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Genetic analysis of *Chrysanthemum* hybrids based on RAPD molecular markers

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Abstract. Forty-five random primers were screened, of which twenty-two primers were selected to detect the molecular marker in three hybrid combinations of *Chrysanthemum* by using random amplified polymorphic DNA (RAPD). From this study, the patterns of molecular markers could be classified into seven types: Type I markers shared bands in both parents, and offspring; Type II markers shared bands in male and female parents; Type III markers shared bands in male parent and offspring; Type IV markers shared bands in female parent and offspring; Type V markers were presented in the male parent only; Type VI markers were present in the female parent only; Type VII markers were present in offspring only. Of these, only Type III markers were suitable for identifying the true male parent. Different unique markers of Type VII in offspring are quite suitable as identifying markers of new hybrids to protect the rights of plant breeders. In this study, 34.4% to 48.9% of the RAPD markers were found to reveal additivity among parents and offspring in three hybrid combinations of *Chrysanthemum.* However, 38% to 52.6% of markers (Type II, V, and VI) were absent in offspring, but 11.6% to 13.1% of unique markers (Type VII) were present in offspring. Moreover, there were no definite rules as to whether markers in offspring were more similar to female or to male parents by similarity analysis. In two hybrid combinations, the parents were more similar to each other than either was to the offspring. The above results illustrate that the genetics of *Chrysanthemum* are very complex. RAPDs, however, are a powerful tool to detect different molecular markers in hybrid populations of *Chrysanthemum* cultivars.

Keywords: *Chrysanthemum*; Hybrids; Molecular marker; RAPD.

Introduction

Chrysanthemum morifolium Ramat (Asteraceae) has been bred for 3,000 years in China and Japan. It is one of the major horticultural crops in the Netherlands today (Wolff et al., 1994). *Chrysanthemum morifolium* cultivars are polyploids belonging to a hexaploid species with an average chromosome number of 54 (Dowrick, 1953; Langton, 1989), but the exact origin of the hexaploid species is still unknown (Wolff et al., 1994). The species has a strong self-incompatibility system, as do all members of the Asteraceae family (Richards, 1986). It is known that the self-incompatibility in the Asteraceae is determined by a multiallelic sporophytic system. This system is correlated with dry papillate stigmas, trinucleate pollen, and the incompatibility reaction at the stigmatic surface (Richards, 1986), but the genetics of *Chrysanthemum* have not yet been completely revealed (Wolff and Peters-Van Rijn, 1993; Zagorski et al., 1983). Selfing is generally not possible, although some pseudo-self-incompatible plants have been discovered (Anderson et al., 1992). The rate of successful crosses between related and unrelated cultivars is low, usually only 5% to 50% (Zagorski et al., 1983). Nevertheless, breeding of *Chrysanthemum* cultivars has been accomplished by traditional techniques.

However, not all types of markers are suitable for breeding applications. Morphological and cytological markers are not useful for breeding analysis (Roxas et al., 1993). Although isozyme markers are useful to characterize genetic diversity (Fiebich and Henning, 1992; Roxas et al., 1993), and to identify the hybrids of cultivars (Roxas et al., 1993), the paucity of isozyme loci restricts their usefulness in breeding (Helentjaris et al., 1986). DNA markers have been used to manipulate marker-assisted selection (MAS), and to guide the introgression of target genes from related species by restriction fragment length polymorphism (RFLP) in the past several years (Wolff et al., 1994). However, RFLP is labor intensive and costly.

An alternative technique for identifying molecular marks called random amplified polymorphic DNA (RAPD) has been developed (Williams et al., 1990). In this method, by using a single arbitrary primer (10 mer) and amplifying DNA by polymerase chain reaction (PCR), the resulting DNA markers easily can be separated on an agarose gel by electrophoresis (Williams et al., 1990). The advantages of RAPD is its simplicity, rapidity, the requirement for only a small quantity of DNA, and the ability to generate numerous polymorphisms (Cheng et al., 1997). Therefore, it has been a powerful technique for genetic analysis (Chapco et al., 1992; Kiss et al., 1993; Landry et al., 1993; Wight et al., 1993; Williams et al., 1990).

