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Comparative analysis of genetic diversity among species of *Chrysanthemum* and its related genera using inter-simple sequence repeat and sequence-related amplified polymorphism markers

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ABSTRACT. In this study, inter-simple sequence repeats (ISSRs) and sequence-related amplified polymorphism (SRAP) were applied to assess the genetic diversity in 38 species of *Chrysanthemum* and related genera. A total of 204 and 567 bands were amplified by 24 ISSR and 25 SRAP primers, of which 196 (97%) and 557 (99%) were polymorphic, respectively. The ISSR-based genetic similarity ranged from 0.016 to 0.886 and averaged 0.201, while the SRAP-based genetic similarity varied from 0.010 to 0.811 and averaged 0.122. Both the ISSR and SRAP techniques revealed similar clustering patterns and

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grouped species of *Chrysanthemum* and *Ajania* together. The results of principal coordinate analysis corroborated the unweighted pair group method with arithmetic average clustering. Additionally, results from ISSR and SRAP data were significantly correlated ($r = 0.89$, $P < 0.001$). Knowledge about genetic diversity among species can aid the transfer of traits of interest from the wild into cultivated chrysanthemum in future distant interspecific breeding.

Key words: *Chrysanthemum*; Inter-simple sequence repeat; Related genera; Sequence-related amplified polymorphism; Genetic diversity

INTRODUCTION

Species of *Chrysanthemum* and related genera, the gene pool of cultivated chrysanthemum, possess great ornamental values and are therefore of great economic importance for commercial utilization. Moreover, some of the species are endowed with novel traits of horticultural interest and strong stress and disease resistance that are not present in chrysanthemum cultivars (Sun et al., 2010a; Deng et al., 2010, 2011, 2012). Hence, many new cultivars of chrysanthemum with improved target traits possibly will emerge once hybrid parents are expanded to wild species of *Chrysanthemum* and related genera. The future success of these events, however, depends on accurate information about the genetic diversity and relatedness among the species of *Chrysanthemum* and related genera. So far, considerable interest has focused on phylogenetics among species of *Chrysanthemum* and related genera (Bremer and Humphries, 1993; Watson et al., 2000, 2002; Kondo and El-Twab, 2002; Ohashi and Yonekura, 2004; Zhao et al., 2007, 2009, 2010a,b; Tang et al., 2009), yet their genetic relationship remains a concern.

Inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) have been recognized as useful molecular markers in cultivar identification, marker-assisted selection, and other purposes including analyses of genetic diversity and delimitation across species or genera such as *Celosia* (Feng et al., 2009), *Cynodon* (Gulsen et al., 2009), Cucurbitaceae (Sikdar et al., 2010), *Polygala* (Lüdtke et al., 2010), and *Solanum* (Li et al., 2010). These studies have given useful clues to understand species or genera relationships that assist in implementing a sound breeding program. Recently, ISSR and SRAP have been used to characterize medicinal chrysanthemum (Shao et al., 2010), but no similar reports on genetic diversity among wild species of *Chrysanthemum* and related genera are available.

Here, we reported a comparison of the genetic diversity among species of *Chrysanthemum* and related genera using both ISSR and SRAP markers. The objectives of the study were to 1) reveal genetic diversity among species of *Chrysanthemum* and related genera using ISSR and SRAP markers, 2) compare different marker techniques, and 3) address the hybridization potential among species of *Chrysanthemum* and related genera.

MATERIAL AND METHODS

Plant materials and DNA extraction

The plant materials herein comprised 38 species of 10 genera, including 16 species of

Chrysanthemum, seven species each of *Ajania* and *Artemisia*, two species of *Tanacetum*, and one species each of *Glebionis*, *Coleostephus*, *Crossostephium*, *Leucanthemum*, *Nipponanthemum*, and *Argyranthemum* (Table 1). All the materials were maintained at the Chrysanthemum Germplasm Resource Preserving Center, Nanjing Agricultural University, China.

