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Molecular identification of the traditional herbal medicines, Arisaematis Rhizoma and Pinelliae Tuber, and common adulterants via universal DNA barcode sequences

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ABSTRACT. Methods to identify Pinelliae Tuber and Arisaematis Rhizoma are required because of frequent reciprocal substitution between these two herbal medicines and the existence of several closely related plant materials. As a result of the morphological similarity of dried tubers, correct discrimination of authentic herbal medicines is difficult by conventional methods. Therefore, we analyzed DNA barcode sequences to identify each herbal medicine and the common adulterants at a species level. To verify the identity of these herbal medicines, we collected five authentic species (*Pinellia ternata* for Pinelliae Tuber, and *Arisaema amurense*, *A. amurense* var. *serratum*, *A. erubescens*, and *A. heterophyllum* for Arisaematis Rhizoma) and six common adulterant plant species. Maturase K (*mat*K) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) genes were then amplified using universal primers. In comparative analyses of two DNA barcode sequences, we obtained 45 species-specific nucleotides sufficient to identify each species (except *A. erubescens* with *mat*K) and 28 marker nucleotides for each species (except *P. pedatisecta*

with *rbc*L). Sequence differences at corresponding positions of the two combined DNA barcodes provided genetic marker nucleotides that could be used to identify specimens of the correct species among the analyzed medicinal plants. Furthermore, we generated a phylogenetic tree showing nine distinct groups depending on the species. These results can be used to authenticate Pinelliae Tuber and Arisaematis Rhizoma from their adulterants and to identify each species. Thus, comparative analyses of plant DNA barcode sequences identified useful genetic markers for the authentication of Pinelliae Tuber and Arisaematis Rhizoma from several adulterant herbal materials.

Key words: DNA barcode; Maturase K (*mat*K); Pinelliae Tuber; Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L); Arisaematis Rhizoma; Molecular authentication

INTRODUCTION

Pinelliae Tuber and Arisaematis Rhizoma are well-known traditional herbal medicines widely used in East Asian countries including Korea, China, and Japan. Both of these herbal medicines are toxic perennial herbs of the monocot family, Araceae, and are mainly used as antiemetics and analgesics (Dan et al., 2004). During treatments using Asian traditional medicines, both dried tubers are used to treat dampness and transform phlegm, and can be used for phlegm-dampness in the spleen and stomach. Pinelliae Tuber specifically treats spleen and stomach phlegm-dampness while also alleviating nausea and reducing focal distention. However, Arisaematis Rhizoma, which has an acrid flavor, has a far greater dispersing action than does Pinelliae Tuber, and mainly affects liver channel phlegm-dampness. Furthermore, Arisaematis Rhizoma excels in treating wind-phlegm in the channels and collaterals, and also disperses blood and reduces swelling (Maki et al., 1987; Dan et al., 2004; Chen et al., 2012).

In the national pharmacopoeia of Korea, China, and Japan, Pinelliae Tuber is designated as the tuber of *Pinellia ternata* only; however, other *Pinellia* species, as well as some additional species, including those belonging to *Typhonium* and *Arisaema* species, are commonly misused as this herbal medicine (Korea Institute of Oriental Medicine, 2015). In particular, tubers of *Pinellia tripartita*, *Pinellia pedatisecta*, *Arisaema erubescens*, *Arisaema yunnanense*, and *Typhonium flagelliforme* are commonly distributed as Pinelliae Tuber in medicinal markets because of the similarity of the dried tubers of these plants to those of *P. ternata*, as well as their short growing period and high production yield compared to *P. ternata* (Lin et al., 2006; Cui et al., 2008; Liu and Guo, 2010). Arisaematis Rhizoma, another herbal medicine with similar morphological characteristics to those of dried Pinelliae Tuber, is described as the tuberous rhizome of only three plant species, *Arisaema amurense*, *A. erubescens*, and *Arisaema heterophyllum*, in the national pharmacopoeia of Korea, China, and Japan (Korea Institute of Oriental Medicine, 2015). However, young tuberous rhizomes of diverse plant species belonging to the other *Arisaema* species have been incorrectly used for centuries as not only Arisaematis Rhizoma but also Pinelliae Tuber (Lin et al., 2006; Liu and Guo, 2010). Previous reports have suggested that there may be seven species and five varieties of *Arisaema* distributed in Korea, although only two species, *A. amurense* and *A. heterophyllum*, are official medicinal ingredients (Ko and Kim, 1985; Korea Institute of Oriental Medicine, 2015). Thus,

other *Arisaema* species, including *A. takesimense*, *A. serratum*, *A. ringens*, and *A. thumbergii* are unacceptable species for use in herbal medicine. Unfortunately, differentiating between the young tuberous rhizomes of *Arisaema* species and Pinelliae Tuber is difficult when using dried herbal medicines. Thus, as a result of the morphological similarities in the tubers and the lack of visual differences among processed herbal drugs, it is very difficult to determine the original species or adulterants and to distinguish between Pinelliae Tuber and Arisaematis Rhizoma. Therefore, accurate and reliable methods that do not depend on the morphology of the herbal medicine are required to differentiate between authentic and inauthentic herbal materials at a species level.

