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A study of taxonomical relationships among species of Korean *Allium* sect. *Sacculiferum* (Alliaceae) and related species using inter-simple sequence repeat (ISSR) markers

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Abstract. Morphological differences among Korean *Allium* subgenus *Rhizirideum* sect. *Sacculiferum* and some related species are minor, making species delimitation difficult. Inter-simple sequence repeat (ISSR) was employed to assess genetic diversity and relationships. Thirty-four accessions, representing six taxa of *A. thunbergii*, *A. sacculiferum*, *A. deltoides-fistulosum*, *A. cyaneum* var. *cyaneum*, *A. cyaneum* var. *deltoides*, *A. anisopodium*, were sampled in this study. The ISSR markers revealed high polymorphism among taxa studied. In the phenetic analysis, accessions of *A. sacculiferum* and *A. deltoides-fistulosum* are nested within *A. thunbergii* accession group; *A. cyaneum* var. *cyaneum* and *A. cyaneum* var. *deltoides* are separated from the above sect. *Sacculiferum* group; the status of putative *A. anisopodium* is less clear, with a position between sect. *Sacculiferum* and *A. cyaneum*. We propose that *A. deltoides-fistulosum* and *A. sacculiferum* be sunk into *A. thunbergii* and that the putative *A. anisopodium* be recognized as *A. cyaneum* var. *deltoides*.

Keywords: *Allium*; Genetic diversity; Korea; Phenetic analysis; Sect. *Sacculiferum*.

Introduction

Section *Sacculiferum* is small in the large subgenus *Rhizirideum* of *Allium*, but poorly delimited. When Gritzenko (1979) proposed this section, it included two species, *A. sacculiferum* Maxim. and *A. komarovianum* Vved, both characterized by globose to ovate bulbs, simple coriaceous bulb coats, 3-angular or keeled flat leaves, subglobose rose or violet flowers, simple stamens much longer than the tepals, deep nectary grooves at the base of the ovary covered by hood-like projections, and finally flat obovate seeds (Hanelt and Fritsch, 1994). Gritzenko's (1979) two species were subsequently sunk into *A. thunbergii*, a rather broadly circumscribed species (Xu, 1980; Xu et al., 1990). Xu (1980) erroneously placed *A. thunbergii* in sect. *Haplostemen*, together with the commonly cultivated East Asiatic species *A. chinense*, a very close relative. In the most recent infrageneric classification of *Allium*, Hanelt and Fritsch (1994) broadened the concept of sect. *Sacculiferum* by including *A. chinense*, and *A. virgunculae*, a related Japanese species, within it.

In the revision of Korean *Allium* (Yu et al., 1981), eight species and one variety were recognized in subgenus *Rhizirideum*, viz: *A. thunbergii*, *A. sacculiferum*, *A. deltoides-fistulosum* (sect. *Sacculiferum*), *A. cyaneum* var. *cyaneum*, *A. cyaneum* var. *deltoides*, *A. splendens* (sect. *Reticulato-bulbosa*), *A. anisopodium* (sect. *Anisopodia*), *A. senescens* (sect. *Rhizirideum*), *A. victorialis* (sect. *Anguinum*). Morphologically *A. splendens*, with its tooth-like scale of the inner filament, and the latter two species, *A. senescens* with stalk before flowering, and *A. victorialis* having flat ovate shaped leaf, are distinct from others in the Korean taxa of subgen. *Rhizirideum*. For sect. *Sacculiferum*, the species status of *A. sacculiferum* was maintained, and *A. deltoides-fistulosum* was newly described (Yu et al., 1981). The morphological differences among most of those species, however, are minor, making species identification difficult. For example, some populations occurring in the Mt. Dukyu region were labeled *A. cyaneum* var. *cyaneum* with interstamenous appendage and wax powders on the leaf surfaces, and cross section of leaves semi-circular and hollow, while populations in Mt. Kaya and Isl. Cheju were proposed as *A. cyaneum* var. *deltoides* without interstamenous appendage and wax-powders on the leaf surfaces, and cross section of leaves triangular and hollow. Populations distributed in Mt. Sorak were recognized as *A. anisopodium* with leaf cross-section narrow and flat and solid (Yu et al., 1981), despite the fact that they resemble *A. cyaneum* var. *deltoides* in many other respects (Woo, 2000).

Molecular markers have proven useful in clarifying genetic relationships within subgenus *Rhizirideum* (Dubouzet et al., 1997; Raamsdonk et al., 200), but sect. *Sacculiferum* remains intact in those studies. Until recently, a preliminary phylogenetic study of sect.

