

Identification of *Arisaema* Species by leaf arrangement and molecular markers

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ABSTRACT

The objectives of this study were to identify three *Arisaema amurense*, *A. ringens* and *A. triphyllum* species based on molecular markers and to select accessions with unique morphological characters. Tentative identification based on leaf morphology was verified using single nucleotide polymorphisms (SNPs) generated from sequence analysis of the nuclear transcribed spacer of rDNA gene (nrITS) and the chloroplast intergenic spacer of *trnL-trnF* gene (cpIS). Specific SNPs from nrITS for *A. amurense* and *A. triphyllum* were identified. However, *A. ringens*-specific SNPs could not be identified because of multiple variations in the nrITS sequences of the species. Species-specific SNPs for the conserved cpIS sequenced in the same species were identified for each species. Morphological characteristics based on the number of leaflets were not sufficient to characterize closely related species such as *A. amurense* and *A. ringens*. *Arisaema amurense* with a unique leaf morphology with variegated, serrated, and denticulate foliage could be selected for further evaluation as a source of new ornamental germplasm.

Keywords: germplasm collection, leaf morphology, molecular analysis, new ornamental plant, sequence analysis

INTRODUCTION

The genus *Arisaema* Mart. has a unique morphology characterized by a fleshy spadix surrounded by a leaf-like spathe and leaflets that has a great potential as a landscape plant. Eight species of *Arisaema*, including the most common *A. amurense* Maxim., *A. ringens* (Thunb.) Schott., *A. heterophyllum* Blume, and *A. thunbergia* Blume occur in Korea. *Arisaema ringens* is characterized by a broadly ovate 3-leaflet with acuminate apices, and *A. amurense* is characterized by a 5-leaflet with entire, denticulate, or serrate leaflet margins. *Arisaema thunbergii* has leaves with 9-21 lanceolate to linear-lanceolate leaflets with entire margins and acuminate apices (Figure. 1). *Arisaema heterophyllum* has over a dozen leaflets, entire margins and acute to short acuminate apices (Ko and Kim, 1985; Gusman and Gusman, 2006).

The phenotypes are influenced by their environments (Joung and Roh, 2005; Joung *et al.*, 2010) and this required use of molecular markers to identify germplasm. *Arisaema* species has been investigated using nucleotide molecular markers (Lee *et al.*, 2011; 2013) and the

chloroplast intergenic spacer genes (Renner *et al.*, 2004). Single nucleotide polymorphisms (SNPs) of the nuclear internal transcribed spacer 1, 2 regions in the rRNA gene (nrITS) or the chloroplast intergenic spacer of *trnL-trnF* gene (cpIS) have proven useful to examine speciation in natural populations of *Kolkwitzia amabilis* Graebn. in the genus of *Hemerocallis* germplasm, and 11 species of *Lilium* native to Korea including *L. hansonii* Leichtlin ex D.D.T. Moore (Park *et al.*, 2014; 2022; Suh *et al.*, 2013).

