2		AB769959	*	T	*	*	----------YCC*TC	*	*	**	*	A*	*	**	**	*	*	----	R*	*	*					
C. terniflora var. *robusta* (Ctr)	tr3		AB775153	*	T	*	*	----------*CC*TC	*	*	**	*	A*	*	**	**	*	*	----	**	*	*				168	
	tr4		AB775154	*	T	*	*	----------YCS*TC	*	*	**	*	A*	*	**	**	*	*	----	**	*	*					
	tr5		AB775155	*	T	*	*	----------YCC*TC	*	*	**	*	A*	*	**	**	*	*	----	G*	*	*					
	tr6		AB775156	*	T	*	*	----------YCC*TC	*	*	**	*	A*	*	**	**	*	*	----	**	*	*					
	tr7		AB775157	*	T	*	*	----------TCC*TC	*	*	**	*	A*	*	**	**	*	*	----	**	*	*					
C. terniflora var. *koreana* (Ctk)	tk1, tk2		AB769960	*	*	*	*	----------TT**TC	C	*	**	*	A*	*	TC	G*	A	*	----	**	G	*				168	
C. brachyura (Cb)			AB120204	*	*	G	*	----------*T**TC	*	*	**	*	A*	*	*C	**	A	*	----	**	*	*				168	
	br1, br2		AB775158	*	*	G	*	----------*T**TC	*	*	**	*	A*	*	*C	**	A	*	----	**	*	*					
C. patens (Cp)			AB120184	A	*	*	G	CGCCGCCCCGGCGGCGC*CTC	C	T	CG	*	AC	*	**	**	*	G	----	*G	T	T				183	
	p1, p2		AB775159	A	*	*	G	CGCCGCCCCGGCGGCGC*CTC	C	T	CG	*	AC	*	**	**	*	G	----	*G	T	T					
C. florida (Cf)			AB120186	A	*	*	G	CGCCGCCCCGGCGGCGC*CTC	C	T	AG	G	AC	A	**	**	*	G	----	*G	T	T				183	
	f1		AB775160	A	*	*	G	CGCCGCCCCGGCGGCGC*CTC	C	T	AG	G	AC	A	**	**	*	G	----	*G	T	T					

The nucleotide numbers refer to sequence AB120204 in the DNA Data Bank of Japan (DDBJ). Asterisks (*) indicate the same nucleotide as the top sequence; hyphens (-) indicate nucleotide deletions; W denotes A or T, R denotes A or G, and Y denotes C or T. GU732597, JF778872, GU732584, AB120183, AB120204, AB120184, and AB120186 were obtained from the DDBJ. For each species, we chose the ITS sequence closest to our result if two or more ITS sequences were recorded for the species in the DDBJ. All sequences we obtained in this study were recorded in DDBJ. Abbreviations: Ch: *Clematis hexapetala*; Cm: *C. mandshurica*, Cc: *C. chinensis*, Ctr: *C. terniflora* var. *robusta*, Ctk: *C. terniflora* var. *koreana*, Cb: *C. brachyura*, Cp: *C. patens*, Cf: *C. florida*, and TP: type.