Total genomic DNA was extracted from young leaves of each species according to the cetyltrimethylammonium bromide-based method that was described by Murray and Thompson (1980) with minor modifications. DNA concentrations were 25 ng/μL for polymerase chain reaction (PCR) analysis and were determined by comparison with known concentrations of Lambda DNA.

Table 1. Thirty eight species of *Chrysanthemum* and related genera, along with their resource and distribution, used for genotyping analysis in this study.

No.	Code	Species	Collection site	Distribution
1	C1	*Chrysanthemum zawadskii*	Huangshan, Anhui, China	Eastern Europe through Russia to China and Japan
2	C2	*Chrysanthemum japonense*	Kyushu, Japan	Japan
3	C3	*Chrysanthemum indicum*	Nanjing, Jiangsu, China	Eastern Asia
4	C4	*Chrysanthemum lavandulifolium*	Beijing, China	China
5	C5	*Chrysanthemum nankingense*	Nanjing, Jiangsu, China	China
6	C6	*Chrysanthemum dichrum*	Neiqiu, Hebei, China	China
7	C7	*Chrysanthemum vestitum*	Tianzhushan, Anhui, China	China
8	C8	*Chrysanthemum boreale*	Honshu, Japan	China, Korea, Japan
9	C9	*Chrysanthemum japonicum*	Honshu, Japan	Japan
10	C10	*Chrysanthemum hypargyrum*	Taibaishan, Shaanxi, China	China
11	C11	*Chrysanthemum yoshinaganthum*	Shikoku, Japan	Japan
12	C12	*Chrysanthemum okiense*	West Honshu, Japan	Japan
13	C13	*Chrysanthemum ornatum*	Kyushu, Japan	Japan
14	C14	*Chrysanthemum crassum*	Kyushu, Japan	Japan
15	C15	*Chrysanthemum yezoense*	North Honshu, Japan	Japan
16	C16	*Chrysanthemum weyrichi*	Nikko, Japan	Far East, Japan
17	Aj1	*Ajania pacificum*	Tsukuba, Japan	China (Taiwan)
18	Aj2	*Ajania shiwogiku* var. *kinokuniense*	Tsukuba, Japan	Japan
19	Aj3	*Ajania myriantha*	Jinchuan, Sichuan, China	China
20	Aj4	*Ajania remotipinna*	Taibaishan, Shaanxi, China	China
21	Aj5	*Ajania fruticulosa*	Helanshan, Ningxia, China	China, Central Asia, Mongolia, Eastern Siberia
22	Aj6	*Ajania salicifolia*	Jingyuan, Gansu, China	China
23	Aj7	*Ajania tripinnatisecta*	Hongyuan, Sichun, China	China
24	Ar1	*Artemisia sericea*	Nanjing, Jiangsu, China	China, Russia, Siberia, Mongolia
25	Ar2	*Artemisia scoparia*	Nanjing, Jiangsu, China	China, Mongolia, Russia, Japan, Korea, India, Europe
26	Ar3	*Artemisia japonica*	Nanjing, Jiangsu, China	Asia
27	Ar4	*Artemisia vulgaris*	Nanjing, Jiangsu, China	Eurasia, North America, Northern Africa
28	Ar5	*Artemisia abrotanum*	Tsukuba, Japan	North America
29	Ar6	*Artemisia absinthium*	Tsukuba, Japan	North America
30	Ar7	*Artemisia sieversiana*	Yuntaishan, Henan, China	China, Korea, Japan, Mongolia
31	T1	*Tanacetum parthenium*	Chiba, Japan	Cultivated
32	T2	*Tanacetum vulgare* L.	Tsukuba, Japan	Europe, Asia
33	G	*Glebionis coronaria*	Nanjing, Jiangsu, China	Cultivated
34	Co	*Coleostephus multicaulis*	Dafeng City, Jiangsu, China	China
35	Cr	*Crossostephium chinense*	Xiamen, Fujian, China	China, Japan, Philippines
36	L	*Leucanthemum vulgare*	Beijing, China	Cultivated
37	N	*Nipponanthemum nipponicum*	Chiba, Japan	Japan
38	A	*Argyranthemum frutescens*	Tokyo, Japan	Cultivated

ISSR and SRAP profiling

A set of 77 ISSR primers and 23 SRAP primers (9 forward and 14 reverse primers)

was synthesized by Invitrogen (Shanghai, China). All primers were initially used to detect polymorphism between five arbitrarily selected species, and only those primers generating reproducible polymorphisms were applied to all 38 species for further polymorphic analysis (Table 2).