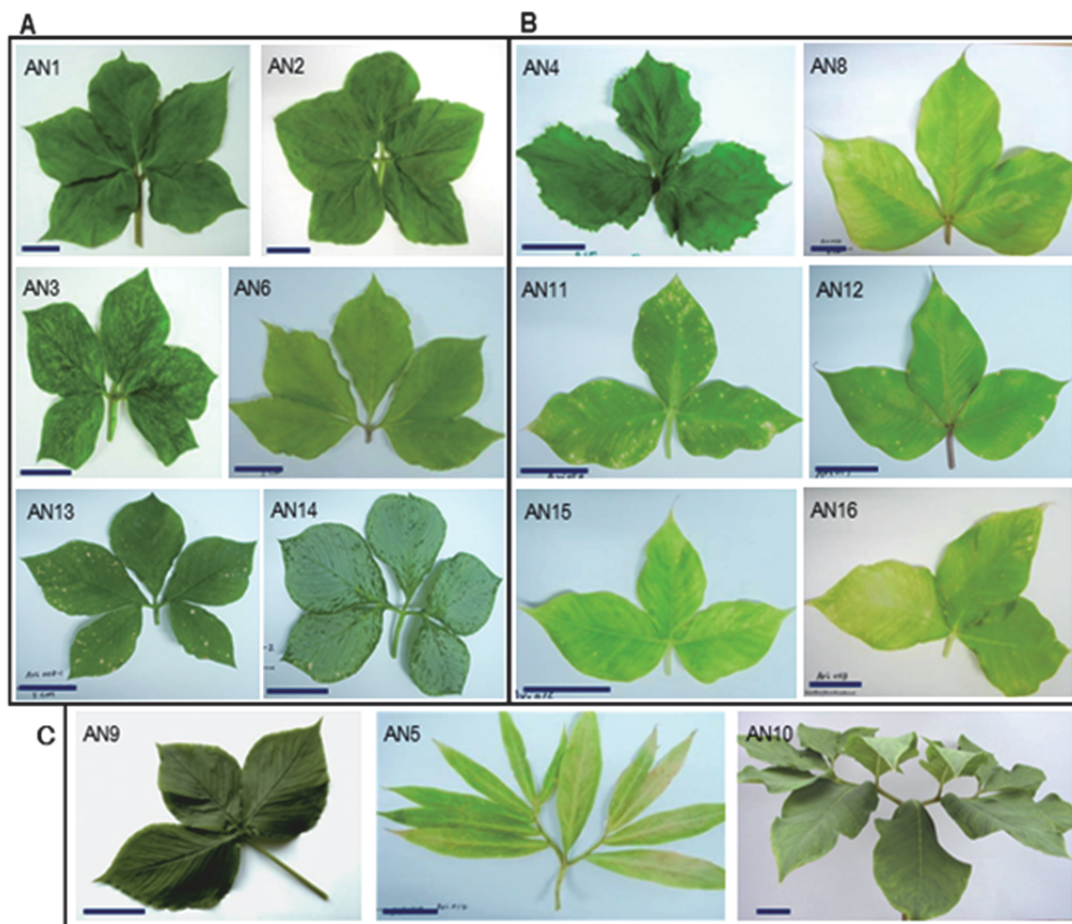


Figure 1. Leaf morphology and arrangement of 15 *Arisaema* accessions (AN) showing an acute (AN1) and round (AN2) leaflet apex and serrated leaflet margins (AN4); (A), 5-leaflet identified as *A. amurense*, (B), 3-leaflet identified as *A. ringens*, and (C), *A. triphyllum* (AN9), *A. thunbergii* (AN5), and *A. heterophyllum* (AN12). Refer to Table 1 for collection information (Bar=5 cm).

The objectives of this research were to compare and confirm accessions based on the leaf morphology and sequences of nrITS and cpIS for *A. amurense*, *A. ringens*, and closely related species, and to potentially recognize new germplasm with unique leaf morphology for use as a landscape ornamental plant.

MATERIALS AND METHODS

Plant materials. Accessions (AN) of *A. amurense* including accession with serrated and denticulate leaflet margin from Pungdo, Korea [accession no. (AN) 4] and with entire leaflet with silver variegation from Jeju, Korea (AN 14) and of *A. ringens* and *A.*

heterophyllum was evaluated (Table 1). Accessions of *A. triphyllum* (AN17, 18, 19) were also included (Lee *et al.*, 2011). Additionally, accessions of *A. triphyllum* collected from the USA were included.

DNA extraction and PCR. Genomic DNA was extracted as described (Lee *et al.*, 2011). For nrITS, primers ArITS-1 (5'-TTT CCG TAG GTG AAC CTG CG-3') and ArITS-2 (5'-CCT GAC CTG GGG TCG CAA T-3') were designed. The PCR reaction mix and the PCR amplification conditions were as described (Park *et al.*, 2014). The PCR products which showed double peaks in the sequencing chromatogram of AN 5, AN 10, AN 15, and AN 16 were cloned into a T-Blunt vector (SOT01-K020, SolGent, Daejeon, Korea) following the manufacturer's instructions. The ligation mix was transformed into *E. coli* DH5α cells. More than ten clones were selected from each accession and sequenced.