DNA barcoding has been suggested for use in phylogenetic studies, species identification of diverse flowering plants, and authentication of herbal medicines (Techen et al., 2004; Guo and Ge, 2005; Zhang et al., 2007; Hollingsworth et al., 2011). The development of polymerase chain reaction (PCR)-based methods that use a diverse range of DNA polymerases and sequencing technology has enabled the discrimination of inter- and intra-species differences by comparative sequence analysis of various DNA barcode regions in the chloroplast genome (Kress et al., 2005; Sucher and Carles, 2008). Among the diverse DNA barcode candidates, maturase K (*mat*K) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) are universal plant DNA barcodes that are used by the Consortium for the Barcode of Life (CBOL, 2009). These candidates are widely used as genetic markers to identify plant species and to analyze phylogenetic relationships (Kato et al., 1998; Kress and Erickson, 2007; Saarela et al., 2013). Chloroplast *mat*K and *rbc*L DNA barcode regions have been used to identify species of diverse plant taxa, including herbal materials, and to clarify taxonomic origins (Cabrera et al., 2008; Sucher and Carles, 2008; Guo et al., 2011; Chao et al., 2014). In particular, DNA barcoding can provide objective tools that permit the authentication and identification of herbal medicines, especially for medicines that are often sold as dried slices or powders. However, there are no reports of molecular identification tools (and phylogenetic relationships) used to authenticate official plant species of the important traditional medicines Pinelliae Tuber and Arisaematis Rhizoma.

In this study, we analyzed the sequences of the *mat*K and *rbc*L universal DNA barcode regions to identify 10 plant species and one variety, which have been used as authentic herbal medicines (Pinelliae Tuber and Arisaematis Rhizoma), as well as common adulterants. Their phylogenetic relationships were also elucidated. In a comparative analysis of the two DNA barcode sequences, we obtained several marker nucleotides sufficient to authenticate each species and clarified the phylogenetic relationships of the analyzed plant species. These results provide useful information and tools that can be used to distinguish Pinelliae Tuber and Arisaematis Rhizoma and for the accurate discrimination and authentication of herbal materials and adulterants at a species level.

MATERIAL AND METHODS

Plant materials

A total of 60 genotypes from 10 species and one variety, including seven from *A. amurense*, six from *A. amurense* var. *serratum*, three from *A. erubescens*, six from *A. heterophyllum*, six from *A. takesimense*, seven from *A. serratum*, six from *A. ringens*, five from *P. ternata*, four from *P. tripartita*, six from *P. pedatisecta*, and four from *T. flagelliforme*, were used in the analysis (Table 1). Samples were collected from different native habitats in Korea and China and stored at -70°C after freezing in liquid nitrogen. All plant materials were given accession numbers, and specimens were

preserved in the Korean Herbarium of Standard Herbal Resources (KHSHR) at the Korea Institute of Oriental Medicine. Species identification was performed by the Classification and Identification Committee of the KIOM, which comprises nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology.

Preparation of genomic DNA

Genomic DNA was extracted from fresh leaves stored at -70°C and from herbal medicines using a DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer protocol. DNA concentrations and purities were determined by spectrophotometry (Nanodrop ND-1000, Nanodrop, Wilmington, DE, USA) and 1.5% agarose gel electrophoresis with known standards. For PCR amplification, the final concentration of each DNA sample was approximately 20 ng/μL in TE buffer.

Amplification of DNA barcodes

The *mat*K and *rbc*L genes were amplified using matK AF (5'-CTA TAT CCA CTT ATC TTT CAG GAG T-3') and matK 8R (5'-++AAA GTT CTA GCA CAA GAA AGT CGA-3') primers for ~1.3 kb of the *mat*K region, and rbcL F (5'-ATG TCA CCA CAA ACA GAA ACT AAA GC-3') and rbcL R (5'-TCC TTT TAG TAA AAG ATT GGG CGG AG-3') primers for ~1.5 kb of the *rbc*L region, as previously described (Olmstead and Reeves, 1995; Kato et al., 1998). PCRs were performed in 50-μL reaction solutions containing 10 mM Tris-HCl, pH 9.0, 2.5 mM $MgCl_2$, 200 μM each dNTP, 10 mM $(NH_4)_2SO_4$, 0.5 U *Taq* DNA polymerase (Solgent, Daejeon, Korea), 0.6 μM each primer, and 10-20 ng template DNA. DNA amplification was performed on a DNA Engine Dyad® PTC-0220 (Bio-Rad, Foster City, CA, USA). The parameters used were 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C, and 2 min at 72°C, and a final extension for 10 min at 72°C. PCR products were separated on 1.5% agarose gels with a 1-kb plus DNA ladder (Solgent) and visualized with ethidium bromide staining under ultraviolet light.