Table 3 Continued

Species	Sample No.	Clone No.	Nucleotide No. 5.8S rDNA 265	288	295	314	317, 318	331	333, 334	344	Length of 5.8S rDNA	Nucleotide No. ITS2 373	411	421	485	493	502, 503	521	525, 526	528	544, 545	551-555	560, 561	564	Length of ITS2	Length of ITS	TYPE
Ch	GU732597		C	C	C	C	TT	C	TC	G		C	C	C	C	G	CG	A	GT	A	CC	-----	GG	T			
	h1, h2, h3, h4		*	*	*	*	**	*	**	*	159	*	*	*	*	*	**	*	**	*	**	-----	**	*	220	551	
	h5		*	*	*	*	**	*	**	*		*	*	Y	*	*	**	*	**	*	**	-----	**	*			
	h6, h7		*	*	*	*	**	*	**	*		*	*	Y	*	*	**	*	**	*	**	-----	**	Y			
Cm	JF778872		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	*			
	m1		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	Y			
	m2		*	*	*	*	**	*	**	*	159	*	*	Y	*	*	**	*	**	*	**	-----	**	*	220	551	
	m3		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	*			
	m4		*	*	*	*	**	*	**	*		*	*	Y	*	*	**	*	**	*	**	-----	**	*			
	m5		*	*	*	*	**	*	**	*		*	*	Y	*	*	**	*	**	*	**	-----	**	*			
Cc	GU732584		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	C	220	547	
		15	*	*	*	*	**	*	**	*		*	*	*	_	*	**	*	**	*	**	-----	**	C	219	548	TP1
		7, 10, 12	*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	C			
		2	T	*	T	*	**	*	**	A		A	*	*	*	*	**	*	*C	G	**	-----	**	C			
		1, 6, 14	*	*	*	*	**	*	**	*		*	*	*	*	*	T*	*	*C	G	**	-----	**	C	220	547	
	c1	13	*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	*C	G	**	-----	**	C			
		4	*	*	*	*	**	*	**	*		*	*	*	*	T	**	*	**	*	**	-----	**	C			TP2
		9	*	*	*	*	**	*	**	*	159	*	*	*	_	T	**	*	**	*	**	-----	**	C			
		3	*	*	*	*	**	*	**	*		*	*	*	_	T	**	*	**	*	**	-----	**	C	219	546	
		11	*	*	*	*	**	*	**	*		*	*	*	_	*	**	*	**	*	**	-----	**	C			
		5, 8, 16	*	*	*	*	**	*	**	*		*	*	*	_	*	**	*	**	*	**	-----	**	C			
		4, 8	*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	C	220	549	TP1
		1	*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	C	220	547	TP2
	c2	6	*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	*C	G	**	-----	**	C			
		2, 3, 5, 7	*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	C	220	551	TP3
Ctr	AB120183		*	T	*	T	**	A	*T	*		*	*	*	*	*	**	*	AC	G	**	-----	**	*			
	tr1		*	*	*	*	**	*	**	*		*	*	Y	*	*	**	*	*Y	R	**	-----	**	*			
	tr2		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	RC	G	**	-----	**	*			
	tr3		*	*	*	*	**	*	**	*	159	*	*	*	*	*	**	*	RC	G	**	-----	**	*	220	547	
	tr4		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	RC	G	**	-----	**	*			
	tr5		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	RC	G	**	-----	**	*			
	tr6		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	*C	G	**	-----	**	*			
	tr7		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	*C	G	**	-----	**	*			
Ctk	tk1, tk2		T	*	T	*	**	*	**	A	159	A	*	*	*	*	**	*	*C	G	**	-----	**	C	220	547	
Cb	AB120204		*	*	*	*	**	*	**	*	159	*	*	*	*	*	**	*	**	*	**	-----	**	*	220	547	
	br1, br2		*	*	*	*	**	*	**	*		*	*	*	*	*	**	*	**	*	**	-----	**	*			
Cp	AB120184		*	*	*	*	-C	*	C*	*	158	*	A	*	*	*	**	*	**	*	--	ACCAC	--	C	221	562	
	p1, p2		*	*	*	*	-C	*	C*	*		*	A	*	*	*	**	*	**	*	--	ACCAC	--	C			
Cf	AB120186		*	*	*	*	-C	*	C*	*	158	*	A	*	*	*	*A	R	**	*	--	ACCAC	--	C	221	562	
	f1		*	*	*	*	-C	*	C*	*		*	A	*	*	*	**	*	**	*	--	ACCAC	--	C			

In conclusion, *Clematis* plants examined in this study were successfully divided into 9 taxa according to their ITS1, 5.8S rDNA, and ITS2 nucleotide sequences. Furthermore, three types of ITS sequences were observed in *C. chinensis* through DNA cloning.

Identification of the crude drug samples by ITS sequence

Among the 11 Clematidis Radix samples collected from Japanese, Chinese, and Korean markets (w1-11), 7 samples covering all 3 countries displayed ITS sequences identical or almost identical to Cm, and so were deduced to be derived from *C. mandshurica*. Although some of these samples displayed nucleotide differences compared with plant sample m4 of Cm at some positions as shown in Table 4, e.g., at position 33, the same variations were found at the corresponding sites in other Cm plant samples. Similarly, w9 was identified as *C. terniflora* var. *robusta*, while w7 and w8 were identified as *C. brachyura.*

The sequencing results obtained from crude drug sample w2 were unclear, as were those observed in samples c1 and c2 of Cc, due to the presence of overlapping sequences. However, we detected the type 3 sequence of Cc in w2 by extracting the higher signals from relatively readable sequence profiles. Moreover, the macroscopic characteristics of the underground parts of this crude drug and the black dried leaves[1)] found in the packet of w2 also supported the identification that this sample was derived from *C. chinensis*.