Table 1 Leaflet morphology of *Arisaema* accessions and tentative identification based on leaflet morphology and identification by molecular markers of nrITS and cpIS. Characteristics of used amendment

Accession (AN)	Location ^z	Leaf morphology			Identification of <i>Arisaema</i> species based on		
		basal connection	leaf apex	margin	Leaf ^y	nrITS ^x	cpIS ^w
1	Pungdo, Korea	petiolate	mucronate	entire	<i>amurenses</i> (5)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
2	Pungdo, Korea	petiolate	round	entire	<i>amurenses</i> (5)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
3	Pungdo, Korea	petiolate	acute	Entire, variegated	<i>amurenses</i> (5)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
4	Pungdo, Korea	sessile	acute	Serrated, denticulate	<i>ringens</i> (3)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
5	HBG, Korea	pedate	pointed, narrow	entire	<i>thunbergii</i> (10)	<i>thunbergii</i> (V)	<i>thunbergii</i> (C)
6	Jeju, Korea	petiolate	acute	entire	<i>amurenses</i> (5)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
8	Jeju, Korea	sessile	acute	entire	<i>ringens</i> (3)	<i>ringens</i> (III)	<i>ringens</i> (B)
9	Ann Arbor, MI, USA	sessile	acute	smooth veins	<i>triphyllum</i> (3)	<i>triphyllum</i> (IV)	<i>triphyllum</i> (E)
10	Jeju, Korea	pedate	pointed	entire	<i>Heterophyllum</i> (12)	<i>heterophyllum</i> (II)	<i>Heterophyllum</i> (D)
11	Jeju, Korea	sessile	acute	entire	<i>ringens</i> (3)	<i>ringens</i> (III)	<i>ringens</i> (B)
12	Jeju, Korea	sessile	acute	entire	<i>ringens</i> (3)	<i>ringens</i> (III)	<i>ringens</i> (B)
13	Jeju, Korea	petiolate	acute	entire	<i>amurenses</i> (5)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
14	Jeju, Korea	petiolate	acute	Entire, silver marks	<i>amurenses</i> (5)	<i>amurenses</i> (I)	<i>amurenses</i> (A)
15	Jeju, Korea	sessile	pointed	entire	<i>ringens</i> (3)	<i>ringens</i> (V)	<i>ringens</i> (B)
16	Jeju, Korea	sessile	pointed	entire	<i>ringens</i> (3)	<i>ringens</i> (III)	<i>ringens</i> (B)
17	Carroll Valley, PA, USA	Sessile green petiole	acute	smooth	<i>triphyllum</i> (3)	<i>triphyllum</i> (IV)	<i>triphyllum</i> (E)
18	Beltsville, MD, USA	Sessile black petiole	acute	smooth	<i>triphyllum</i> (3)	<i>triphyllum</i> (IV)	<i>triphyllum</i> (E)
19	Easthampton, MA, USA	sessile	acute	smooth	<i>triphyllum</i> (3)	<i>triphyllum</i> (IV)	<i>triphyllum</i> (E)

^z Accessions from Jeju were collected within a 500 m radius from 33-22-40 N latitude, 126-25-13 E longitude, and 721 m altitude. Accessions from Pungdo were collected within a 300 m radius from 37-06-65 N latitude, 126-23-20 E longitude, and 50 m altitude. HBG (Hantaek Botanical Garden, Korea). ^y the tentative identification of these accessions followed the description key for the *Arisaema* native to Korea (Ko and Kim, 1985) and Gusman and Gusman (2006). Leaflet numbers were represented in

parentheses. ^x Clades of nrITS (Figure 2) are presented in parenthesis. ^w Clades of cpIS (Figure 3) are presented in parenthesis.