Table 2. Sequences of the inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) primers used in this study.

ISSR primer	Primer sequence (5'→3')	SRAP primer*	Primer sequence (5'→3')
ISSR04	ACACACACACACACAG	Me13	TGAGTCCAAACCGGTAA
ISSR05	ACACACACACACACAT	Me17	AGCGAGCAAGCCGGTGG
ISSR08	ATGATGATGATGATGATG	Me19	CAAATGTGAACCGGATA
ISSR12	GAGGAGGAGGAGGAGGAG	Me20	GAGTATCAACCCGGATT
ISSR17	GACAGACAGACAGACA	Me21	GTACATAGAACCGGAGT
ISSR22	ACACACACACACACACAA	Me22	TACGACGAATCCGGACT
ISSR23	ACACACACACACACACTA	Me23	CACAGTCATGCCGGAAT
ISSR24	ACACACACACACACACTC	Me24	GACCAGTAAACCGGATG
ISSR32	AGAGAGAGAGAGAGAGAC	Me25	CAGGACTAAACCGGATA
ISSR33	AGAGAGAGAGAGAGAGTA	Em1	GACTGCGTACGAATTAAT
ISSR35	AGAGAGAGAGAGAGAGTA	Em2	GACTGCGTACGAATTTGC
ISSR42	ACACACACACACACACCG	Em4	GACTGCGTACGAATTTGA
ISSR43	ACACACACACACACACCT	Em5	GACTGCGTACGAATTAAC
ISSR48	TGTGTGTGTGTGTGTGAA	Em6	GACTGCGTACGAATTGCA
ISSR49	TGTGTGTGTGTGTGTGAC	Em7	GACTGCGTACGAATTATG
ISSR52	TGTGTGTGTGTGTGTGGA	Em8	GACTGCGTACGAATTAGC
ISSR53	TGTGTGTGTGTGTGTGGC	Em9	GACTGCGTACGAATTACG
ISSR57	AGAGAGAGAGAGAGAGTG	Em10	GACTGCGTACGAATTTAG
ISSR58	AGAGAGAGAGAGAGAGGA	Em11	GACTGCGTACGAATTTCG
ISSR59	AGAGAGAGAGAGAGAGGC	Em14	GACTGCGTACGAATTCAG
ISSR61	AGAGAGAGAGAGAGAGGT	Em15	GACTGCGTACGAATTCTG
ISSR62	AGAGAGAGAGAGAGAGCA	Em16	GACTGCGTACGAATTCGG
ISSR64	AGAGAGAGAGAGAGAGCG	Em19	TGTGGTCCGCAAATTTAG
ISSR75	AGTGAGTGAGTGAGTG		

*Me = forward primers; Em = reverse primers.

For ISSR analysis, the PCR procedures followed Zhang et al. (2010), and PCR products were separated by electrophoresis on 1.5% agarose gels run at 100 V for 50 min in 1X Tris-acetate-ethylenediaminetetraacetic acid buffer and visualized by ethidium bromide staining. For SRAP analysis, both PCR and electrophoretic procedures were carried out as described by Zhang et al. (2011).

Data scoring and statistical analysis

Amplified bands were scored 1/0 as presence/absence of homologous bands across the 38 species for each primer or primer combination. Dendrograms were constructed based on Nei's genetic distance using unweighted pair group method with arithmetic average (UPGMA) with the SAHN module of NTSYS-pc 2.2 (Rohlf, 2005). The COPH and MXCOP modules were used to determine the goodness-of-fit between the cluster analysis and original matrix. The principal coordinate analysis (PCoA) was conducted to construct a two-dimensional array of eigenvectors using the DCENTER and EIGEN modules of NTSYS-pc. Finally, the Mantel *Z*-test (Mantel, 1967) was used with 1000 permutations to determine the significance level between the ISSR- and SRAP-based similarity matrices.