The above-mentioned results were summarized as follows: Three out of 4 samples from the Japanese

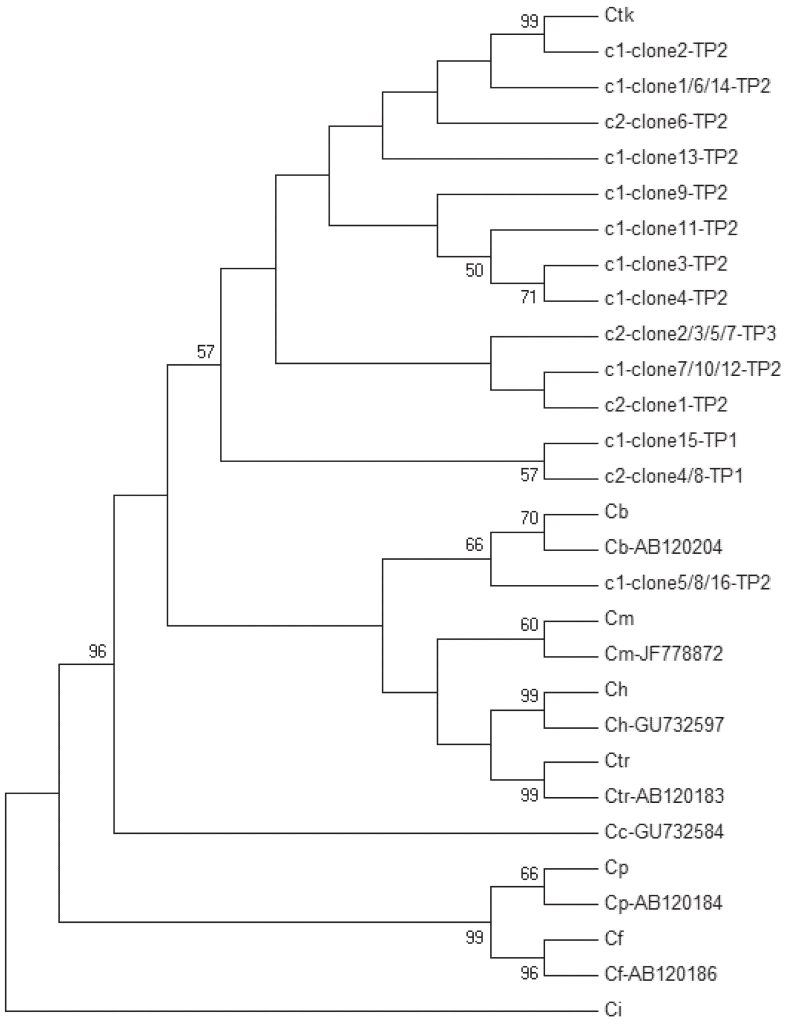

Fig. 1 Phylogenetic tree constructed based on the ITS sequence results of 9 *Clematis* taxa
The sequence with the smallest nucleotide variations in each taxa and all clones of Cc samples c1 and c2 obtained in this study were used to construct the phylogenetic tree. The number of the clone of Cc and its clone type is shown after the sample names c1 and c2. AB120204, JF778872, GU732597, AB120183, GU732584, AB120184, and AB120186 were obtained from the DDBJ. These sequences were comparatively similar to our sequences of corresponding taxa. Numbers beside the branches indicate bootstrap values (≥50%) of 1000 replications. Abbreviations: Ctk: *C. terniflora* var. *koreana*, Cc: *C. chinensis*, Cb: *C. brachyura*, Cm: *C. mandshurica*, Ch: *C. hexapetala*, Ctr: *C. terniflora* var. *robusta*, Cp: *C. patens*, Cf: *C. florida*, Ci: *C. ianthina*, and TP: type.