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In *Chrysanthemum*, genetic variation is very high between cultivars. These cultivars can be distinguished by using only two different primers based on RAPDs. High levels of polymorphisms at the DNA level in *Chrysanthemum* have been determined (Wolff and Peters-Van Rijn, 1993), and the identical DNA patterns from different accessions of the same *Chrysanthemum* cultivar can be detected by using RAPDs (Wolff et al., 1995). Furthermore, sporting and chimerism of *Chrysanthemum* also revealed different DNA patterns among cultivars in two families and among the layers of one cultivar by RAPD analysis (Wolff, 1996).

The purpose of this study is to set up a MAS system by using RAPDs in *Chrysanthemum* hybrid combinations including parents and offspring. In addition, the potential application of parentage analysis in the identification of genetic markers is discussed.

Materials and Methods

Plant Materials

Four commercial *Chrysanthemum* cultivars (A, B, C, D) and three hybrid combinations were used in this study. These individuals are "Cold Homae" (A), "Red Gafe" (B), "Red Gafe (♀) x Cold Homae (♂)" (BxA), "Yellow Shuho" (C), "Yellow Shuho (♀) x Cold Homae (♂)" (CxA), "White Shuho" (D), and "Yellow Shuho (♀) x White Shuho (♂)" (CxD). These cultivars were grown in Taichung District Agricultural Improvement Station. The flower characteristics are summarized in Table 1.

Preparation of Total Cellular DNA

Total cellular DNA from the leaves of *Chrysanthemum* was prepared by using an extraction technique modified from Shure et al. (1983). 0.5 grams of fresh leaves were harvested and ground to powder with liquid nitrogen in a mortar and pestle, then transferred to a 1.5 ml centrifuge tube (preheated in 60°C water) containing 700 µl of urea buffer (8.0 M urea, 0.05 M NaCl, 0.05M Tris-HCl pH 7.5, 0.02 M EDTA, 1% sarcosyl), mixed thoroughly and incubated in water bath at 60°C for 10 min. The tube was inverted periodically. To this was added 700 µl phenol:chloroform = 1:1 (v/v, Tris pH 8.0 saturated), and the tube was gently inverted repeatedly. The tube was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a new centrifuge tube, and 0.7 volume of 2-propanol and 1/10 volume 4.4 M NH_4OAc were added. The tube was centrifuged at 10,000 rpm 10 min at 4°C to collect precipitated DNA. The DNA pellet was resuspended with 400 µl TE (10 mM, Tris-HCl pH 8.0, 1 mM EDTA) and incubated with 5 µg DNase-free RNase (Sigma) for 10 min at 65°C. The RNase and the remaining protein were extracted with an equal volume of phenol:chloroform = 1:1 (v/v, Tris pH 8.0 saturated) and centrifuged at 10, 000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube, and the DNA was precipitated by the addition of a 1/10 volume 4.4 M NH_4OAc and three volumes of 95% ethanol. Precipitated DNA was collected by centrifugation at 10,000 rpm for 10 min at 4°C, washed with 70% ethanol twice, and dried before redissolving in 200 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Approximate DNA yields were calculated by a spectrophotometer (Hitachi U-2001), and DNA samples were stored at -20°C.

RAPD Reaction

Forty-five decamer oligonucleotide primers (Operon Technologies Inc., Alameda, California) were screened by polymerase chain reaction (PCR). PCR reactions were performed by using a 25 µl mixture, containing 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 0.01% BSA, with four dNTPs (0.2 mM each), 0.2 µM primers, 1.25 units of *Taq* DNA polymerase (Virogene) and 2 ng genomic DNA, and 25 µl mineral oil (Williams et al., 1990). For DNA amplification, the DNA thermocycler (Biometra) was programmed as follows: incubation at 94°C for 3 min; 45 cycles at 94°C for 45 sec, 40°C for 45 sec and 72°C for 1 min 30 sec, followed by one final extension cycle of 3 min at 72°C. The amplification products were separated by electrophoresis in 1.5 % (w/v) agarose (FMC Bioproducts) gels with 0.5 x TBE buffer, stained by 0.5 µg/ml of ethidium bromide (EtBr) and photographed under exposure to UV light.

