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ASSESSMENT OF GENETIC VARIABILITY IN DIFFERENT CHRYSANTHEMUM VARIETIES OF PAKISTANUSING RAPD MARKERS

S. Naz[1], I. Shehzadi[1], K. Shahzadi[2] and S. Ilyas[1]

[1]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan.
[2] Department of Botany, Lahore College for Women University, Lahore, Pakistan.
Corresponding Author e.mail:drsnaz31@ hotmail.com

ABSTRACRT

Twenty one Chrysanthemum cultivars of three groups i.e. Single chrysanthemum (Pompon, Korean, Spoon shaped), Semi double chrysanthemum, Double Chrysanthemum (Anemone, Reflex, Intermediate incurved, Irregular incurved, Regular incurved, Quill) were characterized by RAPD to understand the extent of genetic diversity and relatedness among them. Out of 25 RAPD primers screened, 10 were selected that produced a total of 87 bands, of which 71 were polymorphic with 8.7 bands per primer.The Jaccard Coefficients was used to calculate the genetic similarity. UPGMA was used to generate the dendrogram that clearly separated 21 Chrysanthemum cultivars into 3 main clusters. The average genetic similarity observed across all the cultivars was 0.57.Two types of Quill (Off-white and Dark brown) showed maximum similarity value of 0.95.Hence in the present study RAPDs proved them to be the marker of choice for the detection of variants in different hybrid combination of chrysanthemum including parents and offspring and in marker assisted selection in chrysanthemum breeding programs.

Key words: Chrysanthemum cultivars, RAPD, genetic diversity

INTRODUCTION

In the trade of global flower market, Chrysanthemum is the second largest cut flower after rose. It is being cultivated both as cut flower and potted plant.Chrysanthemum is originated from Greek word "chryos" means Gold and "anthos" mean flower. According to Dwivedi and Banerji, (2009) it was first cultured in China about 3500 years ago.

Chrysanthemum belongs to family Asteraceae and bears characteristic inflorescence, the capitulum with one or two types of florets i.e. ray florets and disc florets. Flower color ranges from white to yellow, pink, bronze, red, deep purple and green. It is short day herbaceous perennial plant that propagates naturally by stolons; asexually by stem cuttings taken from basal shoots and vegetativelyby cuttings and suckers. Plant height ranges from 1/3 to 1m. The plants are erect with lower pannified and upper oblong, deeply incised and dentate leaves (Akbar and Mir, 2006). *Chrysanthemum sp.* grows in semi dry soils but mostly prefers loamy soils with almost neutral pH (Barakat *et al*., 2010).

Due to its medicinal value, its tea from leaves is used to cure bad cold, indigestion and diarrhea. Dried flowers are useful to make menstrual flow, bring about abortion and to treat intestinal worms and indigestion (Kazuhiko *et al*., 2005). The extracts of *C. coronarium* flower have antibacterial, insecticidal and antiparasitic activities (Oka *et al*., 2000).It also helps to reduce indoor air pollution (Hasan *et al*., 2012).

Morphological study is commonly used to find out the genetic diversity as it is simple technique to measure the genetic variation. Genetic variability among *Chrysanthemum sp.* has been assessed traditionally by morphological markers (Fu *et al*., 2008)but a number of molecular markers have alsobeen used to identify the variation in ornamental plants (Rout &Mohapatra, 2006). Molecular markers have a number of advantages over the morphological measurement in order to measure the genetic diversity (Liu *et al*., 2008) as they suggest a first Screening, a more accurate discriminatory authority and are independent of the environmental factors (Vicente and Fulton, 2003).RAPD markers are produced from the amplification of PCR of genomic DNA fragments have advantages including quickness, requirement of low amount of DNA and the ability to generate various polymorphisms (Williams *et al*., 1990).The objective of present research was to determine the genetic diversity and development of phylogenetic tree of different Chrysanthemum cultivars by RAPD markers.

MATERIALS AND METHODS

Plant material: A total of 21 chrysanthemum genotypes (Table 1) used in this study were collected from Bagh-e-Jinnah, Lahore, Pakistan.

Morphological and molecular characterization of chrysanthemum: Morphological characters were studied in selected chrysanthemum by already set standards. These Characters include the plant height, stem, size, shape, arrangement, venation of leaf and types of

inflorescence. For Molecular characterization, RAPD (Random Amplified Polymorphic DNA) analysis selected for the estimation of genetic diversity and phylogeny among these chrysanthemum cultivars.

DNA isolation: Total genomic DNA was isolated from fresh and healthy leaves using the CTAB (hexadecyl trimethyl ammonium-bromide) method (Murray and Thompson, 1980) with few modifications as described earlier by Naz *et al*.,(2014).

Polymerase Chain Reaction (PCR): The RAPD primers were purchased from Invitrogen product (Invitrogen, USA). A total of 25 decamer oligonucleotides of arbitrary sequence were tested for PCR amplification. The basic protocol reported by William *et al*. (1990) for PCR was performed in a total volume of 15 µl, containing 20ng/$\mu L^{-1}$of template DNA, 0.4µM of single primer, 0.6UTaq DNA polymerase (Invitrogen, USA) 0.20µMdNTPs, 1.5mMMgCl_2, 10mMTris-HCl and 50mMKCl.