Table 4 Comparison of the ITS sequences of Clematidis Radix and plant materials

Sample No.	Nucleotide No. of ITS																		Market	Producing area	Results of identification
	ITS1														ITS2						
	33	57	71-77	79, 80	83	99	143	150, 151	155, 156	159	165-168	171	182	421	525, 526	528	564				
Ch (h1)	A	A	TTAGCGG	GA	T	C	G	GT	AG	G	----	A	C	C	GT	A	T				
Cm (m4)	W	*	***A***	TC	Y	*	A	*C	**	A	----	*	*	Y	**	*	*				
w1	*	*	***A***	TC	Y	Y	A	*Y	**	A	----	*	*	*	**	*	*	Japan	Liaoning, China	*C. mandshurica*	
w3	*	*	***A***	TC	Y	*	A	*C	**	A	----	*	*	*	**	*	*	Japan	China	*C. mandshurica*	
w4	W	*	***A***	TC	Y	*	A	*C	**	A	----	*	*	Y	**	*	*	Japan	China	*C. mandshurica*	
w5	*	*	***A***	TC	Y	*	A	*C	**	A	----	*	*	Y	**	*	*	Shanghai, China	Hebei, China	*C. mandshurica*	
w6	W	*	***A***	TC	Y	Y	A	*Y	**	A	----	*	*	*	**	*	*	Shanghai, China	Jiangxi, China	*C. mandshurica*	
w10	*	*	***A***	TC	Y	*	A	*C	**	A	----	*	*	*	**	*	*	Korea	China	*C. mandshurica*	
w11	*	*	***A***	TC	Y	*	A	*Y	**	A	----	*	*	*	**	*	Y	Korea	China	*C. mandshurica*	
Ctr (tr2)	T	*	----YCC	TC	*	*	A	**	**	*	----	R	*	*	RC	G	*				
w9	T	*	----YCC	TC	*	*	A	**	**	*	----	*	*	*	RC	G	*	Korea	Korea	*C. terniflora* var. *robusta*	
Cb (b1)	*	G	----*T*	TC	*	*	A	*C	**	A	----	*	*	*	**	*	*				
w7	*	G	----*T*	TC	*	*	A	*C	**	A	----	*	*	*	**	*	*	Fujian, China	Unknown	*C. brachyura*	
w8	*	G	----*T*	TC	*	*	A	*C	**	A	----	*	*	*	**	*	*	Jiangxi, China	Dongbei, China	*C. brachyura*	
Cc (c2-clone2)	*	*	----*T*	TC	C	*	A	TC	GA	A	GACC	*	G	*	**	*	C				
w2	*	*	----*T*	TC	C	*	A	TC	GA	A	GACC	*	G	*	**	*	C	Japan	Anhui, China	*C. chinensis*	

The nucleotide numbers refer to sequence AB120204 in the DDBJ. Asterisks (*) indicate the same nucleotides as the top sequence; hyphens (-) indicate nucleotide deletions; W denotes A or T, R denotes A or G, and Y denotes C or T. Abbreviations: Ch: *Clematis hexapetala*; Cm: *C. mandshurica*, Ctr: *C. terniflora* var. *robusta*, Cb: *C. brachyura*, Cc: *C. chinensis*. The number of samples used in the comparisons with the crude drugs is written within the brackets ( ).

markets were categorized as *C. mandshurica*, and the rest as *C. chinensis*; 2 out of 4 samples from the Chinese markets were identified as *C. mandshurica*, and the other two as *C. brachyura*; and of the 3 samples from Korean markets, two were identified as *C. mandshurica,* and the other as *C. terniflora* var. *robusta.*

Discussion

1. Ch and Cm, as well as Cp and Cf, could not be differentiated using their ITS2 sequences, even though Zeng *et al*.[21] reported that ITS2 could be used to identify Clematidis Radix. Our results showed that both ITS2 and ITS1 sequences are necessary for the differentiation of *Clematis* species. In addition, we detected more variations in the ITS1 sequence than in the ITS2 sequence of *Clematis* plants.

2. The ITS sequences of the Cc samples we examined were considerably different from those recorded in the DDBJ; in other words, all of our Cc samples had unclear overlapping sequences, indicating that Cc might be of hybrid origin. The overlapping always began around position 60. In addition, most of the other species also showed deletions around the same position (Table 3). Thus, it seems that the nucleotides in the region around position 60 are easily alterable and that this area might be key for the differentiation of *Clematis* species. Furthermore, the high similarity between the ITS sequences of clone No. 2 from sample c1 of Cc and Ctk indicates that Ctk might be an ancestor of the Cc hybrid.