Analysis of nucleotide sequences for the identification of marker nucleotides and phylogenetic relationships

The *mat*K and *rbc*L DNA fragments amplified from 60 individual plant samples were retrieved from agarose gels with a Gel Extraction Kit (Solgent) and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Nucleotide sequences of the inserted DNA fragments were determined from both strands via dideoxynucleotide chain termination using an automatic DNA sequence analyzer (ABI 3730, Applied Biosystems Inc., Foster City, CA, USA). The nucleotide sequences of the *mat*K and *rbc*L genes of each species were registered in NCBI GenBank (Table 1).

To identify marker nucleotides that could be used to authenticate each species using *mat*K and *rbc*L gene sequences, the 60 sample sequences were multiple sequences aligned and edited using ClustalW implemented in biological sequence editing software (BioEdit, version 7.2.5) (Hall, 1999). The resulting species-specific indels and substitutions were selected as marker nucleotides from *mat*K and *rbc*L, respectively, and the combination of two DNA barcode regions for the discrimination of each species.

For analysis of sequence variability between and within species, 60 complete sample sequences of *mat*K and *rbc*L were compared using the DNADist DNA distance matrix in the BioEdit software. For the generation of phylogenetic trees, 60 complete *mat*K and *rbc*L sequences were analyzed with the DNADist neighbor-joining and unweighted pair group methods using arithmetic algorithm (UPGMA) methods in BioEdit (Hall, 1999).

RESULTS

Characteristics of DNA barcode regions

Fragments of the *mat*K and *rbc*L genes were successfully amplified at the expected lengths of approximately 1300 and 1500 bp, respectively, in all 60 samples. The sequences of the resulting DNA fragments were determined using the universal vector primers, T7 and SP6, after subcloning into the pGEM-Teasy system, and individual sequences were registered with GenBank (Table 1). The complete *mat*K and *rbc*L genes were 1286 and 1517 bp in length in all of the analyzed samples except for *T. flagelliforme* in which *mat*K was 1292 bp (Table 2). In the comparative alignment of *mat*K sequences, all samples were aligned with a length of 1292 bp, and showed sequence variability that ranged from 0.0000-0.0047 and 0.0000-0.0393 at inter- and intra-species levels, respectively (Table 2). The *rbc*L sequences were aligned in the same length with sequences (1517 bp) showing 0.0000-0.0046 and 0.0000-0.0194 inter- and intra-species sequence variability, respectively (Table 2).

Identification of species-specific marker nucleotides

To determine species-specific sequences for the identification of individual species, a comparative analysis was performed that depended on 60 complete samples from both the *mat*K and *rbc*L gene regions. In the sequence comparisons, nucleotide substitutions that were observed only at an inter-species level were found at 11 positions in *A. erubescens*, 21 positions in *A. heterophyllum*, 8 positions in *A. serratum* and *A. takesimense*, 10 positions in *A. ringens*, 35 positions in *P. ternata*, 38 positions in *P. tripartita*, 34 positions in *P. pedatisecta*, and 44 positions in *T. flagelliforme*, against *A. amurense* and its variety in the maximized alignment of each *mat*K region (Table 2 and **Figure S1**). However, we did not identify any distinct sequence variability between *A. amurense* and *A. amurense* var. *serratum*, and between *A. serratum* and *A. takesimense* at the species level, for both of the DNA barcodes examined. These results strongly support the view that *A. amurense* var. *serratum* and *A. takesimense* is a taxonomic synonym of *A. amurense* and *A. serratum*, respectively, in The Plant List (2013). Therefore, we classified and further analyzed *A. amurense* var. *serratum* and *A. takesimense* as the same species as *A. amurense* and *A. serratum*, respectively.

In the maximized alignment of each *rbc*L region, substitutions were observed in 13, 12, 5, 8, 19, 19, 18, and 23 positions versus *A. amurense*, with respect to the same species order mentioned above for the *mat*K region (Table 2 and **Figure S2**). Of these, species-specific marker nucleotides that could be used to identify each species were obtained at six positions specific to *A. amurense* and its variety, five positions for *A. heterophyllum*, one position for *A. serratum* and *A. takesimense*, two positions for *A. ringens*, three positions for *P. ternata*, five positions for *P. tripartita*, two positions for *P. pedatisecta*, and 22 positions (substitutions) and a 6-bp insertion for *T. flagelliforme* (Table 3 and **Figure S1**).

Table 1. Summary of information regarding plant materials.