For amplification of the cpIS, gene specific primers IS-F (5'-TAC TCT TTC ACA AAA AGA TTC G-3') and IS-R (5'-GAT TTT CAG TCC TCT GCT CTA C-3') were designed. The PCR conditions were the same as for the nrITS amplification, except annealing was 50°C.

The PCR products were purified, cloned, and sequenced as described (Park et al., 2014). Sequences were registered in National Center for Biotechnology Information GenBank (NCBI) (<http://www.ncbi.nlm.nih.gov/>).

Data analysis. Phylogenetic trees for the nrITS including a selected clone of each accession and cpIS sequences were constructed with the neighbour-joining (NJ) method using MEGA 6.0 software (Tamura *et al.* 2013). Bootstrap values, as indicated at each node, were obtained after 500 replications.

To identify the SNP markers, the sequence data of each accession were aligned with the ClustalW method (Mesquite version 3.04 <http://mesquiteproject.wikispaces.com/>).

RESULTS AND DISCUSSIONS

The nrITS sequences in the most of accessions were direct sequenced of the PCR amplicons. However, the nrITS of AN5, 10, 15 and 16, showed double peaks at position 236 and other positions or sequence shifts resulting from sequence substitution and in/del sites (data not presented); therefore, PCR amplicons were cloned and sequenced for each accession and consensus sequences were selected. For nrITS, accessions formed five clades (Figure 2). In clade I, all *A. amurense* collected from Pungdo (AN 1, 2, 3) and Jeju (AN 6, 13) and AN 14 with an entire leaflet with silver variegation (AN 14), as well as the three-leaflet with serrated denticulate leaf margin *A. ringens* collected from Pungdo (AN 4), were clustered together and the nrITS sequences of these accessions showed 100% homology among these accessions. Three clones of AN10 with twelve leaflets identified as *A. heterophyllum* were clustered into clade II. In clade III, all *A. ringens* with three leaflets collected from Jeju, except AN 15, were clustered together (Figure 2). In clade IV, all *A. triphyllum* (AN 9, 17, 18, 19) with three leaflets were clustered together. The AN15 sequences that were clustered into clade V that included AN 5 were identified as *A. thunbergii* with ten leaflets.

The sequences of clone 1 of AN 5 (AN 5-1) and AN 5-3 were 100% identical to *A. thunbergii* Genbank accession KT634007 and KT634002, respectively, and showed high homology with several *A. thunbergii* accessions such as Genbank accession KT634006, KT634005 and KT634004. For cpIS, PCR amplicons of all accessions were direct sequenced. Accessions formed five clades by the clustering analysis created by the NJ method (Figure 3).

In clade A, all *A. amurense* collected from Pungdo (AN 1, 2 and 3), Jeju (AN 13 and 14) and *A. ringens* collected from Pungdo (AN 4) were clustered together.

The cpIS sequence of these accessions showed 100% homology. All *A. ringens* collected from Jeju (AN8, 11, 12, 15 and 16) were included in clade B, and the sequences of these accessions also showed 100% homology. All *A. thunbergii* were clustered together in clade E.