3. The phylogenetic tree showed that Cb is closely related to the branch containing Cm and Ctr. Wang[22] reported that Cb might derived from the sect. *Clematis*, which includes Cm and Ctr, because Cb grows in similar habitats and exhibits similar flower structures to both species. Our genetic results supported this opinion.

4. Both Cp and Cf have been classified into sect. *Viticella* of the genus *Clematis*, and herbological studies have suggested that they were used as sources of Clematidis Radix in ancient times.[23,24] However, they were hardly found in the modern markets. In fact, both species, having big beautiful flowers, are endangered in these days because they have been excessively collected from the wild due to their ornamental value.

5. As for the locations where the market samples were produced, all 7 Clematidis Radix samples identified as *C. mandshurica* were produced in China. Of the 7 samples, one was labeled as being produced in Liaoning, while the other 6 did not provide any information on their areas of producition. No Korean produced Cm-derived Clematidis Radix was obtained from the markets visited in the present study, although such products were found in Korean markets in the 1970s.[14] In addition, it is known that some of the Clematidis Radix sold in Chinese markets is produced in North Korea. In our study, 2 samples derived from *C. brachyura*, a unique species growing only in the Korean Peninsula, were found in Chinese markets; one was labeled as being produced in northeast China, and no information was provided for the other one. Both samples were thought to have been imported from North Korea because this species does not grow in China. As for the 2 samples identified as Cm, whose labels stated that they were produced in Hebei and Jiangxi, we supposed that these descriptions were also incorrect considering the distribution of this species. Such mistakes can easily occur when crude drugs circulate in big markets, where they are assembled and divided repeatedly.

6. In this study, we did not find any Clematidis Radix derived from *C. hexapetala*, one of the three officially sanctioned sources of Clematidis Radix, although they were found in Chinese markets in the 1970s.[15] Thus, Clematidis Radix derived from *C. hexapetala* might be rare in modern markets. One possible reason for this is the considerably different morphology of this species: i.e., both *C. mandshurica* and *C. chinensis* have 4 perianth lobes and ovate leaflets while *C. hexapetala* has 5 to 7 perianth lobes and linear lanceolate leaflets. Moreover, the roots of the latter species are much thinner than those of the former two.

7. The Japanese produced Ireisen derived from *C. terniflora* var. *robusta* was sold in Japanese markets in the 1970s.[12] However, we did not find any Japanese produced Ireisen in the modern markets, and all of the Ireisen samples circulated in Japan were imported from China and belonged to *C. mandshurica* or *C. chinensis*. The latter sample was produced in Anhui, one of the main habitats of Cc.

8. The Wiryongseon (the Korean name for Clematidis Radix) sold in Korean markets has been reported to be mainly derived from Cb or, rarely from Cm or Ctk.[14] In

addition to these taxa, Ctr-derived Wiryongseon produced in Korea was also found in this study. This variety grows in the southern part of the Korean Peninsula. Since the Korean Pharmacopeia describes Wiryongseon as the roots of *C. mandshurica* Maxim. (*C. mandshurica* Rupr.) or other species of the same genus, this variety can also be used as Wiryongseon.