Data Analysis

Amplified RAPD markers were scored as present (+) or absent (-) for each sample. Ambiguous bands that could not be easily distinguished were not scored (Williams et al., 1990). The similarity of samples was calculated as follows: Similarity = $2\ N_{AB}/N_A+N_B$, N_{AB} is the number of

Table 1. Flower characteristics of seven *Chrysanthemum* cultivars.

Parents & cross	Cultivar name	Flower characteristics		
		Size	Color	Petal shape
A	Cold Homae	Small	Purple	Straight
B	Red Gafe	Small	Purple	Twist
C	Yellow Shuho	Large	Yellow	Twist
D	White Shuho	Large	White	Twist
(BxA)	Red Gafe x Cold Homae	Small	Red	Straight
(CxA)	Yellow Shuho x Cold Homae	Small	Yellow	Straight
(CxD)	Yellow Shuho x White Shuho	Large	White	Straight

bands shared by individuals A and B, and N_A and N_B are the number of bands in individuals A and B, respectively (Chapco et al., 1992; Wilde et al., 1992).

Results and Discussion

Among the forty-five primers screened, twenty-two primers, which were selected, yielded the best product for RAPD analysis (Table 2). Among three hybrid combinations of *Chrysanthemum* BxA, CxA, and CxD, 313, 311 and 308 RAPD markers were revealed, respectively. The RAPD markers could be classified into seven types (Figure 1) according to the presence/absence of bands (Table 3). Among RAPD markers, the band patterns in the hybrids were found to be not completely additive. A similar phenomenon also appeared in the interspecific hybridization in *Cyrtandra* (Smith et al., 1996), and intraspecific crosses of sugarcane varieties (Huchett and Botha, 1995). In the hybrid combinations of BxA, markers of offspring revealed only 48.9% shared markers with parents, including Type I, III, and IV. The hybrid combinations of CxA and CxD revealed 39.2% and 34.4% bands shared with parents, respectively.

Arnold et al. (1991) identified the natural hybrids of Louisiana irises by bands shared with both species. Therefore, Type I, III, and IV markers are good markers to identify the new hybrid from parents to ensure effective selection by plant breeders. In addition, Type III markers are especially important markers to identify the true male parent.

Sources of polymorphisms in RAPD assay may include base change within priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a DNA fragment without preventing its amplification (Williams et al., 1990). In addition, the polymorphisms of RAPD markers were observed as different-sized DNA fragments from amplification. Therefore, differences in markers from parents to offspring may be the result of DNA recombination, mutation, or random segregation of chromosome in meiosis processing during hybridization (Huchett and Botha, 1995; Darnell et al., 1990). In this study, 38.0%, 49.2%, and 52.6% markers from parents, including type II, V, and VI markers were not found in hybrid combinations of BxA, CxA, and CxD, respectively. In *Chrysanthemum*, the strict outcrossing results in higher levels of heterozygosity (Wolff et al., 1994). The high number of bands not shared with parents in offspring of *Chrysanthemum* is probably due to segregation

Table 2. Primers used for the genetic analysis of C*hrysanthemum* hybrids.

Primers used	Sequence (5'→3')
OPA-01	CAGGCCCTTC
OPA-05	AGGGGTCTTG
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPB-01	GTTTCGCTCC
OPB-02	TGATCCCTGG
OPB-04	GGACTGGAGT
OPB-05	TGCGCCCTTC
OPB-06	TGCTCTGCCC
OPB-07	GGTGACGCAG
OPB-08	GTCCACACGG
OPB-10	CTGCTGGGAC
OPB-11	GTAGACCCGT
OPC-01	TTCGAGCCAG
OPC-05	GATGACCGCC
OPC-06	GAACGGACTC