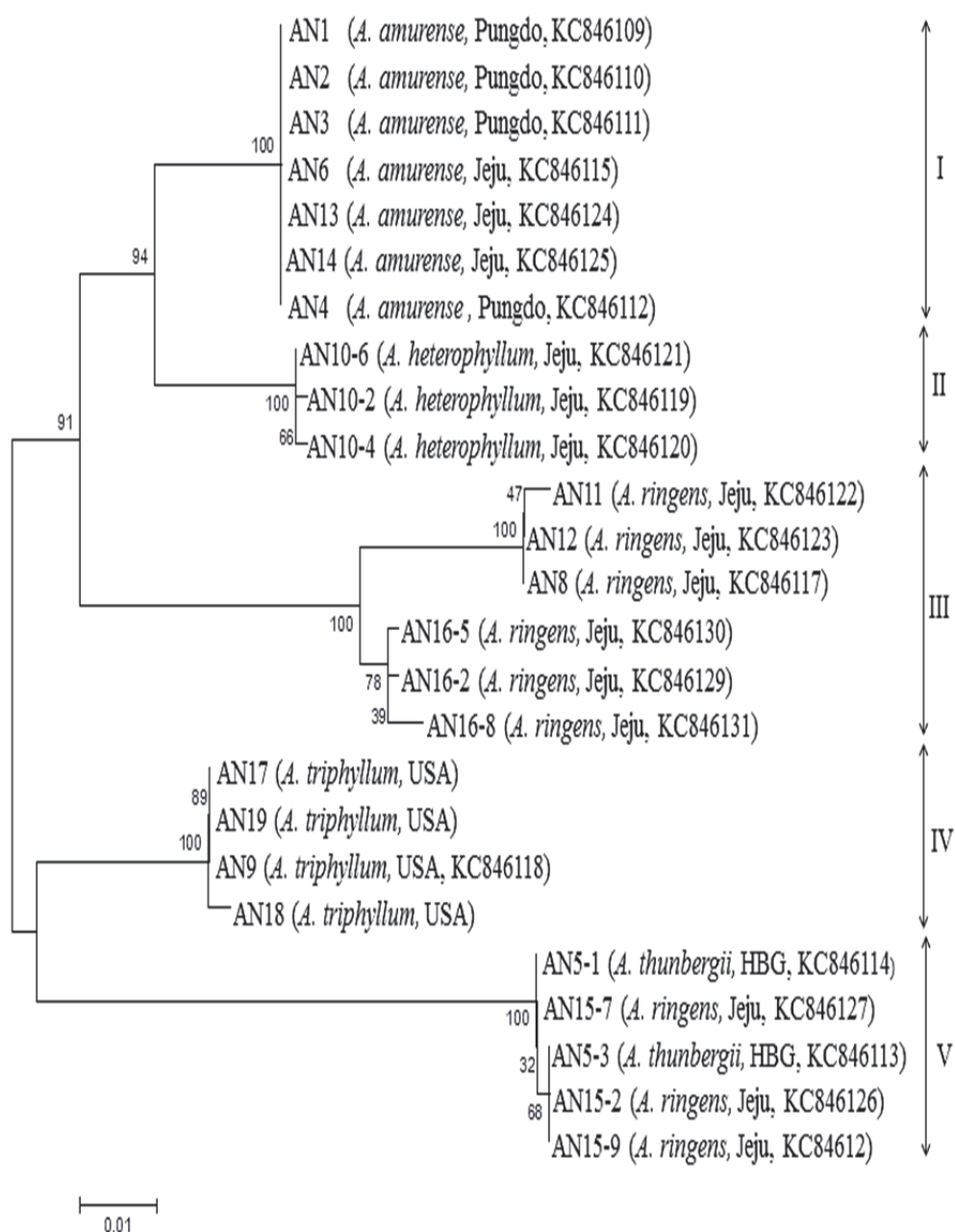


Figure 2. Clustering of *Arisaema* accessions with the NJ method based on a sequence analysis of nrITS. The sequences of nrITS for AN5-1 and -3, 10-2, -4 and -6; 15-2, -7 and -9; and 16-2, -5 and -8 were from clones. NCBI registry numbers are presented in the parenthesis.

The SNPs were detected at 150 positions in the nrITS region; among them, ten SNP markers were *A. amurense* specific, 9 markers were *A. heterophyllum* specific and another 9 markers were *A. triphyllum* specific (Table 2). However, an *A. ringens*-specific SNP marker could not be confirmed because the nrITS sequences were highly variable between the accessions. Furthermore, the nrITS sequence of AN 15 was closely related to that of *A. thunbergii*. In the cpIS region, the sequences were highly conserved among accessions in the same species and SNPs were detected only in 9 positions, including a long in/del sequence at positions of 128-150 (Table 3).