Names		Source	Sample name	GenBank accession No.	
Scientific name	Herbal			*mat*K	*rbc*L
Arisaema amurense Maxim.	Arisaematis Rhizoma (*Tian Nan Xing*, *Cheon Nam Seong*)	Gwangyang, Jeonnam, Korea	AA_KY	KT025764	KT025714
		Yangsan, Gyeongnam, Korea	AA_YS	KT025765	KT025715
		Jeju, Jeju, Korea	AA_JJ	KT025766	KT025716
		Tongyeong, Gyeongnam, Korea	AA_TY	KT025767	KT025717
		Inje, Gangwon, Korea	AA_IJ	KT025768	KT025718
		Jangsu, Jeonbuk, Korea	AA_JS	KT025769	KT025719
		Ansan, Gyeonggi, Korea	AA_AS	KT025770	KT025720
Arisaema amurense var. *serratum* Nakai		Wonju, Gangwon, Korea	AAS_WJ	KT025771	KT025721
		Jeju, Jeju, Korea	AAS_JJ	KT025772	KT025722
		Tongyeong, Gyeongnam, Korea	AAS_TY	KT025773	KT024723
		Yeongcheon, Gyeongbuk, Korea	AAS_YC	KT025774	KT025724
		Gongju, Chungnam, Korea	AAS_GJ	KT025775	KT025725
		Ansan, Gyeonggi, Korea	AAS_AS	KT025776	KT025726
Arisaema erubescens (Wall.) Schott		Guigang, Guangxi, China	AE_QS1	KT025777	KT025727
		Guigang, Guangxi, China	AE_QS2	KT025778	KT025728
		Guigang, Guangxi, China	AE_QS3	KT025779	KT025729
Arisaema heterophyllum Blume		Sancheong, Gyeongnam, Korea	AH_SC	KT025780	KT025730
		Gwangyang, Jeonnam, Korea	AH_KY	KT025781	KT025731
		Tongyeong, Gyeongnam, Korea	AH_TY	KT025782	KT025732
		Cheongyang, Chungnam, Korea	AH_CY	KT025783	KT025733
		Ansan, Gyeonggi, Korea	AH_AS	KT025784	KT025734
		Gapyeong, Gyeonggi, Korea	AH_GP	KT025785	KT025735
Arisaema serratum (Thunb.) Schott	*a*	Gwangyang, Jeonnam, Korea	AS_KY	KT025786	KT025736
		Jangsu, Jeonbuk, Korea	AS_JS	KT025787	KT025737
		Geoje, Gyeongnam, Korea	AS_GJ	KT025788	KT025738
		Jeju, Jeju, Korea	AS_JJ	KT025789	KT025739
		Inje, Gangwon, Korea	AS_IJ	KT025790	KT025740
		Ulleung, Gyeongbuk, Korea	AS_UR	KT025791	KT025741
		Yanbian, Jilin, China	AS_JL	KT025792	KT025742
Arisaema takesimense Nakai	*a*	Ulleung, Gyeongbuk, Korea	AT_BR1	KT025793	KT025743
		Ulleung, Gyeongbuk, Korea	AT_BR2	KT025794	KT025744
		Ulleung, Gyeongbuk, Korea	AT_NR1	KT025795	KT025745
		Ulleung, Gyeongbuk, Korea	AT_NR2	KT025796	KT025746
		Ulleung, Gyeongbuk, Korea	AT_NSJ1	KT025797	KT025747
		Ulleung, Gyeongbuk, Korea	AT_NSJ2	KT025798	KT025748
Arisaema ringens (Thunb.) Schott	*a*	Geoje, Gyeongnam, Korea	AR_GJ	KT025799	KT025749
		Tongyeong, Gyeongnam, Korea	AR_TY	KT025800	KT025750
		Jeju, Jeju, Korea	AR_GJA	KT025801	KT025751
		Jeju, Jeju, Korea	AR_JC	KT025802	KT024752
		Seogwipo, Jeju, Korea	AR_SGP	KT025803	KT025753
		Jeju, Jeju, Korea	AR_CJ	KT025804	KT025754
Pinellia ternata (Thunb.) Makino	Pinelliae Tuber (*Ban xia*, *Ban Ha*)*b*	Chengdu, Sichuan, China	PI1	KT025805	KT025695
		Seogwipo, Jeju, Korea	PT2	KT025806	KT025696
		Sacheon, Gyeongnam, Korea	PT3	KT025807	KT025697
		Sanmenxia, Henan, China	PT4	KT025808	KT025698
		Quiyang, Guizhou, China	PT5	KT025809	KT025699
Pinellia tripartita (Blume) Schott	*a*	Geoje, Gyeongnam, Korea	PTP_GJ	KT025810	KT025700
		Seiong, Chungnam, Korea	PTP_SJ	KT025811	KT025701
		Jeonju, Jeonbuk, Korea	PTP_JEJ	KT025812	KT025702
		Tongyeong, Gyeongnam, Korea	PTP_TY	KT025813	KT025703

Continued on next page

Table 1. Continued.