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Sacculiferum and related species employing ITS sequences (Woo, 2000) revealed that *A. thunbergii*, *A. sacculiferum*, *A. deltoides-fistulosum* and the Dukyu accession of *A. cyaneum* var. *cyaneum* were closely related and indistinguishable in the ITS tree; they constituted a polytomy, with *A. cyaneum* var. *deltoides* as the sister group, and the Bisundae accession of putative *A. anisopodium* furthermore became their sister group, whereas the Russian accession of *A. anisopodium* (material from Royal Botanical Garden, Edinburgh) deviated notably and was placed on another clade. A succinct taxonomy based on morphological characters and ITS sequence data is not available for sect. *Sacculiferum* and related species.

In this study we report an inter-simple sequence repeat (ISSR) analysis on the genetic diversity and relationships of sect. *Sacculiferum* and some related species. ISSR analysis is a PCR- based technique, similar to RAPDs and AFLPs (Wolfe and Liston, 1998). With primers composed of microsatellite sequences and using higher annealing temperature, ISSR markers have better reproducibility than RAPDs (Fang and Roose, 1997; Ratnaparke et al., 1998; Ge and Sun, 1999), and the procedures are simpler than AFLPs. ISSR markers may offer considerable variation among varieties and have been widely used in cultivated species (reviewed in Wolfe and Liston, 1998). The aims of the present study are: (1) to demonstrate the utility of ISSR markers for taxonomical relationship studies among closely related species; (2) to clarify the specific status of taxa in sect. *Sacculiferum* and related taxa, and (3) to elucidate the identity of some uncertain taxa by ISSR analysis.

Materials and Methods

Plant Materials

Thirty four accessions, representing six taxa as recognized by Yu et al. (1981) (*A. thunbergii*, *A. sacculiferum*, *A. deltoides-fistulosum*, *A. cyaneum* var. *cyaneum*, *A. cyaneum* var. *deltoides*, *A. anisopodium*), were sampled in this study (Table 1). *Allium spendens* of sect. *Reticulato-bulbosa*, *A. senescens* of sect. *Rhizirideum*, and *A. victorialis* of sect. *Anguisnum* were not included due to their relative distinctiveness. Sect. *Sacculiferum* has been chosen asthe core group, as well as some other

Table 1. Taxa included in the ISSR analysis. *RBGE* Royal Botanical Garden, Edinburgh.

Abbreviation	Taxon	Origin
	Allium thunbergii G. Don	
THU1		Mt. Hwangmae, Korea
THU2		Mt. Hwangmae, Korea
THU3		Mt. Hwangmae, Korea
THU4		Youngsil, Isl. Cheju, Korea
THU5		Mt. Waryong, Korea
THU6		Mt. Waryong, Korea
	A. sacculiferum Maxim.	
SAC1		Mt. Sokri, Korea
SAC2		Mt. Sokri, Korea
SAC3		Mt. Bulkok, Korea
SAC4		Doe-Heuksan Isl., Korea
SAC5		Pochon, Korea
SAC6		Pochon, Korea
	A. deltoides-fistulosum S. Yoo & W. Lee & S. Lee	
DEL1		Mt. Segol, Korea
DEL2		Mt. Segol, Korea
DEL3		Koksung, Korea
DEL4		Koksung, Korea
DEL5		Koksung, Korea
DEL6		Koksung, Korea
CYAE	*A. cyaneum* Regel var. *cyaneum* 1	China (cultivated in RBGE)
	A. cyaneum Regel var. *cyaneum* 2	
CVC1		Mt. Duckyu, Korea
CVC2		Mt. Duckyu, Korea
CVC3		1100 gogi, Isl. Cheju, Korea
CVC4		1100 gogi, Isl. Cheju, Korea
CVC5		1100 gogi, Isl. Cheju, Korea
	A. cyaneum Regel var. *deltoides* S. Yoo & W. Lee & S. Lee	
CVD1		Mt. Kaya, Korea
CVD2		Mt. Kaya, Korea
CVD3		Sangumbri, Isl. Cheju, Korea
CVD4		Sangumbri, Isl. Cheju, Korea
CVD5		Youngsil, Isl. Cheju, Korea
	A. anisopodium Ledebour	
ASP1		Bisundae, Mt. Sorak, Korea
ASP2		Bisundae, Mt. Sorak, Korea
ASP3		Bisundae, Mt. Sorak, Korea
ASP4		Biryong, Mt. Sorak, Korea
ASP5		Biryong, Mt. Sorak, Korea

Table 2. Intraspecific ISSR polymorphism and the expected heterozygosity (H_E). Not applicable to *A. cyaneum* var. *cyaneum* 1, which has only one accession.