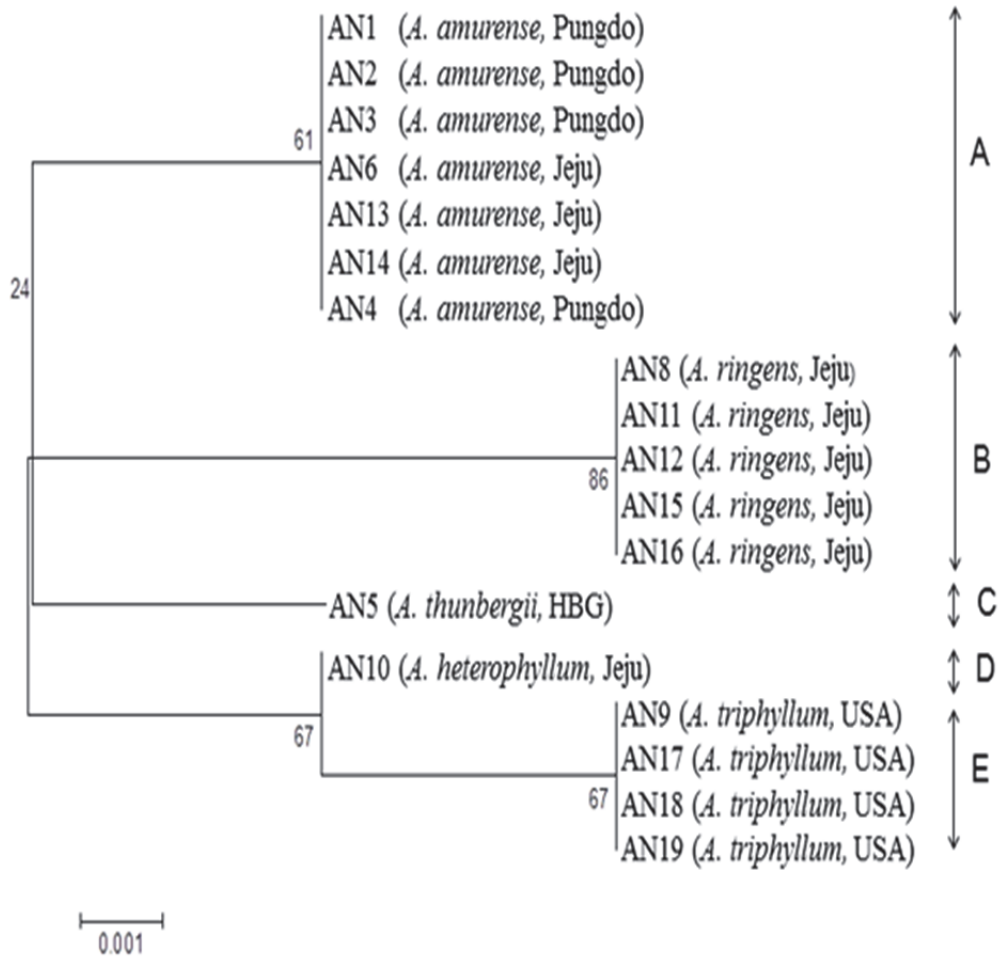


Figure 3. Clustering of *Arisaema* accessions with the NJ method based on sequence analysis of cpIS. NCBI registry numbers are presented in parenthesis: AN1 (KT719373), AN8 (KT719375), AN5 (KT719376), AN9 (KT719377), and AN10 (KT719374)

Table 2 Summary of position numbers from multiple alignments of the nrITS sequence

Clade ^z	Position ^y																			
	<u>1</u>	<u>6</u>	<u>3</u>	<u>8</u>	<u>9</u>	<u>5</u>	<u>6</u>	<u>1</u>	<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>3</u>	<u>7</u>	<u>2</u>	<u>6</u>	<u>3</u>
I	C	T	G	G	G	G	A	T	A	T	T	C	C	C	C	C	T	C	G	C
II	T	T	A	A	-	-	A	C	A	C	-	-	T	C	C	T	G	T	G	C
III	T	T	A	A	-	-	A	T	A	T	-	-	C	C	C	C	G	T	G	C
IV	T	C	A	A	-	-	G	T	G	T	-	-	C	T	T	C	G	T	A	T
V	T	T	A	A	-	-	A	T	A	T	-	-	C	C	C	C	G	T	G	C

