J. Agric. Sci. Mansoura Univ., 32 (6): 4155 - 4164, 2007

GENETIC DIVERSITY AND RELATIONSHIPS IN *Chrysanthemum morifolium* AS REVEALED BY RAPD MOLECULAR MAKER, AND SOME MORPHOLOGICAL CHARACTERS

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ABSTRACT

Chrysanthemum morifolium is a perennial flowering plants of high economic value , that is Propagated vegetatively by taking cuttings since it has a strong sporophytic self- incompatibility system. For differentiating between cultivars, traditional methods,morphological characters were established. PCR based molecular marker assays including RAPD,were employed to study genetic diversity and phylogenetic relationships among ten *Chrysanthemum* cultivars.

RAPD analysis using 10 random primers generated 112 discrete markers ranging from, (280-2660 bp) in size. Ninety-one of these were polymorphic (81.3%), with an average of 11.2 markers per primer. The primers; OPA-12,OPD-08,OPB-08, and OPC-14 , could be specific markers for the cultivars; Bari, Bari, Delianne and Grand-white,respectively.

Cluster analysis of RAPD-data using the software program; SPSS version 10 , revealed that the cultivars belonging to the same type, were genetically related more than the others.

These results suggest that RAPD marker is a useful method for *Chrysanthemum* genetic diversity analysis and phylogenetic relationships.

INTRODUCTION

Chrysanthemum is a genus of about 30 species of perennial flowering plants in the family Asteraceae. It has been bred for 3.000 years in China and Japan (Huang, *et. al.* 2000). It is one of the major horticultural crops in the Netherlands today (Wolf, *et. al.*, 1994). *Chrysanthemum morifolium* is a popular cut flower and pot plant of high economic value that is propagated vegetatively by taking cuttings since it has a strong sporophytic self- incompatibility system (Martin, *et. al.*, 2002). *C. morifolium* cultivars are polyploids belonging to hexaploid species with an average chromosome number of 54 (Langton, 1989), but the exact origin of the hexaploid species is still unknown (Wolff, *et. al.*, 1994). The genetics of *Chrysanthemum* have not yet been completely revealed (Zagorsski, *et. al.*, 1983).

Obtaining new varieties showing different characteristics is one of the aims in the commercial strategies of this species (Wolff & Peters-van Rijn, 1993). Identification of varieties or breeding lines is very important in agricultural species, and is particularly interesting in *Chrysanthemum*, when in many cases the origin of varieties is unknown. Traditionally identification

has been based on morphological characters, however the development of new techniques has allowed basing these analyses on DNA information as the PCR based technique RAPD (Williams, *et. al.*, 1990). Random amplified polymorphic DNA (RAPD) has been developed and proved to be a powerful technique for genetic analysis (Chapco, *et. al.*, 1992). There are many advantages of RAPD, it is simple, rapid, needs only a small amount of DNA and it is able to generate numerous polymorphisms (Cheng, *et. al.*, 1997). Also, it is suitable for the analysis of non-studied species, and can be applied at any stage of plant growth (Ling, *et. al.*, 1997 and Badr, *et. al.*, 2006).

Using RAPD-PCR, high levels of polymorphism between the cultivars of *Chrysanthemum* were determined (Wolff & Peters-Van Rijn., 1993), and the identical DNA patterns from different accessions of the same *Chrysanthemum* cultivar can be detected by RAPDs (Wolff, *et. al.*, 1995). Furthermore, sporting and chimerism of *Chrysanthemum* also reveale different DNA patterns among cultivars in two families and among the layers of one cultivar by RAPD analysis (Wolff, 1996).

This study aimed to detect polymorphism and phylogenetic relationships, between ten *Chrysanthemum* cultivars at the DNA level, and also at the level of some morphological characters.

MATERIALS AND METHODS

Plant Materials

Ten commercial Chrysanthemum cultivars were used in this study (table 1). These cultivars presents different types according to the flower structure.

Morphological characters

Four important economic characters were studied; Ray/disc; Response (W) = the number of weeks

Vigour; the value 7 is used for the most vigorous varieties and a lower number for the less vigorous ones, and Vase life (table 1). Each character was scored as present (1), for the desirable state and as absent (0), for the non- desirable one.

Molecular genetic marker

-GenomicDNA extraction

Total cellular DNA was prepared from the leaves of *Chrysanthemum* cultivars according to the procedure of (Saghai-Maroof, *et. al.*, 1984).

-Determination of DNA concentration

between the beginning of the short day period and the flowering date,

DNA concentration was determined by gel electrophoresis according to (Sambrook, *et. al.*, 1989). The DNA concentration of each sample was adjusted to 5µg / µl for RAPD analysis.