RESULTS

Molecular marker analysis

A set of 24 primers of the 77 total ISSR primers were selected. These produced a total of 204 bands across the 38 species of *Chrysanthemum* and related genera (Table 3). The number of amplified bands per primer ranged from 4 to 14 with an average of 8.5. Of the 204 scored bands, 196 were polymorphic (97%), varying from five (ISSR35 and ISSR43) to 12 (ISSR57) polymorphic bands per primer and a mean of 8.2 polymorphic bands (Table 3). A profile obtained from ISSR23 was shown in Figure 1A.

Table 3. Amplification results of ISSR analysis.

Primer	Bands scored	PB	PPB (%)	Primer	Bands scored	PB	PPB (%)
ISSR04	13	12	92.0	ISSR48	7	7	100.0
ISSR05	6	6	100.0	ISSR49	11	10	91.0
ISSR08	10	10	100.0	ISSR52	6	6	100.0
ISSR12	8	8	100.0	ISSR53	7	7	100.0
ISSR17	8	8	100.0	ISSR57	14	12	86.0
ISSR22	9	8	89.0	ISSR58	8	8	100.0
ISSR23	12	11	92.0	ISSR59	9	9	100.0
ISSR24	7	7	100.0	ISSR61	8	8	100.0
ISSR32	10	10	100.0	ISSR62	4	4	100.0
ISSR33	13	12	92.0	ISSR64	6	6	100.0
ISSR35	5	5	100.0	ISSR75	7	7	100.0
ISSR42	11	10	91.0	Total	204	196	-
ISSR43	5	5	100.0	Average	8.5	8.2	97.0

PB = polymorphic bands; PPB = percentage of polymorphic bands among the total scored bands.

A total of 118 SRAP primer combinations derived from 9 forward and 14 reverse primers were first screened for polymorphism. Only 25 primer combinations that generated informative bands were chosen for genotyping analysis. As a result, a total of 567 bands were produced, with an average of 21.8 per primer combination. Of the 567 amplified bands, 557 were polymorphic (99.0%). The number of polymorphic bands for individual primer combinations ranged between 11 (Me21Em4) and 36 (Me21Em15) and averaged 31 (Table 4). A profile obtained from Me25Em19 was shown in Figure 1B.

Cluster analysis

Using the ISSR binary matrix, the genetic similarities based on Jaccard's coefficient among all species ranged from 0.016 to 0.886, with a mean of 0.201. For the ISSR-based dendrogram, cophenetic correlation was calculated at r = 0.94, which corresponded to a very good fit. The dendrogram grouped all species into two major clusters and four separate clades (Figure 2A). Cluster I included all species of *Chrysanthemum* and *Ajania* but *Ajania tripinnatisecta* and *Crossostephium chinense*. The other species of related genera were grouped into cluster II, except that *Artemisia vulgaris*, *Artemisia absinthium*, *Artemisia sieversiana*, and *Argyranthemum frutescens* clustered as four separate clades.

The SRAP-based genetic similarity varied from 0.010 to 0.811 and had an average of 0.122. The UPGMA dendrogram based on Nei's genetic distance for the 38 species was

depicted in Figure 2B. The cophenetic correlation was estimated at 0.91, indicating a good fit between the dendrogram and the original matrix. The dendrogram grouped the 38 species into four major clusters. Cluster a consisted of all species of *Chrysanthemum* and species of *Ajania* excluding *Ajania salicifolia* and *Ajania tripinnatisecta*. The other species of related genera were grouped together in cluster b, and *Crossostephium chinense* and *Artemisia sieversiana* were grouped as two separate clusters, c and d.