^z Clade I: *A. amurense* (AN1, 2, 3, 4, 6, 13 and 14), II: *A. heterophyllum* (AN10-2, -4, and -6), III: *A. ringens* (AN8, 11, 12 and 16), IV: *A. triphyllum* (AN9), V: *A. ringens* (AN15), and *A. thunbergii* (AN5)

^y Position numbers are from multiple alignments of the nrITS sequence. Dash represents a deletion in an in/del site. Underlined nucleotide letters indicate species-specific SNP markers.

Table 3 Summary of position numbers from multiple alignments of the cpIS sequence

Clade (species)	Position ^z								
	35	119	128-150	159	180	194	216	519	<u>224</u>
A (<i>A. amurense</i>)	C	C	-	C	-	A	T	A	<u>C</u>
B (<i>A. ringens</i>)	C	<u>T</u>	-	C	-	-	<u>C</u>	A	T
C (<i>A. thunbergii</i>)	C	C	ATACACTT	<u>A</u>	-	-	T	A	T
D (<i>A. heterophyllum</i>)	C	C	TTTGTTAGGTTATCCTTACACTT	C	-	A	T	C	T
E (<i>A. triphyllum</i>)	<u>A</u>	C	-	C	<u>T</u>	A	T	C	T

^z Position numbers are from multiple alignments of the cpIS sequence. Dash represents a deletion in an in/del site. Underlined characters indicate species-specific SNP markers.

Based on the SNPs of nrITS and cpIS, the 3-leaflet AN4 collected from Pungdo, tentatively identified as *A. ringens* based on the morphology, should now be identified as *A. amurense*. The clustering analysis of nrITS and cpIS showed that *A. triphyllum* was separated from *A. amurense* and *A. ringens*. Between nrITS and cpIS, the clustering analysis showed incongruent patterns. The *A. heterophyllum* clade was closely related to *A. amurense* clade in nrITS, but it was not closely related to *A. amurense* in terms of cpIS; however, it was rather closely related to *A. triphyllum*. This difficulty to identify AN 4 could be related to the sex which is not determined at the time of collection. A male plant of *A. amurense* has 3-leaflet and a female plant has 5-leaflet (Gusman and Gusman, 2006). The characterization of species in scet. Pistillata in *Arisaema* based on morphology is rejected by the molecular data based on the combined chloroplast genes (Renner *et al.*, 2004). Constructing specific primers to identify and separate *A. amurense* AN 4 or AN 14 may not be feasible using sequences based on nrITS since the leaflet morphologies could not be distinguished from the typical *A. amurense* (AN 1, 2, 3, 6, 13). Also, sequences of AN 4 and AN 14 showed 100% homologies with other typical *A. amurense*. In the previous report (Lee *et al.*, 2013), several primer sets including *trnL-IS-trnF* gene specific (DQ400875) and two other nuclear genes could not separate *A. amurense* from *A. ringens*.

A new form of *Arisaema* with variegated, serrated, and denticulate leaves will be desirable to increase diversity. *Arisaema amurense* (AN 14) with silver variation in leaves is unique morphologies and could have some potential as new germplasm to utilize in a landscape.

CONCLUSIONS

Germplasm identification and characterization based on the morphology is the result of phenotypic evaluation under certain environmental conditions and must be completed and corroborated with molecular analysis. Therefore, single nucleotide polymorphisms from nrITS and cpIS generated with current primers could not be effectively used to differentiate *A. amurense* closely related germplasm. Morphological characteristics based on the number of leaflets were not sufficient to characterize closely related species such as *A. amurense* and *A. ringens*. *Arisaema amurense* (AN 4) collected from Pungdo with serrated and denticulate leaves tentatively identified as *A. ringens* should further be evaluated. *Arisaema amurense* (AN 14) with silver variation in leaves could be considered as having some potential for further evaluation.

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