-Random Amplified Polymorphic DNA (RAPD)

Polymerase chain reaction (PCR) program:

PCR mixture (25 µl) consisted of 5µl 10 X PCR buffer, 5 µl dNTPs, 5µl primer and 1 unit Taq polymerse. The PCR is an accurate method for DNA amplification to reach measurable levels of the nucleic acid. A modified

PCR with higher temperature for annealing was used according to Abdel-Ghany and Zaki (2003). Amplification was carried out in a thermocycler (Eppendorf, Germany) programmed for 35 cycles. Primary denaturation was given 5 min. at 95 °C, denaturation occurred for 30 sec. at 95 ° C, annealing was allowed for 30 sec. and the reaction temperature varied and was dependent on each primer (table 2).

Table (1) *Chrysanthemum* cultivars, their Characteristics and some morphological characters.

Type	Characteristics	Cultivar	Ray/disc (mm)	Response (W)	Vigour	Vase life(day)
Single	Have disc florets that form Daisy or Marguerite "eye" in the center of the flower. The ray florets are arranged in not more than 5 rows.	Bacardi	65/15	7	6	13
		Bari	75/18	7.5	5.5	12
		Lineker	60/15	7	7	16
		Reagan	75/15	7	5	10
Decorative	Ray florets are regularly or irregularly reflexed. This is the most common garden class; the blooms measure 5-10 cm. The ray florets can be long.	Euro	80/20	5.5	5.5	12
		Fiji	90/20	4.5	4.5	10
		I bis	60/10	5	5	11
Pompon	Blooms are usually spherical, though sometimes they are almost flat when small. They are usually not over 10 cm wide. Some Pompon varieties have incurved ray florets in the center of the flower head.	Feeling white	35/05	7	6	12
Anemone	The ray florets are in 5 or fewer rows and shorter petaloid disc flowers form a cushion-like center.	Grand white	90/15	7	7	18
Spider	Have relatively long, thin and tubular ray florets of irregular length, sinuous or hooked at the tips.	Delianne	45/25	7.5	5.5	11

Data analysis

To identify and characterize the polymorphism and phylogenetic relationships between the ten *Chrysanthemum* cultivars, the polymorphic fragments of electrophoretic DNA produced by RAPD-PCR amplification, with ten random primers, were scored as present (1), and absent (0), then were used to produce a dendrogram. Another linkage distance dendrogram on the basis of morphological characters, and a third one on the basis of both DNA and morphology together were constructed, using the software program SPSS version10.

Table (2): Primers' sequences employed in the RAPD-PCR, their annealing temperatures and G-C Contents.

Primer	Sequence 5`-3`	Annealing Tm (°C/30Sec)	G-C Content (%)
OPA-10	GTGATCGCAG	32	60
OPA-12	TCGGCGATAG	32	60
OPA-17	GACCGCTTGT	32	60
OPB-08	GTCCACACGG	35	70
OPC-10	TGTCTGGGTG	32	60
OPC-12	TGTCATCCCC	32	60
OPC-14	TGCGTGCTTG	32	60
OPD-05	TGAGCGGACA	32	60
OPD-07	TTGGCACGGG	35	70
OPD-08	GTGTGCCCCA	35	70

RESULTS AND DISCUSSION

RAPD-DNA analysis and polymorphism

Genetic polymorphism and phylogenetic relationships between 10 *Chrysanthemum* cultivars were detected according to four morphological characters(table 1), and RAPD-PCR analysis using 10 random primers, which reflects wide variations in the numbers of total DNA fragments and the numbers of amplified polymorphic bands (table 3). The primers OPA-10 and OPC-12 gave the highest number of amplified products (15 bands for each), while the lowest number of PCR fragments was found with the primer OPA-17, which had only 7 bands. As for the band numbers of PCR products with other primers, they ranged from 8 bands resulted by OPD-05 till 14 bands from using OPA-12.

The total number of amplified PCR banding patterns after using ten primers was 112, with sizes ranging from 280 bp to 2660 bp (Fig. 1), which presented all RAPD amplification products of the ten *Chrysanthemum* cultivars, (a) shows the DNA profile with the primer OPA-10, which had high polymorphism, but also produced a specific band (300 bp) with the cultivars Lineker and Ibis. (b) presents results with primer OPA-12 that linked using all samples, and gave the highest degree of polymorphism (92.9 %). Also, it produced a specific band (300 bp) in each of Reagan, Delianne, and Feeling-white, beside another one (1050 bp) with Fiji, and Delianne, also, a specific band (380 bp) in Bari. The results of primer OPA-17 are in (c), in which all samples gave a pattern with low polymorphism. In (d)results of primer OPB-08 had high polymorphism, and a specific band in Delianne (450 bp), another one (400 bp) in Feeling-white, and Euro.(10) OPC-10 showed the lowest percent of polymorphism (70%), and a specific band (280 bp) with Ibis, Bacardi, and Lineker.(f) OPC-12 was of high polymorphism, with a specific band (300 bp) in Grand white and Lineker. Primer OPC-14, in (g) had a