Names		Source	Sample name	GenBank accession No.	
Scientific name	Herbal			*mat*K	*rbc*L
Pinellia pedatisecta Schott	*a*(*Hu Zhang*, *Ho Jang Nam Seong*)*b*	Dingxi, Gansu, China	PP_Cn_GS	KT025814	KT025704
		Changsha, Hunan, China	PP_Cn_HN	KT025815	KT025705
		Quiyang, Guizhou, China	PP_Cn_QY	KT025816	KT025706
		Kaili, Guizhou, China	PP_Cn_KL	KT025817	KT025707
		Anguo, Hebei, China	PP_Cn_AG	KT025818	KT025708
		Harbin, Heilongjiang, China	PP_Cn_HB	KT025819	KT025709
Typhonium flagelliforme (Lodd.) Blume (= *Arum flagelliforme* Lodd.)	*a*(*Shui Ban Xia*, *Su Ban Ha*)*b*	Pingnan, Guangxi, China	TF_Cn_PN1	KT025820	KT025710
		Pingnan, Guangxi, China	TF_Cn_PN2	KT025821	KT025711
		Baoxing, Sichuan, China	TF_Cn_PN1	KT025822	KT025712
		Baoxing, Sichuan, China	TF_Cn_PN2	KT025824	KT025713

[a]There is no appropriate official herbal name. [b]Common inauthentic herbal names used in China and Korea, respectively.

Table 2. Analysis of the two DNA barcode regions used in this study.

Barcode	Species	Constant length (bp)	Aligned length (bp)	Intra-species variability	Inter-species variation	
					Indels	Substitutions
*mat*K	*A. amurense*	1286	1292	0-0.0008	0	0
	A. amurense var. *serratum*	1286	1292	0-0.0016	0	0
	A. erubescens	1286	1292	0	0	11
	A. heterophyllum	1286	1292	0	0	21
	A. serratum	1286	1292	0	0	8
	A. takesimense	1286	1292	0	0	8
	A. ringens	1286	1292	0	0	10
	P. ternata	1286	1292	0-0.0016	0	35
	P. tripartita	1286	1292	0-0.0008	0	38
	P. pedatisecta	1286	1292	0-0.0023	0	34
	T. flagelliforme	1292	1292	0.0015-0.0047	6	44
*rbc*L	*A. amurense*	1517	1517	0-0.0007	0	0
	A. amurense var. *serratum*	1517	1517	0	0	0
	A. erubescens	1517	1517	0-0.0007	0	13
	A. heterophyllum	1517	1517	0	0	12
	A. serratum	1517	1517	0	0	5
	A. takesimense	1517	1517	0	0	5
	A. ringens	1517	1517	0	0	8
	P. ternata	1517	1517	0-0.0007	0	19
	P. tripartita	1517	1517	0.0013-0.0033	0	19
	P. pedatisecta	1517	1517	0	0	18
	T. flagelliforme	1517	1517	0.0013-0.0046	0	23

Furthermore, we also obtained two marker nucleotides specific for *A. amurense* and its variety, six for *A. erubescens*, two for *A. heterophyllum*, three for *A. serratum* and *A. takesimense*, four for *A. ringens*, four for *P. ternata*, one for *P. tripartita*, and seven for *T. flagelliforme* from the

*rbc*L gene (Table 4 and **Figure S2**). Thus, we identified 45 nucleotide substitutions and a 6-bp insertion, which were sufficient to identify each species, excluding *A. erubescens*, using the *mat*K gene and 28 positions, excluding *P. pedatisecta*, using the *rbc*L gene (Tables 3 and 4). These species-specific marker nucleotides from *mat*K and *rbc*L indicate that both of these DNA barcode regions are useful and sufficient for distinguishing between Arisaematis Rhizoma and Pinelliae Tuber as well as medicinal plant species in general.

Table 3. Summary of marker nucleotides obtained from the comparison of maturase K (*mat*K) gene sequences

Sequence position	33	81	90	92	107	111	131	162	167	239	265	289	323	329	339	379	381	406 401	430	451	512	552
A. amurense	C	C	T	A	A	A	T	G	A	T	G	T	C	C	A	C	G	------	G	C	A	A
A. amurense var. *serratum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-----	.	.	.	.
A. erubescens	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	------	.	.	.	.
A. heterophyllum	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	T	A	------	.	A	.	.
A. serratum	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	------	.	.	.	G
A. takesimense	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	------	.	.	.	G
A. ringens	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	------	.	.	.	.
P. ternata	.	T	C	.	.	.	.	.	.	.	.	.	.	A	.	T	A	------	.	.	G	.
P. tripartita	.	T	.	.	G	.	.	T	.	.	.	.	.	.	G	T	A	------	.	.		.
P. pedatisecta	.	T	.	.	.	.	.	.	.	.	.	.	A	.	.	T	A	------	.	.		.
T. flagelliforme	T	T	.	.	.	T	C	.	T	C	A	C	.	.	.	T	A	GTGTAT	T	.		.