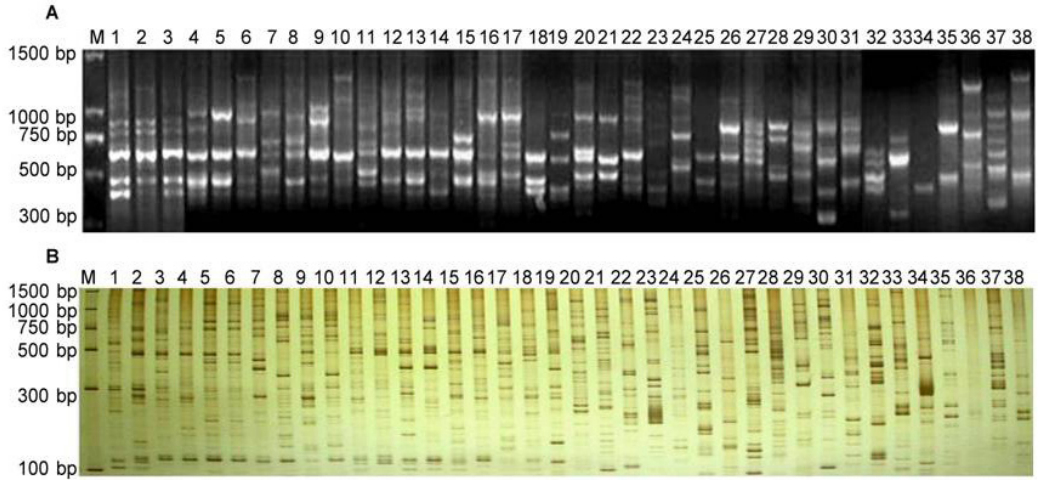

Figure 1. Inter-simple sequence repeat (ISSR) profiles obtained from ISSR 23 (**A**) and sequence-related amplified polymorphism (SRAP) profiles obtained from Me25Em19 (**B**) of the 38 species of *Chrysanthemum* and related genera. *Lanes 1-38* = species listed in Table 1. *Lane M* = DNA marker ladder.

Table 4. Amplification results of sequence-related amplified polymorphism (SRAP) analysis.

Primer combination	Bands scored	PB	PPB (%)	Primer combination	Bands scored	PB	PPB (%)
Me13Em4	18	18	100.0	Me22Em7	19	19	100.0
Me17Em9	19	19	100.0	Me22Em8	28	28	100.0
Me19Em15	14	14	100.0	Me22Em9	29	27	93.0
Me20Em4	31	29	94.0	Me22Em11	31	31	100.0
Me20Em5	15	15	100.0	Me22Em14	22	22	100.0
Me20Em10	35	35	100.0	Me22Em15	19	19	100.0
Me21Em1	19	18	95.0	Me23Em5	12	12	100.0
Me21Em2	16	16	100.0	Me23Em6	34	33	97.0
Me21Em4	11	11	100.0	Me22Em5	19	19	100.0
Me21Em6	17	17	100.0	Me24Em15	25	24	96.0
Me21Em11	16	16	100.0	Me24Em16	13	13	100.0
Me21Em15	36	35	97.0	Me25Em19	29	29	100.0
Me22Em1	23	22	96.0	Total	567	557	-
Me22Em4	17	16	94.0	Average	21.8	21.0	99.0

PB = polymorphic bands; PPB = percentage of polymorphic bands among the total scored bands.

PCoA and Mantel test

Two-dimensional plots of PCoA according to the ISSR- and SRAP-based genetic distance, respectively, were shown in Figure 3A and B. PCoA results generally reinforced the

dendrograms. The first two ordinates explained 39.6 and 41.7% of the total variations for ISSR and SRAP, respectively. By the Mantel test, a significant correlation ($r = 0.89$, $P < 0.001$) was observed between the ISSR- and SRAP-based Jaccard's similarity matrices, implying that the two marker systems have a linear relationship with a high determination coefficient ($R^2 = 0.79$).