polymorphic pattern with high molecular weight specific bands, (2642 bp) in Reagan, Bacardi Lineker and Euro, also a unique band (2400 bp) in Grand white.(h) OPD-05 had a pattern with low polymorphism, and a specific band (400 bp), with Euro and Feeling white.(i) demonstrates Primer OPD-07 presented medium percent of polymorphism, and a specific band (400 bp) with Fiji, and Bacardi While the primer OPD-08 in (j) had a high polymorphic pattern and only one specific band (400 bp) with Bari.

This results agreed with the findings of Martin *et. al.* 2002 and Wolf 1996, who reported that *Chrysanthemum* cultivars show high levels of genetic variability , when RAPD was used . It proved to be a very useful and rapid , even if the scored number of markerswas not very high .

It can be concluded that RAPD markers successed in differentiating *Chrysanthemum* cultivars. Also, each of the primers; OPA-12, OPD-08, OPB-08, and OPC-14, showed a specific band in only one cultivar from the following ; Bari (380 bp), Bari (400 bp), Delianne (400 bp) and Grand white (2400 bp) respectively, therefore they could be specific markers for these cultivars only.

RAPD banding patterns presented a total number of 112 markers, being 91 of them polymorphic (table 3).This analysis revealed high diversity between the cultivars, the primer OPA-12,gave the highest percent of polymorphism (92.9%), followed by the primers OPC-12, and OPB-08, which presented high percentages of polymorphism (86.7%), and (84.6) respectively.

The lowest percent of polymorphism was produced by the primer OPC-10 (70%) followed by the primer OPA-17 (71.4%). The other primers showed medium percentages of polymorphism ranged from (75% - 81.8%).

Sources of polymorphisms in RAPD assay may include base change within priming site sequence,deletion of priming site, or priming sites are too distant to support amplification , and deletion or insertions that chang the size of a DNA fragment without preventing its amplification(Wiliams *et al.* 1990).

Different thermal cyclers, temperature profiles, the type of DNA polymerase ,and the concentration of Mg Cl_2 , primer and templateDNAcan affect the reproducibility of RAPD assay (Mac Pherson *et. al* .1993, Meunier and Grimont, 1993).Thus a standardized methodology should be devised for RAPD assay to obtain identical RAPD patterns (Huang *et.al.*,2000)

Phylogenetic Relationships

A dendrogram tree was constracted at the basis of four morphological characters (Fig 2). It revealed two clusters ; the first had two sub-clusters ,one of them contained allthe cultivars of the type Decorative ; Fiji, Euro, and Ibis . While the cultivars of the type Single, showed relatedness to each other ; Bacardi& Lineker,or with other types ; Bari & Delianne(Spider) , also, Reagan with Feeling-white (Pompon).

The dendrogram tree of the polymorphic DNA fragments(Fig 3) from all the ten cultivars produced a cluster ,with two sub-clusters. Two cultivars from type Decorative were related ; Ibis and Figi, while the third one Euro was distant, but each two of the cultivars from type Single were genetically related ; Lineker &Bari also, Reagan & Bacardi .As for Delianne (Spider), Feeling-

white (Pompon) and Grand-white(Anemone), were related to Bacardi, Euro ,and Reagan respectively.

The phylogenetic relationships according to the combined data of morphology, and RAPD products together (Fig 4) revealed a great similarity to the genetic relationships constructed due to the RAPD products only . This similarity may be due to the fact that morphological character alone could not be a true measure for phylogenetic relationships and RAPD technique proved to be an accurate method for that (Martin *et. al.*, 2002,and Wolf, 1996). This also may be due to the limited number of genes controlling the studied morphological characters ,while the DNA fingerprinting detected the differences in the whole genome (Murphy *et. al.*, 1990).

Table (3): Amplified polymorphic bands, and percent polymorphism in DNA of the ten Chrysanthemum cultivars using ten primers.