References

1) Nanjing University of Chinese medicine: Great Dictionary of Chinese Medicine, Shanghai People's Publishing House, Shanghai, pp. 2274-2278, 2006.
2) Didry, N., Dubreuil, L. and Pinkas, M.: Antibacterial activity of vapours of protoanemonin. *Pharmazie,* **46**, 546-547, 1991.
3) Zhang, Y.Y., Zhang, H.W., Li, P.F., Chen, B.L. and Fu, J.: Antispasmodic, anti-inflammatory and analgesic effect of *Clematis, Chin. Trad. Patent Med.,* **23**, 808-811, 2001. (Abstr. in English)
4) Zhou, X.S. and Yi, D.B.: Studies on the analgesic and anti-inflammatory effects of *Clematis chinensis* Osbeck in mice and rats. *Chin. Clin. Med.,* **4**, 12-13, 2003. (Abstr. in English)
5) Qiu, G.Q., Zhang, M. and Yang, Y.J.: The anti-tumor activity of total saponin of *Clematis chinensis. Chin. Med. Mat.,* **22**, 351-353, 1999. (Abstr. in English)
6) Chinese Pharmacopoeia Commission: Pharmacopoeia of the Peoples's Republic of China (Chinese Edition), vol. 1, Chemical Industry Press, Beijing, pp. 234-235, 2010.
7) Ministry of Health, Labour and Welfare: The Japanese Pharmacopoeia 16th Edition. Hirokawa Publishing Co., Tokyo, pp. 1449-1450, 2011.
8) Xie, Z.W.: Discussion of Varieties of Traditional Chinese Medicine. Shanghai Science and Technology Press, Shanghai, pp. 221-228, 1990.
9) Mimaki, Y., Yokosuka, A., Hamanaka, M., Sakuma, C., Yamori, T. and Sashida, Y.: Triterpene Saponins from the Roots of *Clematis chinensis. J. Nat. Prod.,* **67**, 1511-1516, 2004.
10) Shi, S.P., Jiang, D., Dong, C.X. and Tu, P.F.: Triterpene Saponins from *Clematis mandshurica. J. Nat. Prod.,* **69**, 1591-1595, 2006.
11) Dong, C.X., Wu, K.S., Shi, S.P. and Tu, P.F.: Flavanoids from *Clematis hexapetala. J. Chin. Pharm. Sci.,* **15**, 15-20, 2006.
12) Namba, T. and Mikage, M.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (I): Histological Studies on the Roots of *Clematis terniflora* DC. var. *robusta* (CARR.) TAMURA and "Wei-ling-xian" from Japan. *Shoyakugaku Zasshi,* **37**, 307-316, 1983.
13) Mikage, M. and Namba, T.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (II): On the Botanical Origin of "Wei-ling-xian" from Taiwan and Liang-guang. *Shoyakugaku Zasshi,* **37**, 317-324, 1983.
14) Mikage, M. and Namba, T.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (III): On "Wei-ling-xian" from Korean Peninsula. *Shoyakugaku Zasshi,* **37**, 325-333, 1983.
15) Mikage, M. and Namba, T.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (IV): On the Botanical Origin of "Wei-ling-xian" from Northeast China. *Shoyakugaku Zasshi,* **37**, 334-341, 1983.
16) Mikage, M. and Namba, T.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (V): On the Botanical origins of "Wei-ling-xian" from Central Taiwan and the Province of Fuchien in Continental China. *Shoyakugaku Zasshi,* **37**, 342-350, 1983.
17) Kitaoka, F., Kakiuchi, N., Long, C.F., Itoga, M., Mitsue, A., Mouri, C. and Mikage, M.: Molecular Characterization of *Akebia* Plants and the Derived Traditonal Herbal Medicine. *Biol. Pharm. Bull.,* **32**, 665-670, 2009.
18) Shi, W., Kitaoka, F., Doui, M., Miyake, K., Sasaki, Y., Kakiuchi, N. and Mikage, M.: Quality evaluation of *Chotoko* - Local and specific variations in the alkaloid contents of *Uncaria* plants -. *J. Trad. Med.,* **29**, 63-73, 2012.
19) Tamura, M.: A classification of genus *Clematis. Acta Phytotax. Geobot.,* **38**, 33-44, 1987.
20) Wang, W.T. and Li, L.Q.: A new system of classification of the genus *Clematis* (Ranunculaceae). *Acta Phytotax. Sin.,* **43**, 431-488, 2005.
21) Zeng, X., Li, L., Ye, N., Yao, H., Liu, Z.H. and Qin, M.J.: Molecular identification of radix et rhizoma clematis and its adulterants based on ITS2 DNA barcode. *Glob. Trad. Chin. Med.,* **4**, 264-269, 2011. (Abstr. in English)
22) Wang, W.T.: A revision of *Clematis* sect. *Pterocarpa*

(Ranunculaceae). *Guihaia,* **26**, 341-344, 2006.

23) Mikage, M. and Namba, T.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (VI): Herbological Studies on “Wei-ling-xian”(1). *Shoyakugaku Zasshi,* **37**, 351-360, 1983.

24) Mikage, M. and Namba, T.: Pharmacognostical Studies on the *Clematis* Plants and Related Crude Drugs (VII): Herbological Studies on “Wei-ling-xian”(2). *Shoyakugaku Zasshi,* **37**, 361-366, 1983.