Taxon	Number of accessions	Number of ISSR bands	Number of polymorphic bands	Polymorphim (%)	Expected heterozygosity (SE)
A. thunbergii	6	239	196	82.0	0.268 (0.204)
A. sacculiferum	6	220	143	65.0	0.197 (0.201)
A. deltoides- fistulosum	6	220	112	50.9	0.159 (0.204)
A. cyaneum var. *cyaneum* 1	1	34	–	–	–
A. cyaneum var. *cyaneum* 2	5	152	107	70.4	0.177 (0.194)
A. cyaneum var. *deltoides*	5	197	106	53.8	0.176 (0.207)
A. anisopodium	5	190	131	68.9	0.214 (0.214)

closely related species in subgen. *Rhizirideum*. Wherever possible 5-6 accessions were sampled for each taxon. Taking into account the grouping of Dukyu accession of *A. cyaneum* var. *cyaneum* with sect. *Sacculiferum* in the ITS tree (Woo, 2000), one accession of *A. cyaneum* var. *cyaneum* originating from China was also sampled from Royal Botanical Garden, Edinburgh. Korean plant materials including bulbs were collected in the wild and cultivated in the greenhouse of Ewha Womans University, while *A. cyaneum* var. *cyaneum* from Royal Botanical Garden, Edinburgh was germinated by seeds.

Genomic DNA Extraction and PCR Amplification

Total DNA was extracted from fresh leaf tissue, following the 2 × CTAB method (Doyle and Doyle, 1987). One hundred SSR primers from the Biotechnology Laboratory, University of British Columbia (UBC primer set no. 9) were screened, and 15 primers (Nos. 807, 808, 809, 810, 811, 817, 825, 835, 841, 850, 851, 857, 861, 864, 891) were finally used. Amplification was performed in a volume of 20 µl containing 1.5 mM $MgCl_2$, 2% formamide, 200 nM primer, 0.75 units of *Taq* polymerase, and 20 ng of genomic DNA. The following cycle program was adopted: an initial 5 min at 94°C, followed by 30 s at 94°C, 45 s at 50-55°C (depending on primers used), 1.5 min at 72°C for 35 cycles, and 7 min at 72°C for a final extension. The amplified products were separated on 1.5% agarose gel and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667.

Data Analysis

The amplified unambiguous bands were scored manually to compile a presence/absence matrix. Percentage of polymorphism at the intraspecific level was calculated as the proportion of polymorphic loci to the total number of loci scored in all accessions of the same taxon. The computer program POPGENE (Yeh et al., 1997) was used to estimate the expected heterozygosity (H_E) (Nei, 1973) and Nei's (1972) genetic identity (I). The UPGMA (unweighted pair-group method with arithmetical averages) dendrogram of Nei's (1972) genetic distance was constructed using POPGENE.

Results

ISSR Diversity

A total of 93 loci were scored for the 15 SSR primers in the 34 accessions of *Allium* surveyed. The locus number varied from 3 to 10 per primer (averaging 6.6), with fragment size ranging from 300 to 1,500 bp. Among the taxa studied, *A. thunbergii* has the highest expected heterozygosity (H_E = 0.268) and ISSR polymorphism (82%), while *A. deltoides-fistulosum* has the lowest (H_E = 0.159; ISSR polymorphism = 50.9%) (Table 2). Nei's (1972) genetic identities (I) between taxa range from 0.650 (*A. deltoides-fistulosum* vs. *A. cyaneum* var. *cyaneum* 1) to 0.868 (*A. thunbergii* vs. *A. sacculiferum*), with a mean identity of 0.781±0.061 (Table 3).

Genetic Relationships

In the phenetic analysis based on genetic distances, the 34 accessions of *Allium* cluster into two main groups (Figure 1): one includes 23 accessions and is furthermore divided into two subgroups, one for the three taxa of sect. *Sacculiferum* (*A. thunbergii*, *A. sacculiferum* and *A. deltoides-fistulosum*), the other one for the five accessions

Table 3. Nei's identity (1972) between taxa included in the ISSR analysis.