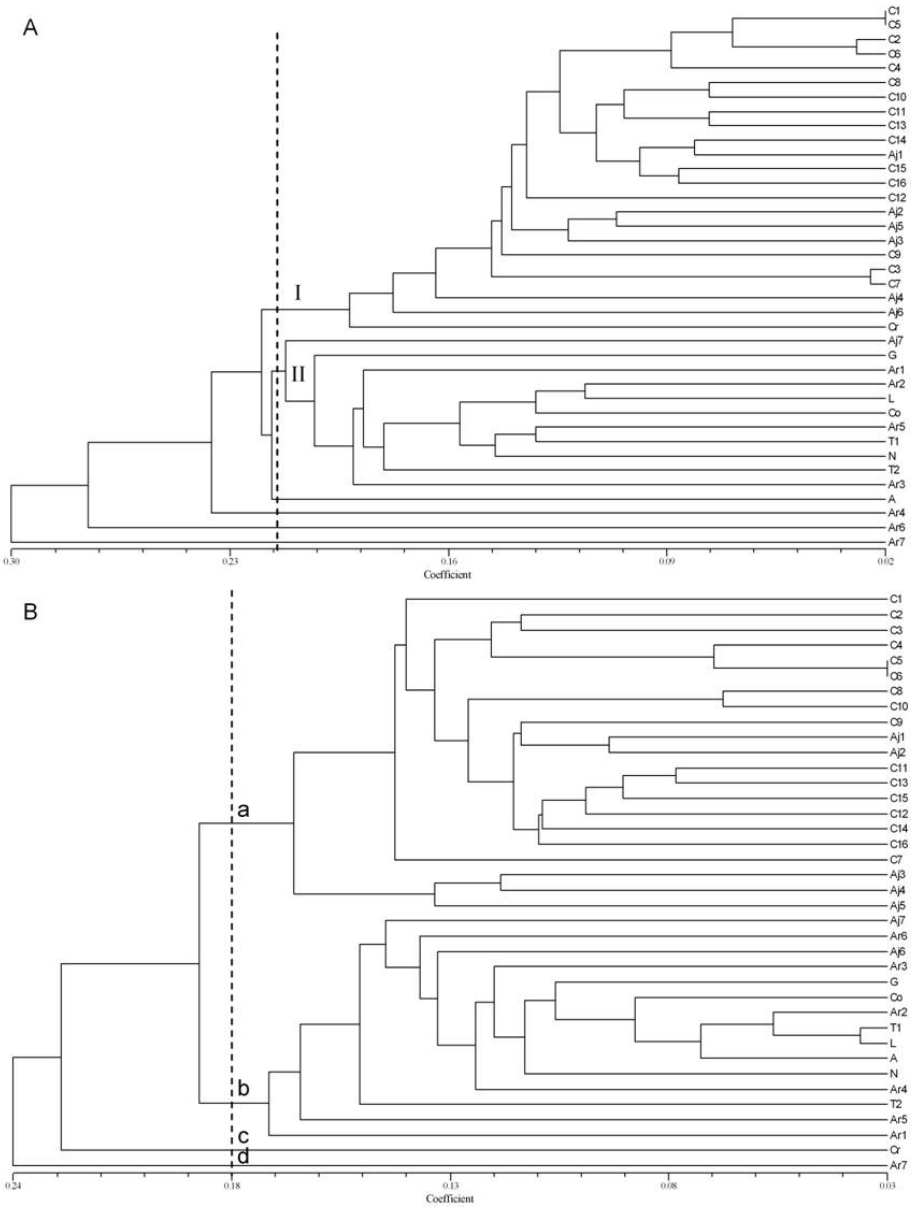

Figure 2. Unweighted pair group method with arithmetic average (UPGMA) dendrogram using ISSR (**A**) and SRAP (**B**) of 38 species of *Chrysanthemum* and related genera. The codes listed on the right of the dendrograms refer to those listed in Table 1.