Primer	No. of total DNA bands	No. of polymorphic bands	% Polymorphism
OPA-10	15	12	80.0
OPA-12	14	13	92.9
OPA-17	7	5	71.4
OPB-08	13	11	84.6
OPC-10	10	7	70.0
OPC-12	15	13	86.7
OPC-14	9	7	77.8
OPD-05	8	6	75.0
OPD-07	10	8	80.0
OPD-08	11	9	81.8
Total	112	91	81.3
Average	11.2	9.1	

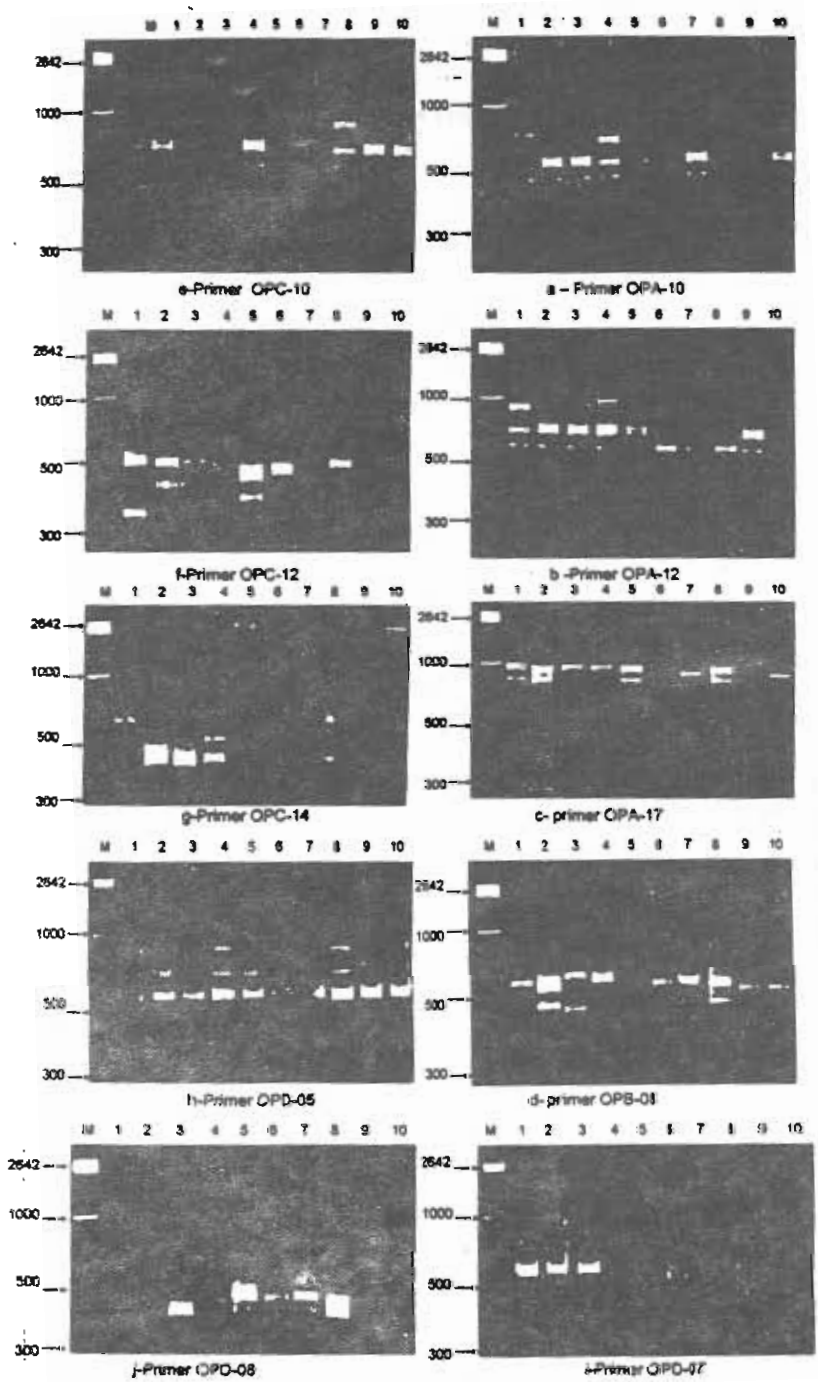

Fig. (1, a-j): Marker: 100 bp DNA Ladder and the PCR products of 10 Chrysanthemum cultivars in Lanes 1-10: Grand white, Reagan, Ibis, Fiji, Bacardi, Dallasne, Lineker, Bart, Feeling white, and Euro, respectively using ten primers.