Taxa	*A. thunbergii*	*A. sacculiferum*	*A. deltoides-fistulosum*	*A. cyaneum* var. *cyaneum* 1	*A. cyaneum* var. *cyaneum* 2	*A. cyaneum* var. *deltoides*	*A. anisopodium*
A. thunbergii	–						
A. sacculiferum	0.868	–					
A. deltoides-fistulosum	0.835	0.863	–				
A. cyaneum var. *cyaneum* 1	0.739	0.669	0.650	–			
A. cyaneum var. *cyaneum* 2	0.843	0.805	0.789	0.801	–		
A. cyaneum var. *deltoides*	0.800	0.789	0.734	0.743	0.813	–	
A. anisopodium	0.825	0.809	0.779	0.674	0.782	0.792	–

of *A. anisopodium*; the other main group consists of 11 accessions of *A. cyaneum* var. *cyaneum* and *A. cyaneum* var. *deltoides*, in which the Chinese accession of *A. cyaneum* var. *cyaneum* groups with the Korean accessions of *A. cyaneum* var. *cyaneum*.

Discussion

ISSR Markers

Essentially, ISSR markers make possible the genome-wide estimation of genetic diversity of individuals, allowing us to produce a large amount of data in a short time. ISSR techniques have been widely applied to assess genetic diversity in several economically important crop and fruit plants, and in biological conservation. Occasionally, it has been used to study relationships at the interspecific level (Huang and Sun, 2000). The present study demonstrates that ISSR can provide a clear portrayal of relationships among closely related congeneric species. In a previous study of sect. *Sacculiferum* using ribosomal DNA ITS gene sequence data (Woo, 2000), the relationships of the three species of sect. *Sacculiferum*, *A. thunbergii*, *A. sacculiferum*, *A. deltoides-fistulosum*, were not completely resolved, largely due to the insufficiency of nucleotide substitution, although ITS sequences have proven useful for resolving relationships at the generic and infrageneric levels (Baldwin et al., 1995). That ISSR markers should be used with caution in systematic study is pointed up by the fact that while ISSR always offers a great deal of variation within and among populations, a high level of polymorphisms may also introduce a high level of homoplasies in some genetically divergent species. A compromise approach is to reduce the number of polymorphic loci per primer by optimizing the amplification condition and to sample a large number of accessions per species.

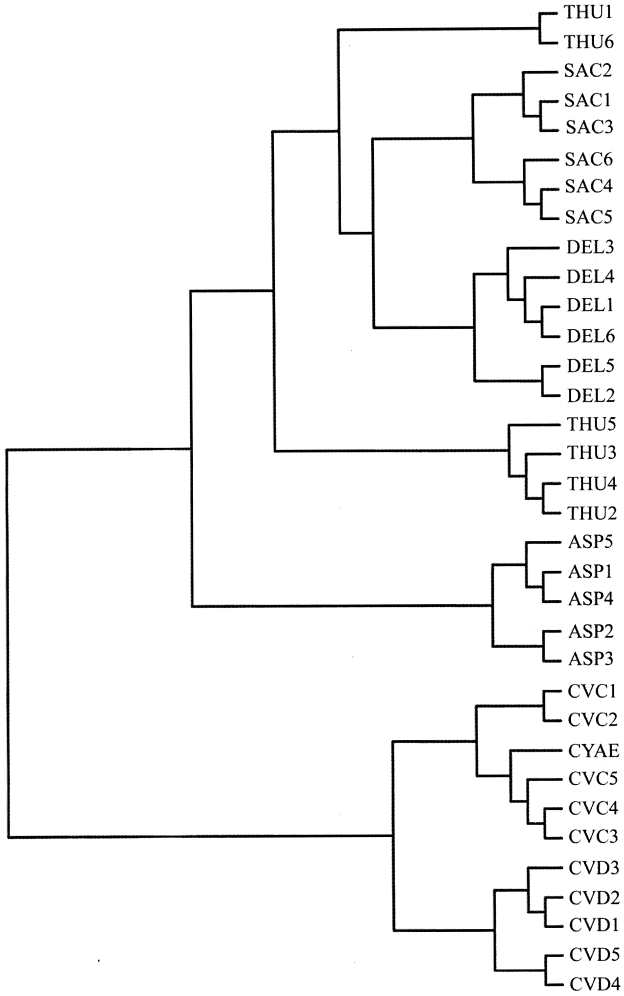

Figure 1. UPGMA dendrogram for 34 accessions representing six taxa in this study based on ISSR data. Taxon abbreviations are given in Table 1.

Genetic Relationships

The naturalness of the species group of *A. thunbergii*-*A. sacculiferum*-*A. deltoides-fistulosum* was somewhat supported by the phenetic analyses (Figure 1). The highest ISSR diversity was found in *A. thunbergii* (Table 2), a species widely distributed in China, Japan, Korea, and Mongolia. The highest diversity of *A. thunbergii* is concordant with the fact that a high genetic diversity value might be related to wide distribution (Hamrick, 1989). ISSR markers indicate that species of *A. sacculiferum* and *A. deltoides-fistulosum*, as now recognized, originated from within *A. thunbergii*. Given that these three taxa have slight morphological differences, this is not unexpected. *Allium sacculiferum* occurs in Korea, Mongolia, and eastern Siberia, differing from *A. thunbergii* by its short leaf sheath, cross section of leaf laminar, and keel. Xu (1980) and Xu et al. (1990) merged *A. sacculiferum* into *A. thunbergii*, although a thorough study of the infraspecific variability was appealed.