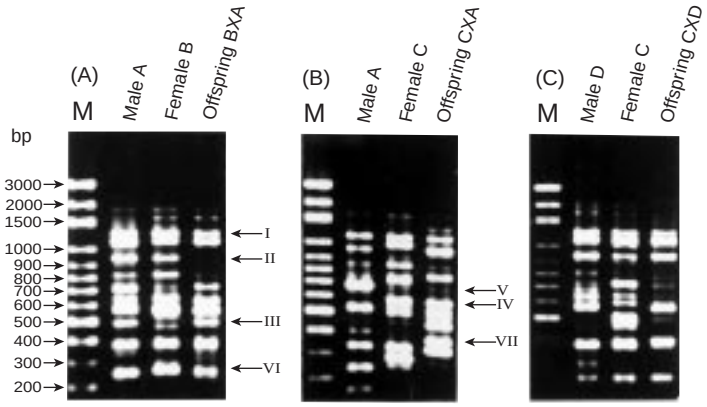

Figure 1. RAPD molecular marker patterns generated (A) with A16 primer in the cross combination of the [*Chrysanthemum morifolium* Ramat "Gold Homae" (A), "Red Gafe" (B) and "Red Gafe (♀) x Gold Homae (♂)" (BxA)]; (B) with B1 primer in the cross combination of ["Cold Homae" (A), "Yellow Shuho" (C) and "Yellow Shuho (♀) x Cold Homae (♂)" (CxA)]; (C) with A16 primer in the cross combination of ["White Shuho" (D), C, and "Yellow Shuho (♀) x White Shuho (♂)" (CxD)]. Roman numerals I through VII denote the following: M = male band, F = female band, O = offspring band; + = present, - = absent; I = M+,F+,O+; II = M+,F+,O-; III = M+,F-,O+; IV = M-, F+,O+; V = M+,F-,O-; VI = M-,F+,O-; VII = M-,F-,O+.

Table 3. The seven types of RAPD markers were identified from three hybrid populations of *Chrysanthemum* cultivars.

Type of markers	Property of markers			RAPD markers of hybrid combinations					
				BxA		CxA		CxD	
	Male	Female	Offspring	(no.)	(%)	(no.)	(%)	(no.)	(%)
I	+	+	+	70	22.4	71	22.8	63	20.5
II	+	+	-	13	4.2	37	11.9	32	10.4
III	+	-	+	53	16.9	38	12.2	15	4.9
IV	-	+	+	30	9.6	13	4.2	28	9.1
V	+	-	-	51	16.3	42	13.5	60	19.5
VI	-	+	-	55	17.6	74	23.8	70	22.7
VII	-	-	+	41	13.1	36	11.6	40	13.0
Total				313		311		308	

+/-: Indicate presence/absence of band, respectively.

of heterozygous chromosomes during meiosis. Chromosomal crossing-over during meiosis may result in the loss of priming sites and thus markers are present in parents but not in offspring (Smith et al., 1996). Furthermore, the phenomenon of non-Mendelian inheritance could be detected because of the existence of competition in RAPD analysis (Lu et al., 1995; Hallden et al., 1996). The aforementioned problem is less serious in the investigation of haploids or completely homozygous material, whereas heterozygous material is more problematic (Hallden et al., 1996). Therefore, it is not surprising to find only a portion of the bands from each parent in the hybrid of *Chrysanthemum*.

Besides, 41 (13.1%), 36 (11.6%), and 40 (13.0%) RAPD markers of type VII (non-parental bands) were detected from offspring of BxA, CxA, and CxD, respectively (Table 3). These non-parental bands may be generated from the recombination and mutation in meiosis processing during hybridization (Darnell et al., 1990; Huchett and Botha, 1995) and may be also created by heteroduplex formation (Ayliffe et al., 1994; Hunt and Page, 1992; Novy and Vorsa, 1996). However, the frequency of non-parental bands of previous reports (Ayliffe et al., 1994; Hunt and Page, 1992; Novy and Vorsa, 1996) is lower than this study.

Of course, unlike two-primer mediated PCR, RAPD assay is performed using low stringency conditions. By interference, mismatches may occur between the primer and its target sequence in the amplification reaction (MacPherson et al., 1993). In fact, different thermal cyclers, temperature profiles, the brand of DNA polymerase, and the concentration of $MgCl_2$, primer and template DNA can effect the reproducibility of RAPD assay (MacPherson et al., 1993; Meunier and Grimont, 1993). Thus, a standardized methodology should be devised for RAPD assay to obtain identical RAPD pattern.