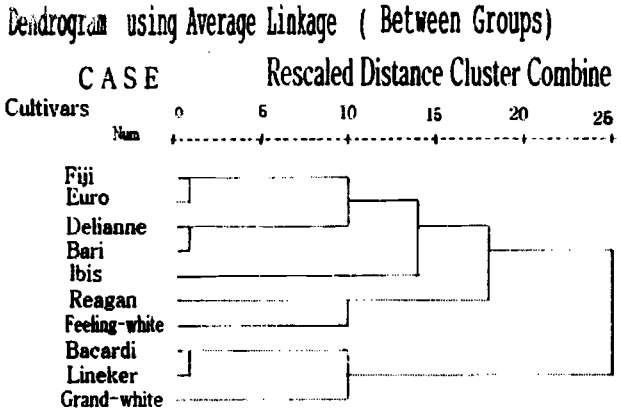

Fig (2: The phylogenetic tree constructed on the basis of four morphological characters.

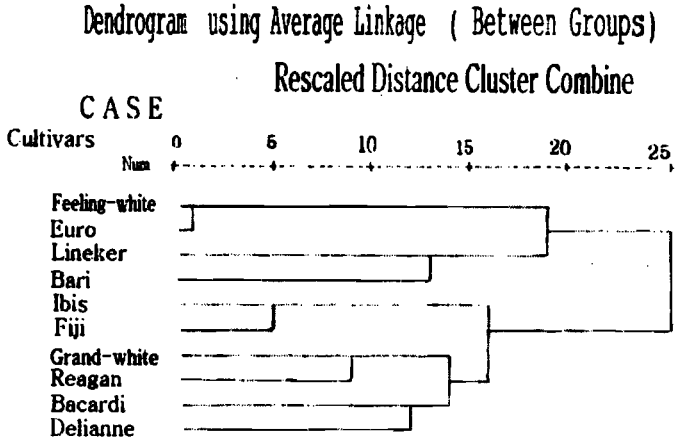

Fig (3 The phylogenetic tree constructed on the basis of RAPD - DNA profiles .

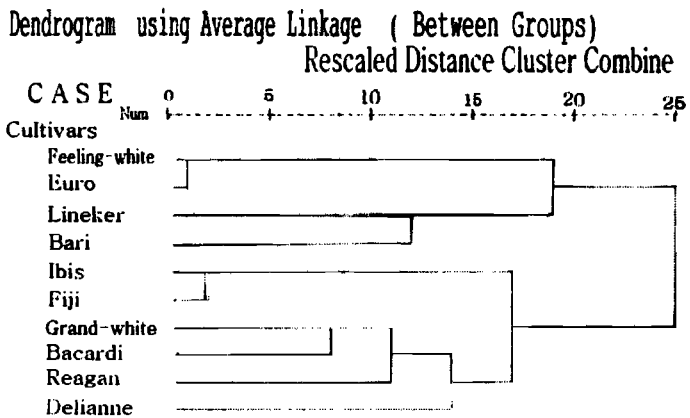

Fig ( ) : 4 he phylogenetic tree constructed on the basis of combined data of RAPD - DNA and morphological characters.

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الإختلافات الوراثية وعلاقات التقارب فى أصناف *Chrysanthemum morifolium* بإستخدام التكنيك الجزيئى RAPD وبعض الصفات الإقتصادية

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يعتبر الكريزانثيم من النباتات الزهريه ذات القيمة الإقتصادية المرتفعة وهو يتكاثر خضريا لوجود ظاهرة عدم التوافق الذاتى به ، ولقد إستخدمت بعض الصفات الإقتصادية الهامة وكذلك التكنيك الجزيئى RAPD للتمييز بين عشرة أصناف من الكريزانثيم ولإظهار الفروق الوراثية وعلاقات التقارب بهدف تطبيقها عند تحسين هذه الأصناف. فى الRAPD إستخدمت عشرة بادئات عشوائية وأظهرت النتائج ١١٢ ممتوم وراثى (marker)،تراوحت أحجامها من ٢٨٠ وحتى ٢٦٦٠ قاعده مزدوجه ، ٩١ ممتوم وراثى كانت متعددة الأنماط بنسبه مئويه ٨١,٣% بمتوسط ١١,٢ ممتوم وراثى لكل بادىء.

كل واحد من البادئات OPC-14, OPB-08 , OPD-08, OPA-12 أعطى ممتوم وراثى خاص يميز واحد فقط من الأصناف وهى Bari , Bari, Delianne , Grand – white

تحليل التقارب الوراثى لنواتج ال RAPD بإستخدام برنامج الحاسب الآلى SPSS version 10 أوضح أن الأصناف التى تنتمى لطراز واحد يوجد بينها تقارب وراثى أكبر من تلك التى تنتمى لطرز مختلفة. وأكدت النتائج على أن التكنيك الجزيئى RAPD هو طريقة مفيدة فى التمييز وتوضيح العلاقات الوراثية والتطورية بين أصناف الكريزانثيم.