Allium deltoides-fistulosum was described by Yu et al. (1981) with the diagnostic characters of a cross section of leaf triangular and the leaf upright. It has apparent resemblance with *A. thunbergii*, and Woo (2000) reduced it to a variety of *A. thunbergii* (although not yet formally described). The ISSR markers provided additional information to clarify taxonomical relationships among the three taxa of sect. *Sacculiferum*. In the phenetic analysis (Figure 1), each of the six accessions of *A. sacculiferum*and *A. deltoides-fistulosum* constitute a division respectively, and are nested within the *A. thunbergii* accession group. ISSR markers indicated that *A. sacculiferum* and *A. deltoides-fistulosum* are not sufficiently divergent to merit species status. The value of genetic identity (I = 0.868) between *A. sacculiferum* and *A. thunbergii* is higher than that of a congeneric level (I = 0.65-0.70), but lower than a conspecific value (I = 0.950) (Crawford, 1990), although the ISSR estimates may not be directly comparable to those obtained with isozymes. The genetic identity between *A. thunbergii* and *A. deltoides-fistulosum* is almost the same as that between *A. sacculiferum* and *A. thunbergii*. We are inclined to

maintain one species for this group of taxa, echoing the treatment of Xu (1980) and Xu et al. (1990). While *A. deltoides-fistulosum* could be reduced to variety status (Woo, 2000), the same delimitation is plausible for *A. sacculiferum*.

While in the ITS study (Woo, 2000) *A. cyaneum* var. *cyaneum* is not distinctive from the species group of sect. *Sacculiferum*, an obvious boundary divides them in the ISSR analysis (Figure 1). The Chinese accession of *A. cyaneum* var. *cyaneum* unexceptionally groups with Korean accessions, and in turn groups with the division of accessions of *A. cyaneum* var. *deltoides*. The status of putative *A. anisopodium*, however, is less clear. In the phenetic analysis (Figure 1) its position is intermediate between sect. *Sacculiferum* and *A. cyaneum* (sect. *Reticulatobulbosa*). Morphologically, Korean *A. anisopodium* resembles *A. cyaneum* var. *deltoides* with respect to ratio of leaf and scape lengths, ratio of perianth and filament lengths, and pedicel. *Allium anisopodium* is well presented in China, central Asia, Mongolia, Siberia, and North Korea. The occurrence of *A. anisopodium* in South Korea was likely to be misidentified. The distinction of ITS sequences between Russian and Korea accessions (Woo, 2000) lent support to this judgment. The putative *A. anisopodium* populations in the Bisundae region may appropriately be recognized as *A. cyaneum* var. *deltoides*. More analyses of the genetic diversity of unambiguous materials of *A. anisopodium* from outside Korea would be desirable before any conclusion is drawn.

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韓國產蔥屬（蔥科）*Sacculiferum* 組及相關種分類學關係的 ISSR 分析

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在外部形態方面，韓國產蔥屬的 *Sacculiferum* 組及一些相關種只具細微差別，這些種之間的界限並不清楚。在本研究中我們運用內間隔簡單序列重複片段（inter-simple sequence repeat, ISSR）來檢測它們的遺傳多樣性和親緣關係。共取樣 34 個體，代表 6 個分類群，分別為：*A. thunbergii, A. sacculiferum, A. deltoides-fistulosum, A. cyaneum* var. *cyaneum, A. cyaneum* var. *deltoides* 和 *A. anisopodium*。ISSR 檢測出所研究分類群均具較高的多態性。表徵分析表明，*A. sacculiferum* 和 *A. deltoides-fistulosum* 的個體 "隱藏" 於 *A. thunbergii* 中，*A. cyaneum* var. *cyaneum* 和 *A. cyaneum* var. *deltoides* 與 *Sacculiferum* 組相分離。*A. anisopodium* 的位置不太確定，我們建議 *A. sacculiferum* 和 *A. deltoides-fistulosum* 應該包含於 *A. thunbergii* 之中，而假想中的 *A. anisopodium* 可能為 *A. cyameum* var. *deltoides*。

關鍵詞：蔥屬；遺傳多樣性；韓國；表徵分析；*Sacculiferum*。