Sequence position	567	579	683	684	689	715	723	741	749	768	792	807	842	945	948	958	960	984	989	1,011	1,046	1,079	1,107	1,146
A. amurense	T	G	C	T	C	A	A	A	G	C	C	A	C	G	T	T	T	C	T	T	T	T	T	T
A. amurense var. *serratum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. erubescens	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A
A. heterophyllum	.	.	.	.	.	G	.	.	.	A	.	T	A	A	.	.	.	.	.	.	.	.	.	A
A. serratum	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G
A. takesimense	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G
A. ringens	.	.	.	.	.	G	.	.	.	.	.	.	.	A	C	.	.	.	.	C	.	.	.	A
P. ternata	.	.	T	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G
P. tripartita	.	.	T	.	.	G	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	G	.	G
P. pedatisecta	.	.	T	.	.	G	.	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	.	G
T. flagelliforme	G	A	A	C	T	G	G	T	A	.	.	.	.	A	.	C	C	.	A	.	C	.	G	G

Sequence positions indicate the number of aligned *mat*K region nucleotide positions of all species. Dots represent sequence residues identical to those in *Arisaema amurense*. Underlined characters indicate unique species-specific marker nucleotides.

Table 4. Summary of marker nucleotides obtained from the comparison of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene sequences

Sequence position	32	33	68	138	243	264	423	498	543	639	651	672	677	696	784	814	825	983	1,050	1,090	1,137	1,245	1,261	1,338	1,345	1,363	1,391	1,416	1,459
A. amurense	C	T	C	C	A	A	T	T	C	T	T	T	A	A	G	A	T	C	T	T	A	T	C	T	A	C	A	A	A
A. amurense var. *serratum*	. .	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. erubescens	.	G	.	T	.	.	.	.	T	.	.	A	.	G	.	.	.	.	C	.	.	.	.	.	T	.	.	.	T
A. heterophyllum	T	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
A. serratum	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	T
A. takesimense	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	T
A. ringens	.	.	.	.	G	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	T
P. ternata	.	.	A	.	.	.	.	.	.	.	.	A	T	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	T
P. tripartita	.	.	.	.	.	.	C	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
P. pedatisecta	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
T. flagelliforme	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	C	C	G	.	.	.	A	.	G	.	T	.	.	T

Sequence positions indicate the number of aligned *rbc*L region nucleotide positions of all species. Dots represent sequence residues identical to those in *Arisaema amurense*, and underlined characters indicate unique species-specific marker nucleotides.

Phylogenetic relationships of Arisaematis Rhizoma- and Pinelliae Tuber-related plant species

A phylogenetic tree was constructed based on each full *mat*K and *rbc*L sequence (from 60 samples obtained from 10 species and one variety) using the neighbor-joining method. All samples were classified into nine distinct groups depending on the species, irrespective of the distribution areas in both *mat*K and *rbc*L (Figure 1). However, *A. amurense* var. *serratum* and *A. takesimense* formed the same group as *A. amurense* and *A. serratum*, respectively (Figure 1). These results strongly support that *A. amurense* var. *serratum* is the same species as *A. amurense*, and that *A. takesimense* is the same species as *A. serratum*. When comparing the two herbal medicines, four species related to Pinelliae Tuber (*P. ternata*, *P. tripartita*, *P. pedatisecta*, and *T. flagelliforme*) formed one clade, but others related to Arisaematis Rhizoma (*A. amurense*, *A. erubescens*, *A. heterophyllum*, *A. serratum*, *A. takesimense*, and *A. ringens*) did not form a distinct clade for both DNA barcode regions (Figure 1). Thus, the results of phylogenetic analyses between the official and inauthentic species of Arisaematis Rhizoma did not correlate with the taxonomic positions and medicinal applications noted in the pharmacopoeia. Furthermore, the results did not provide criteria for distinguishing between the two herbal medicines, Arisaematis Rhizoma and Pinelliae Tuber.