The identification of cultivars or breeding lines is very important in all horticultural and agricultural species in order to protect the rights of plant breeders (Wolff et al., 1995). In *Chrysanthemum*, cultivars are identified in flowering trials, and breeders' rights are presented by cultivar characteristics including flower, leaf and growth morphology (Wolff et al., 1995). The application of isozyme technology can largely improve the identification of *Chrysanthemum* cultivars (Roxas et al., 1993). However, the level of polymorphism obtained is often insufficient to distinguish cultivars, and the growth conditions may influence the quality and quantity of isozymes (Wolff et al., 1995). In this study, it was revealed that several types of markers, especially, Type VII markers are useful in identifying new cultivars. *Chrysanthemum* cultivars are propagated vegetatively by cuttings. The cultivars that are propagated vegetatively must have the same DNA pattern, even after many years of cultivation (Wolff et al., 1995).

Similarity can be used to measure the relatedness of samples (Nybom and Hall, 1991; Welsh et al., 1991). From a similarity matrix of three hybrid combinations of *Chrysanthemum*, it was found that BxA male parent and

Table 4. Similarity matrix of three hybrid combinations of *Chrysanthemum* cultivars.

	A(♂)	B(♀)	BxA	A(♂)	C(♀)	CxA	D(♂)	C(♀)	CxD
A(♂)	1.00								
B(♀)	0.50	1.00							
BxA	0.68	0.55	1.00						
A(♂)				1.00					
C(♀)				0.56	1.00				
CxA				0.63	0.41	1.00			
D(♂)							1.00		
C(♀)							0.52	1.00	
CxD							0.49	0.54	1.00

offspring (0.68) were more similar than female parent and offspring (0.55), and female and male parent (0.50) (Table 4). A similar result also was found in the hybrid combinations of CxA. In the hybrid combinations of CxD, male parent and offspring (0.49) was less similar than female parent and offspring (0.54) as well as between female and male parent (0.52). These results did not match the flower characteristics of *Chrysanthemum* cultivars in Table 1 completely.

In comparing our results with studies on wild species and agricultural cultivars, the *Chrysanthemum* cultivars studied here showed a higher level of genetic variability, probably because of their mating system of strict self-incompatibility (Wolff and Peters-Van Rjin, 1993). Moreover, it was unexpected to find that the similarity between female parent and offspring (0.41) was smaller than between both parents (0.56) of the hybrid combinations of CxA. The similarity between male parent and offspring (0.49) was smaller than between male and female parent (0.52) in the hybrid combination of CxD (Table 4). These phenomena could not be explained well and might be due to the complex and diversified nature of the genotypes of *Chrysanthemum*.

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利用 RAPD 偵測菊花雜交後代遺傳性

黃勝忠　蔡奇助　許謙信

台中區農業改良場

利用 45 條隨機引子，以 RAPD 分析三個菊花雜交組合，其中有 22 條引子可產生有效複製產物。本研究發現 RAPD 的所有分子標誌中可歸為七類。第一類在父、母及子代皆出現的分子標誌；第二類僅出現在父及母代的分子標誌；第三類僅出現在父及子代的分子標誌；第四類僅出現在母及子代的分子標誌；第五類為父親特有的分子標誌；第六類為母親特有的分子標誌；第七類為子代特有的分子標誌。上述七類分子標誌中，第三類分子標誌適合從事鑑別真實父親。第七類標誌為新雜交品種的特有標誌，適合用於保護育種者品種專利的標誌。第一、二及四類標誌為從親代到子代具加成性之標誌。本研究三個菊花雜交組合中，加成性的標誌佔 34.4% 到 48.9%，子代消失之標誌佔 38.0% 到 52.6%，子代特有之標誌佔 11.6% 到 13.1%。此外，由上述三個雜交組合相似性之值發現，子代並不一定與父親或母親相似，甚至部分出現雙親的相似性較親代與子代相似性高的現象。由本試驗之結果可進一部闡明菊花遺傳機制的複雜性。

關鍵詞：菊花；分子標誌；雜交後代；隨機複製多型性 DNA。