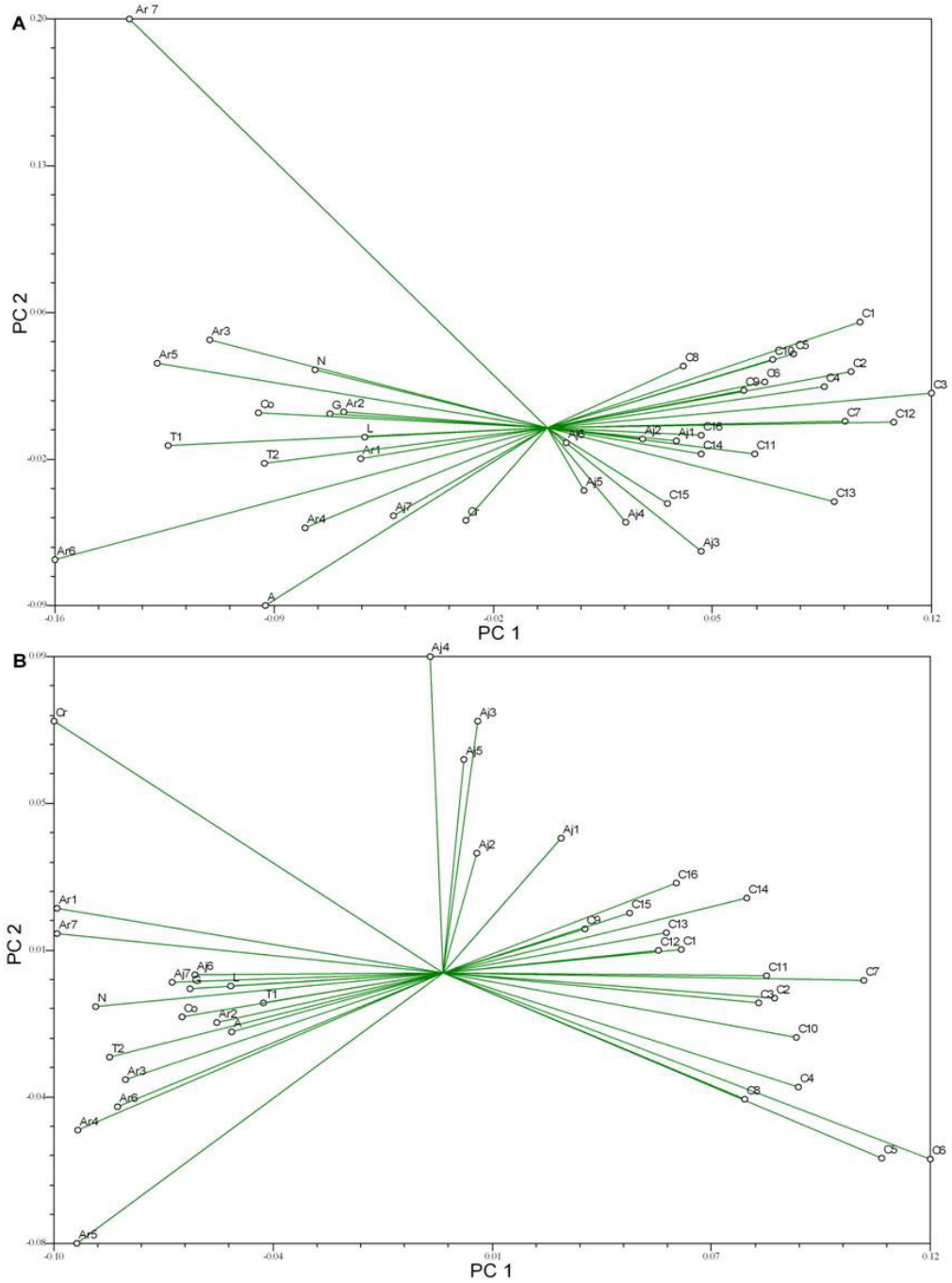

Figure 3. Two-dimensional plot with vectors of principal coordinate analysis (PCoA) of 38 species of *Chrysanthemum* and related genera using ISSR (**A**) and SRAP (**B**) markers. The codes listed in the Figure refer to those listed in Table 1.

DISCUSSION

Complete information about genetic diversity and genetic relationships enable effective conservation and sound utilization of crop germplasm. In this study, ISSR and SRAP markers were applied to assess the level and pattern of genetic diversity in 38 species of *Chrysanthemum* and related genera. Both markers exhibited >95% polymorphism and relatively low genetic similarity, indicative of the wide genetic variability among different species of *Chrysanthemum* and related genera. ISSR and SRAP markers have been widely used to estimate genetic diversity in many crops. Budak et al. (2004) and Fu et al. (2008) reported that SRAP was more informative than ISSR for assessing genetic diversity, but Wang et al. (2012) reported that ISSR was more effective than SRAP for assessing the genetic variation of goat's rues. Song et al. (2010) and Shao et al. (2010) observed that both markers were similarly effective. In this study, SRAP showed more polymorphic bands and lower derived genetic similarity than ISSR. However, a significant correlation ($r = 0.89$, $P < 0.001$) was observed between the similarity matrices obtained based on ISSR and SRAP data. Therefore, the results demonstrated that both ISSR and SRAP were effective and reliable for accurately assessing the level of genetic diversity among species of *Chrysanthemum* and related genera.

Previous studies indicated that many methods, including internal transcribed spacer and intergenic spacer sequences and amplified fragment length polymorphism markers failed to define the boundary between *Chrysanthemum* and *Ajania* (Bremer and Humphries, 1993; Zhao et al., 2010a,b). In this study, UPGMA cluster analyses grouped species of *Chrysanthemum* and most species of *Ajania* together, suggesting that the species of the two genera were more closely related than other related genera. However, the clustering pattern of species within the cluster was not clear, and neither ISSR nor SRAP markers could distinguish species of *Chrysanthemum* and *Ajania*. In addition, most species of *Artemisia* clustered separately, revealing that the species of *Artemisia* might have a monophyletic origin.

PCoA is one of multiple approaches for grouping that is based on similarity coefficients or variance-covariance among the traits of the entries. In this study, the PCoA results generally agreed with those of UPGMA clustering. PCoA sometimes revealed more informative groupings. For instance, in this study, the SRAP-based PCoA unveiled a more unequivocal boundary between *Chrysanthemum* and *Ajania* than the SRAP-based UPGMA clustering.

Species of *Chrysanthemum* and related genera possess excellent genes governing desirable horticultural traits and some tolerance traits that do not appear in cultivated chrysanthemums. The transfer of these excellent genes to cultivated chrysanthemums would be useful to improve cultivated chrysanthemums. Fortunately, several studies have shown the cross compatibility between cultivated chrysanthemums and species of *Chrysanthemum* and related genera (Fukai et al., 2000; Sun et al., 2010b), Thus far, potentially useful F_1 hybrids between cultivated chrysanthemums and species of *Chrysanthemum* and related genera have been produced through inter-specific or inter-generic hybridization with embryo rescue (Watanabe, 1977; Furuta et al., 2004). Recently, Deng et al. (2010, 2012) transferred resistance to aphid and *Alternaria* leaf spot from *Artemisia vulgaris* to cultivated chrysanthemums, and the same authors obtained some F_1 hybrids with enhanced cold tolerance through the hybridization of cultivated chrysanthemum and *Ajania przewalskii* (Deng et al., 2011). Some ornamentally important traits, as well as traits of heat and drought tolerance, were also transferred to chrysanthemum by other authors (Zhao et al., 2007; Sun et al., 2010a; Cheng et al., 2011).

This suggests that the transfer of desirable traits from *Chrysanthemum* and related genera to commercially cultivated chrysanthemums through distant hybridization makes the improved chrysanthemum cultivars possible. In this study, considerable genetic diversity and relatedness were observed by both ISSR and SRAP. This suggests that distant hybridization between species grouped in the same cluster is likely to be successful in future breeding programs.

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