DNA amplification was carried out in advanced Primus-96 Thermal cycler and the thermal cycler conditions for PCR reactions were an initial denaturation cycle of 94°C for 4 minutes was followed by 35 cycles comprising 30 sec at 94°C, 1 min at 36°C and 2 min at 72°C. An additional cycle of 7 min at68°C was used for final extension. Amplified products were separated by electrophoresis on 1.0 % agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination by Gel Doc Apparatus.

Data Analysis: Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying different cultivars were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using 'Simqual' a sub-program of NTSYS-PC (Numerical taxonomy and multivariate analysis system program) (Rohlf, 1993). The dendrogram was constructed by using a distance matrix using the un-weighed pair group method with arithmetic average (UPGMA) subprogram of NTSYS-PC.

RESULTS

Morphological characterization: All 21 selected chrysanthemum accessions were characterized morphologically in this study by comparing the height of plant, leaf length, shape, arrangement and inflorescence type.

This study revealed that maximum height of plant was 117cm in yellow irregular incurved while minimum height of plant was 35 cm in white pompon.The maximum leaf blade length (14cm) was noted in purple irregular incurved and minimum length (3 cm) was found in white Korean. In inflorescence morphology these cultivars showed variety of colors. Similarly Maximum inflorescence size (17 cm) was obtained by yellow reflexwhile minimum inflorescence size (2.5cm) was found in purple semi double (Figure 1A-I).

After screening 25 primers, 10 primers produced polymorphic and repeatable products. The banding profile and polymorphism generated using the one primer (CHP-10) (Figure 3) is shown. PCR amplification of the DNA isolated from 21 selected chrysanthemum cultivars yielded a total of 87 amplified products out of which 71 were polymorphic and 16 were monomorphic (Table 2).

The total number of DNA bands amplified varied between 6 (Primer CHP- 04) and 12 (Primer CHP-02) with the average of 8.7 bands per primer. The maximum number of polymorphic bands (10) was obtained with primer CHP-02 and the minimum number (3) was obtained with primer CHP08. The polymorphism percentage ranged from 42% (Primer CHP-08) to as high as 100% for two primers (CHP-01, CHP-09). Average polymorphism across all 21 varieties was found to be 81.6%.Polymorphism analysis was done for 2 Pompon cultivars (White pompon, Mustard pompon) and RAPD amplification resulted in the total of 90 bands. Out of them, 16 were monomorphic and 74 were polymorphic. Primer CHP-07 generated maximum polymorphic bands and showed 90% polymorphism. A total of 25 amplified products were obtained from Korean (White Korean). Out of these, 10 were monomorphic and 15 were polymorphic. Primer CPH-08 failed to amplify the DNA.Polymorphism analysis was done for 2 spoon shaped chrysanthemum cultivars (maroon, purple) resulting in a total of 40 amplified products, of which 30 were polymorphic and 10 were monomorphic. Primer CPH-09 generated the maximum number of polymorphic bands (8) and primer CPH-04 produced the minimum number of polymorphic bands (1). Primers CPH-01 and CPH-06 failed to amplify the DNA.

A total of 105 amplified products were obtained from 3 cultivars of semi double group of Chrysanthemum (purple, pink, marron). Out of these, 95 were polymorphic and 10 were monomorphic. Primer CPH-07 generated maximum number of polymorphic bands (6) whereas each of the primers CPH-01, CPH-03, CPH-05 and CPH-10 generated only one monomorphic band. Primer CPH-06 generated 100% polymorphism. Polymorphism analysis was done for 3 cultivars of Anemone chrysanthemum (Yellow, Light Pink, Dark pink) resulting in a total of 77 amplified products, out of which 30 were monomorphic and 47 were polymorphic. Primer CHP-07 generated the maximum number of bands and Primer CHP-01; CPH-05 produced the minimum number of bandsamong Anemone chrysanthemum. The highest level of polymorphism was detected with primer

CHP-06 (80%) whereas primer CPH-01 detected the least polymorphism (30%).A total of 66 amplified products were obtained with DNA amplified from 2 Reflex cultivars (Purple reflex, Yellow reflex).Out of them, 50 were polymorphic and 16 were monomorphic. Primer CHP-07 generated the maximum number of polymorphic bands whereas Primer CHP-08 produced minimum number of bands. Polymorphism analysis was done for 2 Intermediate incurved chrysanthemum (Brown, Purple) and RAPD amplification within these cultivars resulted in a total of 40 products, of which 30 were polymorphic and 10 were monomorphic. Primer CHP-07 generated the maximum number of polymorphic alleles while Primer CHP-04 produced the minimum number of alleles among this group.

Polymorphism analysis was done for 2cultivars of irregular incurved (Yellow, Purple) and a total of 45bands were obtained.Of which, 15 were monomorphic and 30 were polymorphic. Primer CHP-06 generated maximum number (4) of polymorphic bands while CHP-04 produced the minimum number (1) of polymorphic bands. The highest level of polymorphism (100%) was detected by primer CHP-07 whereas primer CHP-01 detected the lowest level of polymorphism (33%) among these cultivars.A total of 50 amplified products were obtained from 2 Regular incurved(Dark purple, Yellow). Out of these, 25 were polymorphic and 25 were monomorphic. Primer CPH-06 and CHP-02 generated maximum number of polymorphic bands (5) whereas each of the primers CPH-02, CPH-09and CPH-10 generated only one band. