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Research Report

Genetic Relationships among Diverse Spray- and Standard-type Chrysanthemum Varieties and Their Derived Radio-mutants Determined Using AFLPs

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Abstract. Gamma-ray irradiation is an important tool in ornamental plant breeding, particularly to induce flower-color variation. Standard-type chrysanthemum 'Migok' and spray-type 'Argus' have seven and four gamma-ray mutants, respectively, with novel flower colors. Genetic relationships among 26 chrysanthemum varieties or mutants were analyzed using amplified fragment length polymorphisms (AFLPs) with 20 fluorescently-labeled E+3/M+3 primer combinations (PCs). Clustering analysis was carried out using similarity coefficients and unweighted pair group method with arithmetic averages (UPGMA). The 20 PCs produced 2,837 bands, 95.6% of which were polymorphic. E-ACC/M-CAG had the most polymorphic bands (199) and high polymorphic information content, marker index, and resolving power values. Similarity coefficients ranged from 0.63 to 0.97 overall but were 0.73–0.83 in the 11 radio-mutants. The 26 varieties and radio-mutants were divided into four major UPGMA groups; the 11 radio-mutants formed two subgroups and 10 standard-type varieties or radio-mutants were closely clustered into another. The most promising mutant-specific marker candidates were PC E-ACG/M-CAG (47.5%) and E-ACA/M-CAT (44%) for the 'Migok' and 'Argus' families, respectively.

Additional key words: *Dendranthema grandiflorum*, flower color, gamma ray, molecular marker, mutants

Introduction

Chrysanthemum is one of the most popular ornamentals worldwide, but because market trends continuously change, the development of new varieties is required. Diverse flower types, petal colors, and plant architectures are important to the flower industry (Wang et al., 2004; Zalewska et al., 2007). Generally, chrysanthemums are classified into two types, 'standard' and 'spray', according to how they are cultivated. Standard-type chrysanthemums have a single stem with all the lateral flower buds removed, whereas the spray type has multiple stems with the terminal flower bud removed to allow the lateral buds to flower. Standard- and spray-type chrysanthemums are usually used for cut-flower and pot-crop production, respectively (Dole and Wilkins, 2004).

Mutation breeding is a useful tool to alter specific target traits (Chakrabarty and Datta, 2010). Gamma-ray mutagenesis is the most widely used method for mutation induction to develop new valuable plants to meet market demands (Sung et al., 2013), especially for color spectra and morphological changes (Lamseejan et al., 2000; Yamaguchi et al., 2008).

Molecular markers can be used to distinguish new cultivars more precisely and easily. Many such techniques have been developed, such as restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLPs), and used to screen for genetic diversity. Among these techniques, the AFLP method is one of the most popular for determining genetic relationships (Vos et al., 1995), and the technique has been used to classify many species, including soybean, maize, azalea, and *Brassica* (De Riek et al., 1999; Kwon et al., 2007; Marsan et al., 1998; Maughan et al., 1996).

Kumar et al. (2006) used RAPD to identify the relationships among 11 radio-mutants derived from two chrysanthemum varieties. Similarly, AFLPs were used to identify the relationships among 65 chrysanthemum varieties; the average polymorphism was 72.95% (Wu et al., 2007). Sung et al. (2010) and Kang et al. (2013) reported the genetic relationships among the chrysanthemum 'Argus' and its four derived mutants and among 'Migok' and its gamma-irradiated

plants, respectively. Furthermore, an analysis of genetic diversity and population structure using phenotypic traits and AFLPs was conducted in 48 genotypes of chrysanthemum (Roein et al., 2014). However, little information is available to compare the genetic diversity and relationships in mutagenesis-induced populations derived from standard- and spray-type chrysanthemums.

In the present study, we analyzed genetic relationships among 15 varieties and 11 radio-mutants derived from two varieties and discriminated the mutants from their respective parents using the AFLP technique. The results will help to understand the relationships between morphological differences and genetic diversity in chrysanthemum.

Materials and Methods

Plant materials

A total of 26 chrysanthemum varieties and mutants were used for this study. Among these, 11 radio-mutants were derived from the varieties *Dendranthema grandiflorum* 'Migok' and *Chrysanthemum* × *morifolium* 'Argus'. In 2005, 'Migok' and 'Argus' stems were irradiated by 40 Gy of gamma-ray, respectively. 'Migok', 'Argus', and each derived mutants were propagated vegetatively (stem cutting) for three generations in a greenhouse at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup, Korea.

DNA extraction and AFLP analysis

Fresh young leaves of each of the 15 chrysanthemum varieties and 11 radio-mutants were sampled, and DNA was extracted using the CTAB method with minor modification (Doyle 1987). The total genomic DNA was adjusted to 100 ng·μL^{-1} using a UV/Vis spectrometer (Jenway 6505; Essex, UK).

The AFLP analysis was carried out with the AFLP^{TM} Analysis System I (Invitrogen, Carlsbad, CA, USA) according to Kim et al. (2013) with some modifications. Genomic DNA (500 ng) was digested with *Eco*RI and *Mse*I restriction enzymes at 37°C for 3 h. The double-stranded *Eco*RI/*Mse*I adaptor was ligated to digested DNA by incubating at 37°C for 2 h. Pre-amplification involved 72°C for 2 min, 30 cycles of 94°C for 30 s (denaturation), 65°C for 60 s (annealing), 72°C for 60 s (extension), and 60°C for 10 min using the AFLP Pre-Amp Primer Mix Kit (Invitrogen). The PCR products were checked on 1.5% agarose gel, and then pre-amplified products were diluted (1:10) and used for selective amplification with 24 *Eco*RI+3/*Mse*I+3 primer combinations with three fluorescent dye-labeled (FAM, HEX, and NED; Applied Biosystems, Foster City, CA, USA) selective *Eco*RI primers. Selective amplification PCR conditions were as follows: 12 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min, annealing at $\Delta = -0.7$°C per cycle, and an additional 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min. The sample was fractioned using a capillary electrophoresis system (ABI 3130xl Genetic Analyzer; Applied Biosystems).

Statistical analyses

Statistical analysis was conducted as previously described (Kim et al., 2009, 2013), with minor modifications. We analyzed the genetic diversity based on AFLP data among the 26 chrysanthemum varieties and radio-mutants using the similarity coefficient and unweighted pair group method with arithmetic average (UPGMA) in the NTSYS-pc program (Tamura et al., 2011).

The discriminative power of AFLP primer combinations was evaluated by calculating the gene diversity, polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolving power (Rp). The gene diversity and PIC values for each primer combination were calculated using the summary statistics software Power Marker ver. 3.25 (Liu and Muse, 2005). PIC was averaged over the fragments for each primer combination (Sathyanarana et al., 2011). MI was calculated as described by Varshney et al. (2007) and Sathyanarana et al. (2011): MI = PIC × EMR where, EMR is defined as the product of the total number of loci/fragments per primer (n) and the fraction of polymorphic loci/fragments (β) (E = n. β) (Sathyanarana et al., 2011). The Rp value was calculated as $Rp = \Sigma I_b$ where I_b is the band informativeness, $I_b = 1 - [2 \times |0.5 - P|]$ where, P represent the proportion of the varieties containing the band/fragment (Prevost and Wilkinson, 1999; Sathyanarana et al., 2011).

Results and Discussion

We obtained seven and four mutants induced by gamma-ray mutagenesis from chrysanthemum 'Migok' and 'Argus', respectively (Fig. 1). We then characterized 15 chrysanthemum varieties and 11 radio-mutants derived from two varieties, the standard-type 'Migok' and the spray-type 'Argus', based on their floral morphologies and colors (Table 1). The original standard-type 'Migok' has red-violet ray florets, while its derived mutants, 'Migok'-M1-M7, have various colors of ray florets (red-yellow, red-ivory, red, pink-yellow, yellow-red, pink-yellow, and pink-ivory, respectively). The original spray-type 'Argus' variety has pink-red disc and pink ray florets, whereas its derived mutants, 'Argus'-M1-M4 have violet-red/violet–red, pink-red/pink, yellow/pink, and yellow-red/pink disc/ray florets, respectively. Additionally, the flower type of 'Argus'-M3 changed from anemone to single and they showed phenotypic stability for three generations. During

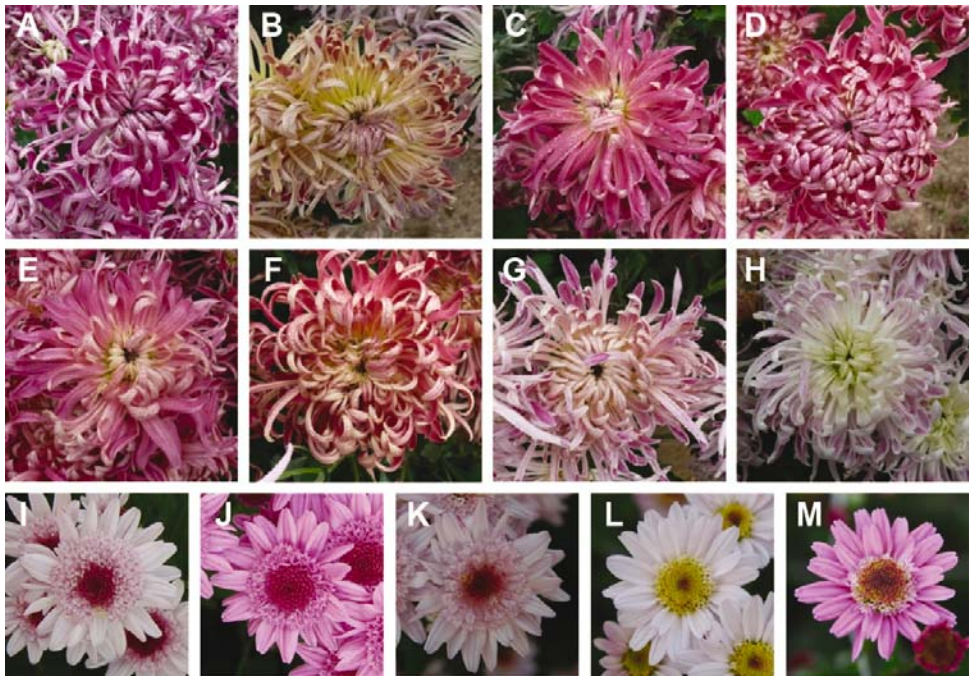

Fig. 1. Flower phenotypes of chrysanthemums and their derived radio-mutants. *Dendranthema grandiflorum* 'Migok' (A) and its seven gamma-ray irradiated mutants: 'Migok'-M1 (B), 'Migok'-M2 (C), 'Migok'-M3 (D), 'Migok'-M4 (E), 'Migok'-M5 (F), 'Migok'-M6 (G), and 'Migok'-M7 (H). *Chrysanthemum* × *morifolium* 'Argus' (I) and its four gamma-ray irradiated mutants: 'Argus'-M1 (J), 'Argus'-M2 (K), 'Argus'-M3 (L), and 'Argus'-M4 (M).

Table 1. Flower phenotypes of 15 chrysanthemum varieties and 11 radio-mutants derived from the standard-type variety 'Migok' and the spray-type variety 'Argus'

Code	Variety or mutant	Flower type A	Flower type B	Color of disc floret	Color of ray floret
V1	Geumsu	Spray	Anemone	yellow	white
V2	Noblewine	Spray	Single	yellow	violet-red
V3	Magic	Spray	Semi-double	yellow	pink-white
V4	Moonlight	Spray	Single	yellow	pink
V5	Migok	Standard	Double	-	red-violet
V5M1	Migok-M1	Standard	Double	-	red-yellow
V5M2	Migok-M2	Standard	Double	-	red-ivory
V5M3	Migok-M3	Standard	Double	-	red
V5M4	Migok-M4	Standard	Double	-	pink-yellow
V5M5	Migok-M5	Standard	Double	-	yellow-red
V5M6	Migok-M6	Standard	Double	-	pink-yellow
V5M7	Migok-M7	Standard	Double	-	pink-ivory
V6	Sangtte	Spray	Semi-double	purple	purple
V7	Argus	Spray	Anemone	pink-red	pink
V7M1	Argus-M1	Spray	Anemone	violet-red	violet-red
V7M2	Argus-M2	Spray	Anemone	pink-red	pink
V7M3	Argus-M3	Spray	Single	yellow	pink
V7M4	Argus-M4	Spray	Anemone	yellow-red	pink
V8	Yesmorning	Spray	Single	yellow	pink-white
V9	Yestogether	Spray	Spider	yellow-green	white
V10	Yelloweye	Spray	Semi-double	yellow-green	yellow
V11	Waterfog	Spray	Semi-double	yellow	white
V12	Westland pink	Standard	Double	-	pink
V13	Eulnyo	Standard	Double	-	yellow
V14	Froggy	Spray	Pompon	-	green-yellow
V15	Pinky	Spray	Semi-double	yellow	pink

V, variety; M, mutant.

Table 2. Polymorphic patterns observed from 20 fluorescently labeled AFLP primer combinations in 15 chrysanthemum varieties and 11 radio-mutants derived from two varieties

Primer combinations	No. of total bands	No. of polymorphic bands	Polymorphism (%)
E-AAC/M-CAA	115	104	90.4
E-AAC/M-CAC	179	165	92.2
E-AAC/M-CAT	61	58	95.1
E-ACA/M-CAA	78	72	92.3
E-ACA/M-CAC	140	132	94.3
E-ACA/M-CAG	130	124	95.4
E-ACA/M-CAT	88	87	98.9
E-ACC/M-CAA	191	187	97.9
E-ACC/M-CAC	198	186	93.9
E-ACC/M-CAG	202	199	98.5
E-ACC/M-CAT	171	170	99.4
E-ACG/M-CAA	110	95	86.4
E-ACG/M-CAG	134	133	99.3
E-ACG/M-CAT	139	130	93.5
E-AGC/M-CAA	163	162	99.4
E-AGC/M-CAC	138	133	96.4
E-AGC/M-CAT	125	120	96.0
E-AGG/M-CAA	137	123	89.8
E-AGG/M-CAC	200	197	98.5
E-AGG/M-CAG	138	136	98.6
Mean	141.9	135.7	95.6
Total	2,837	2,713	-

vegetative growth, no phenotypic difference was found within each variety and their derived mutant (data not shown).

Morphological variation is closely related to DNA differences, confirming that the AFLP technique is an important tool for elucidating relationships among mutants (Lee et al., 2002). To compare the genetic relationship among 15 chrysanthemum varieties and 11 radio-mutants, we conducted an AFLP analysis. A total of 24 *Eco*RI+3/*Mse*I+3 primer combinations (PCs) were used for preliminary screening. Among them, 20 PCs yielded completely identical fragment patterns, with a total of 2,837 bands and an average of 141.9 bands per PC (Table 2). Overall, 2,713 polymorphic bands, with an average 135.7 bands per PC, were detected. The most and fewest polymorphic bands were identified with the E-ACC/M-CAG (199 polymorphic bands) and E-AAC/M-CAT (58) primer pairs, respectively. The percentage of polymorphism was high, varying from 86.4 (E-ACG/M-CAA) to 99.4% (E-AGC/M-CAA).

The marker attributes for the AFLP primer sets were analyzed as PIC, MI, and Rp (Table 3). The PIC values ranged from 0.167–0.223, with an average of 0.202 per fragment. The minimum values of MI (10.972) and Rp (20.308) were observed with the E-AAC/M-CAT combination and the minimum of PIC (0.167) with E-AGC/M-CAT. The E-ACC/M-CAG PC had the maximum values of PIC (0.223), MI (44.281), and Rp (78.308). In addition, MI and Rp were positively correlated ($r^2 = 0.967$, $p < 0.005$) (data not shown). The PIC, MI, and Rp are discriminatory potential parameters used for fingerprinting or estimating genetic diversity in breeding populations (Roldàn-Ruiz et al., 2000; Sathyanarayana et al., 2011; Varshney et al., 2007). The E-ACC/M-CAG combination may be a strong marker candidate for identifying diverse chrysanthemum varieties given the high PIC, MI, and Rp values.

To analyze genetic variation among the varieties and their derived mutants, we determined similarity coefficient values (Table 4). The pairwise genetic distances among the 26 varieties and radio-mutants varied from 0.63 to 0.87, however, the values were as high as 0.73-0.83 in the 11 radio-mutants. The dendrogram based on the similarity matrix using UPGMA revealed four major groups (Fig. 2). Groups A and C contained 'Argus' and its four derived mutants and 'Migok' and its seven derived mutants, respectively. The tree showed evolutionary distance between the standard-type and spray-type chrysanthemums. In particular, the 11 radio-mutants fell within their respective parental groups, and the 10 standard-type varieties or radio-mutants in a total 26 varieties were closely clustered into one group. 'Argus' and its irradiated mutants showed 0.40 to 0.72 range of similarity coefficient using an AFLP, which is similar to other species including rose of

Table 3. Attributes of markers produced by 20 AFLP primer combinations

Primer Combination (PC)	Polymorphic Information Content (PIC)	Marker Index (MI)	Resolving power (Rp)
E-AAC/M-CAA	0.192	19.924	37.308
E-AAC/M-CAC	0.204	33.628	39.308
E-AAC/M-CAT	0.189	10.972	20.308
E-ACA/M-CAA	0.194	13.986	25.923
E-ACA/M-CAC	0.215	28.345	52.923
E-ACA/M-CAG	0.213	26.368	47.077
E-ACA/M-CAT	0.179	15.557	25.769
E-ACC/M-CAA	0.218	40.681	70.923
E-ACC/M-CAC	0.211	39.280	72.385
E-ACC/M-CAG	0.223	44.281	78.308
E-ACC/M-CAT	0.217	36.881	63.000
E-ACG/M-CAA	0.195	18.530	36.846
E-ACG/M-CAG	0.203	26.996	45.462
E-ACG/M-CAT	0.208	27.080	50.308
E-AGC/M-CAA	0.209	33.867	56.923
E-AGC/M-CAC	0.195	25.905	43.231
E-AGC/M-CAT	0.167	20.014	33.231
E-AGG/M-CAA	0.189	23.210	43.308
E-AGG/M-CAC	0.218	42.891	74.231
E-AGG/M-CAG	0.205	27.904	46.846
Max.	0.223	44.281	78.308
Min.	0.167	10.972	20.308
Mean	0.202	27.815	48.181

Table 4. Similarity coefficients among 26 chrysanthemum varieties or radio-mutants

Code	V1	V10	V11	V12	V13	V14	V15	V2	V3	V4	V5	V5M1	V5M2	V5M3	V5M4	V5M5	V5M6	V5M7	V6	V7	V7M1	V7M2	V7M3	V7M4	V8	V9
V1	0.000	0.248	0.319	0.277	0.277	0.268	0.283	0.269	0.238	0.307	0.261	0.278	0.273	0.269	0.262	0.257	0.288	0.263	0.260	0.250	0.302	0.258	0.262	0.305	0.257	0.278
V10		0.000	0.283	0.244	0.257	0.219	0.188	0.231	0.229	0.282	0.258	0.234	0.250	0.237	0.237	0.234	0.277	0.232	0.231	0.243	0.319	0.235	0.251	0.294	0.241	0.234
V11			0.000	0.286	0.275	0.287	0.274	0.287	0.324	0.318	0.330	0.300	0.298	0.298	0.269	0.294	0.314	0.295	0.290	0.304	0.316	0.283	0.301	0.260	0.293	0.290
V12				0.000	0.265	0.252	0.266	0.258	0.273	0.290	0.274	0.247	0.268	0.251	0.240	0.244	0.278	0.251	0.255	0.265	0.316	0.226	0.262	0.287	0.254	0.264
V13					0.000	0.259	0.253	0.255	0.285	0.300	0.291	0.260	0.266	0.264	0.247	0.250	0.289	0.264	0.253	0.254	0.317	0.238	0.246	0.276	0.256	0.262
V14						0.000	0.225	0.246	0.259	0.283	0.270	0.235	0.249	0.240	0.228	0.237	0.265	0.231	0.243	0.237	0.298	0.232	0.234	0.270	0.251	0.245
V15							0.000	0.243	0.252	0.289	0.288	0.246	0.229	0.247	0.231	0.240	0.259	0.253	0.251	0.265	0.303	0.241	0.256	0.273	0.254	0.270
V2								0.000	0.262	0.267	0.272	0.247	0.255	0.245	0.243	0.237	0.286	0.235	0.240	0.246	0.326	0.249	0.257	0.291	0.252	0.245
V3									0.000	0.305	0.254	0.266	0.268	0.266	0.258	0.254	0.281	0.263	0.255	0.266	0.307	0.269	0.263	0.321	0.219	0.259
V4										0.000	0.326	0.296	0.297	0.295	0.283	0.292	0.329	0.302	0.282	0.278	0.357	0.270	0.295	0.324	0.278	0.286
V5											0.000	0.197	0.211	0.182	0.204	0.188	0.226	0.194	0.284	0.263	0.341	0.277	0.281	0.324	0.271	0.283
V5M1												0.000	0.167	0.135	0.150	0.123	0.186	0.148	0.256	0.247	0.319	0.252	0.255	0.292	0.264	0.250
V5M2													0.000	0.165	0.134	0.155	0.168	0.178	0.265	0.258	0.299	0.247	0.273	0.271	0.270	0.281
V5M3														0.000	0.149	0.128	0.185	0.139	0.253	0.244	0.323	0.247	0.246	0.293	0.254	0.262
V5M4															0.000	0.142	0.171	0.168	0.250	0.243	0.287	0.229	0.252	0.262	0.252	0.263
V5M5																0.000	0.166	0.127	0.256	0.236	0.311	0.238	0.250	0.287	0.254	0.251
V5M6																	0.000	0.195	0.292	0.284	0.275	0.284	0.283	0.292	0.294	0.294
V5M7																		0.000	0.257	0.254	0.317	0.257	0.260	0.292	0.256	0.252
V6																			0.000	0.231	0.314	0.238	0.247	0.289	0.247	0.251
V7																				0.000	0.260	0.163	0.172	0.246	0.250	0.263
V7M1																					0.000	0.247	0.244	0.262	0.329	0.332
V7M2																						0.000	0.170	0.229	0.242	0.261
V7M3																							0.000	0.214	0.250	0.244
V7M4																								0.000	0.278	0.294
V8																									0.000	0.260
V9																										0.000

V, variety

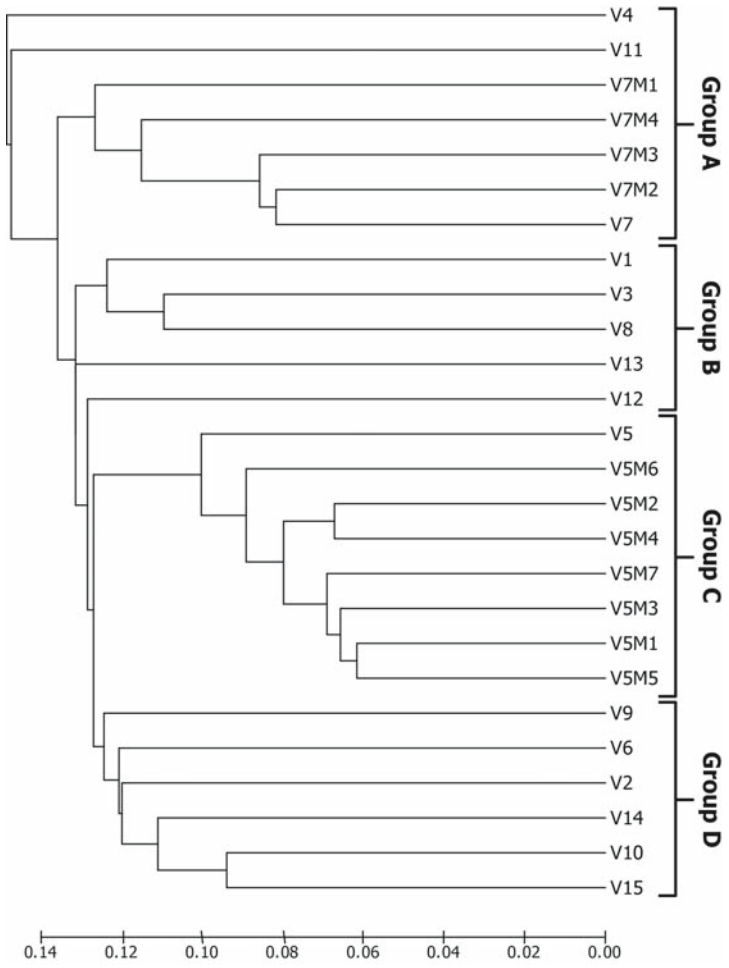

Fig. 2. Dendrogram of 26 chrysanthemum varieties and radio-mutants based on UPGMA clustering analysis of AFLP genotype data.

Sharon and carnation (Sung et al., 2010; Kim et al., 2009; Lee et al., 2002) Recently, a total of 480 Chinese traditional chrysanthemum cultivars were classified based on horticultural traits including petal types, flower head type and flower color, and 204 polymorphic SSR markers (Zhang et al., 2014). However, they recognized the limitation that polyploidy of chrysanthemum makes it hard to analyze the polymorphism results (Zhang et al., 2014)

To identify candidates for mutant-specific markers, we analyzed the AFLP band patterns of standard-type chrysanthemum variety 'Migok', spray-type 'Argus', and their derived radio-mutants. Among 'Migok' and its seven derived radio-mutants, a total of 1,663 bands were produced by the 20 *Eco*RI+3/*Mse*I+3 PCs, and the top five PCs were calculated by the number of mutant specific polymorphism bands per number of total bands (Table 5). Mutant-specific polymorphism varied from 0.9 (E-ACC/M-CAG) to 47.5% (E-ACG/M-CAG). 'Argus' and its four derived radio-mutants produced a total of 2,586 bands, and mutant-specific polymorphism varied from 21.1 (E-AGG/M-CAA) to 44.0% (E-ACA/M-CAT).

The ability to distinguish specific varieties in a population will help breeders to reduce the breeding period. Molecular markers are effective tools for detecting specific varieties (Shao et al. 2010). The discriminatory power of DNA markers is affected by the polymorphism rate among genotypes. AFLP fingerprinting is highly informative for revealing genetic diversity, phylogenetic relationships (Hill et al., 1996; Kardolus et al., 1998; Sharma et al., 1996), and individual variation (Parker et al., 1998). In addition to AFLPs, other molecular markers like RAPD, RFLP, ISSR, SSR, and SNP have also been widely used to analyze genetic diversity or phylogeny in many crop plants. However, very few such DNA markers have been reported for floricultural/ornamental plants (Gupta et al., 2013). Recently, several researchers have tried to elucidate the relationship between morphological characters and genetic diversity using AFLPs in chrysanthemum (Hui et al., 2013; Roein et al., 2014; Qin et al., 2011).

In this study, we analyzed the genetic relationships among diverse chrysanthemums and their derived radio-mutants using the AFLP technique. Two radio-mutants families were

Table 5. List of mutant-specific AFLP marker candidates for standard-type chrysanthemum variety 'Migok' and spray-type variety 'Argus' and their derived radio-mutants

Primer combinations	No. of total bands (A)	No. of polymorphic bands (B)	No. of mutant-specific polymorphic bands (C)	Mutant-specific polymorphism(C/A) *100%	Total polymorphism(B/A) *100%
Standard type chrysanthemum 'Migok' and its derived radio-mutants					
E-ACG/M-CAG	80	73	38	47.5	91.3
E-AGG/M-CAG	89	82	38	42.7	92.1
E-ACC/M-CAT	109	92	24	22	84.4
E-AAC/M-CAT	33	26	3	9.1	78.8
E-AGC/M-CAA	97	94	8	8.2	96.9
Spray type chrysanthemum 'Argus' and its derived radio-mutants					
E-ACA/M-CAT	84	83	37	44	98.8
E-AGC/M-CAT	99	93	41	41.4	93.9
E-AGC/M-CAC	126	120	50	39.7	95.2
E-AAC/M-CAA	104	93	41	39.4	89.4
E-AAC/M-CAT	55	51	18	32.7	92.7

grouped into two major groups (Group A and C), while standard-type varieties or mutant families were clustered in one group (Group C). Given their AFLP parameters (PIC, MI, Rp), the primer combination E-ACC/M-CAG may prove useful for identifying chrysanthemum varieties. Many mutant-specific marker candidates were identified in this AFLP analysis, although some may be false positives. Mutant-specific cleaved amplified polymorphic sequence (CAPS) or sequence characterized amplified region (SCAR) markers linked to mutant traits will be useful to improve the breeding process in the future.

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