Identification of authentic Arisaematis Rhizoma and Pinelliae Tuber

To distinguish authentic herbal medicines on the basis of DNA barcodes, an ideal DNA barcode should exhibit diverse marker nucleotides and an ability to clearly identify species. It should be sufficient to identify authentic herbal medicines by comparing individual DNA barcodes because diverse nucleotide substitutions can be observed between authentic and inauthentic species in both DNA barcodes even though the two plant species, *A. erubescens* and *P. pedatisecta*, did not reveal distinct species-specific marker nucleotides in *mat*K and *rbc*L, respectively (Tables 3 and 4). For the authentication of Arisaematis Rhizoma from inauthentic plant materials and other closely related species such as *Pinellia* and *Typhonium*, we suggest that a comparison of both *mat*K and *rbc*L DNA barcode sequences should be used, because *A. erubescens* could not be clearly differentiated from other species in the *mat*K analysis (Table 3). We also recommend a comparison of both DNA barcode sequences to discriminate authentic Pinelliae Tuber from other adulterant plant materials for similar reasons to those stated above, but with *P. pedatisecta* in the *rbc*L analysis (Table 4). In addition to marker nucleotides, the phylogenetic analysis of individual DNA barcode sequences clearly classified the two herbal medicines according to the species examined. Therefore, phylogenetic analysis using DNA barcodes provided useful information to discriminate authentic herbal medicines at a species level. These combined results obtained from DNA barcode marker nucleotides of both *mat*K and *rbc*L gene sequences, and the phylogenetic relationships among the species, can be used to distinguish between Arisaematis Rhizoma and Pinelliae Tuber and between the other analyzed species.

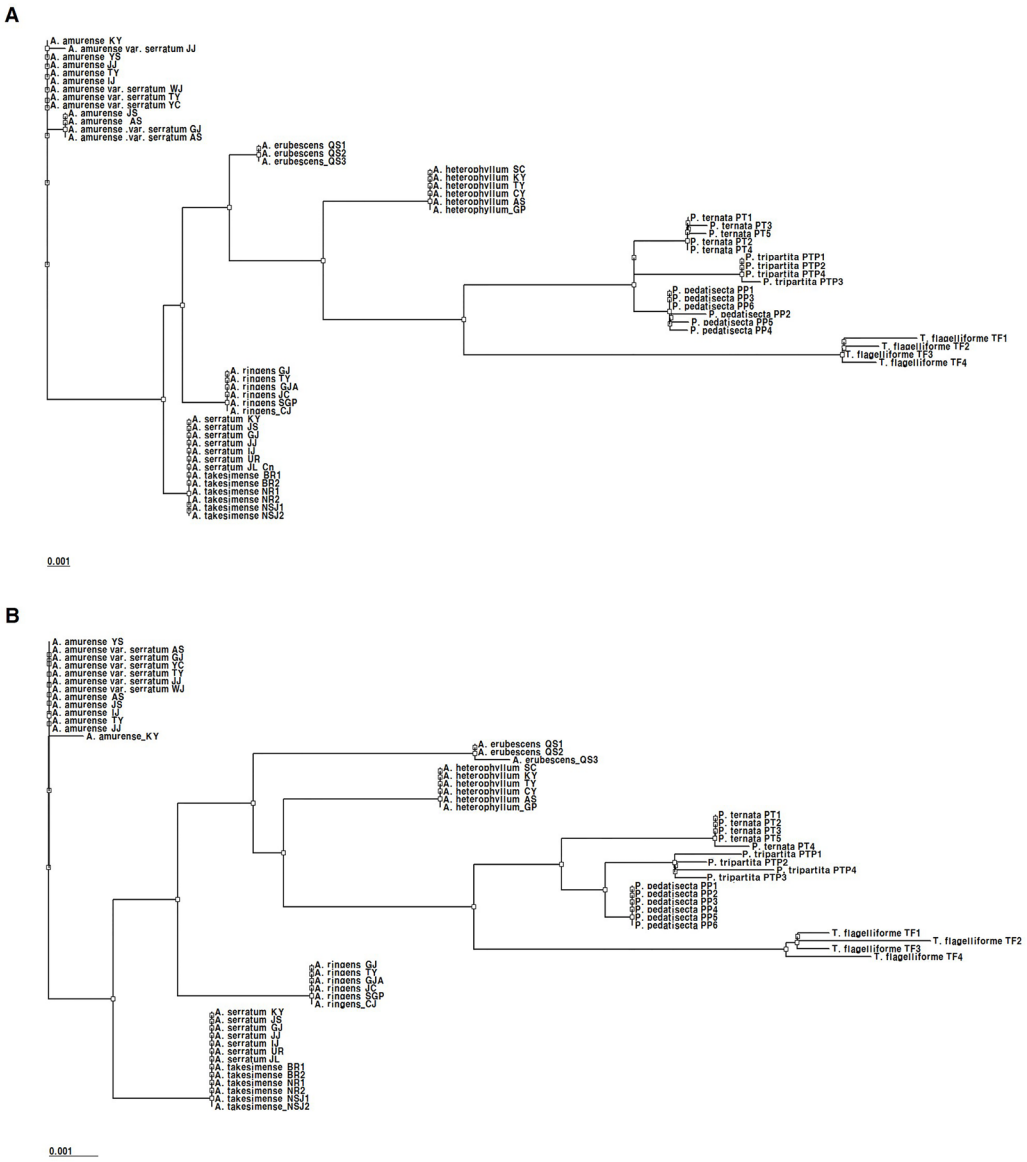

Figure 1. Phylogenetic tree of official Arisaematis Rhizoma and Pinelliae Tuber medicinal plant species and closely related plant species constructed on the basis of matK and rbcL DNA barcode sequences. **A.** Phylogenetic tree of matK barcode sequences. **B.** Phylogenetic tree of rbcL barcode sequences.

DISCUSSION

Accurate authentication of species is important for standardization and quality control of traditional herbal medicines (Sucher and Carles, 2008). Since processed herbal materials, such as dried slices or powders, are very difficult to accurately identify, we performed analyses to

distinguish between Arisaematis Rhizoma and Pinelliae Tuber, and also to authenticate the official species among ten species and one variety. We incorporated original plant species described in the Korean pharmacopoeia, which included common adulterants of Arisaematis Rhizoma and Pinelliae Tuber (Table 1). Thus, we included *A. serratum*, *A. takesimense*, and *A. ringens* for the typical adulterants of Arisaematis Rhizoma, and *P. tripartita*, *P. pedatisecta*, and *T. flagelliforme* for the typical adulterants of Pinelliae Tuber, with the official plant species *A. amurense*, *A. erubescens*, *A. heterophyllum*, and *P. ternata* in our analyses. In addition, we examined all species related to both herbal medicines for clear authentication of these medicinal plants at a species level. Therefore, this study was designed to clarify the differences between Arisaematis Rhizoma and Pinelliae Tuber and to authenticate plant species closely related to these herbal medicines, including common adulterants, on the basis of distinct genetic information for each species.

To provide genetic tools and information for authenticating official herbal medicines, we performed a comparison of DNA barcode sequences using related plant materials. A desirable DNA barcode should contain sufficient sequence variation to permit species discrimination and show significant inter-species differences with minimal intra-species variation, together with convenient flanking regions for PCR amplification and sequencing (CBOL Plant Working Group, 2009; Guo et al., 2011). The CBOL proposed using a combination of *mat*K and *rbc*L as a plant barcode due to the above-mentioned features (Saarela et al, 2013). The two universal DNA barcodes used in this study, *mat*K and *rbc*L, were successfully amplified by the universal primer and were found to exhibit inter-species specific variations that could be used to identify each species with little intra-species sequence divergence (**Figures S1** and **S2**). Therefore, we further analyzed these two regions as potential barcodes for authenticating official Arisaematis Rhizoma and Pinelliae Tuber from adulterant materials.

When comparing individual DNA barcode sequences, *mat*K and *rbc*L showed high sequence divergence, and both regions could be employed as unique barcodes for the authentication of Arisaematis Rhizoma and Pinelliae Tuber (Tables 3 and 4). Although individual DNA barcodes were sufficient to discriminate each species, we strongly recommend using both barcode regions, as suggested for discriminating *Scutellaria baicalensis* from its adulterants in a previous report, because *mat*K and *rbc*L did not exhibit unique marker nucleotides for *A. erubescens* and *P. pedatisecta*, respectively, and multilocus DNA barcodes are more effective for identification of plant species (Stoeckle, 2003; Guo et al., 2011; Purushothaman et al., 2014). In addition to the use of both *mat*K and *rbc*L barcode regions, we also suggest using phylogenetic analyses to authenticate herbal medicines and their adulterants because phylogenetic clustering provides additional information that can be used for species discrimination on the basis of sequence divergence and co-relationships.

The genus *Arisaema*, which is a member of the Araceae family, contains more than 130 species worldwide, and 660 plant names along with various synonyms, are recorded in the Plant List (Gusman and Gusman 2002; The Plant List 2013). *A. amurense* var. *serratum* and *A. takesimense* are suggested as synonyms of *A. amurense* and *A. serratum*, respectively, although these two species are separately classified independent plant taxa in Korea (Ko and Kim, 1985; Kang et al., 2003; The Plant List, 2013). However, both the *mat*K and *rbc*L barcode sequences of *A. amurense* var. *serratum* and *A. takesimense* were identical to those of *A. amurense* and *A. serratum* (Tables 3 and 4, **Figures S1** and **S2**). In this study, therefore, we established genetic tools that can be used for the authentication of two official herbal medicines, Arisaematis Rhizoma and Pinelliae Tuber. Furthermore, we provided objective evidence for the taxonomic classification of Korean *Arisaema* species via species-specific marker nucleotides and phylogenetic analyses.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Figure S1. Comparison of *mat*K barcode sequences of the official Arisaematis Rhizoma and Pinelliae Tuber medicinal plants and closely related plant species. Dots (·) indicate identical sequences to those in *A. amurense* KY, and dashes (-) represent gaps introduced to maximize alignment.

Figure S2. Comparison of *rbc*L barcode sequences of official Arisaematis Rhizoma and Pinelliae Tuber medicinal plants and closely related plant species. Dots (·) indicate identical sequences to those in *A. amurense* YS.

http://www.geneticsmr.com/year2016/vol15-1/pdf/gmr7064_supplementary.pdf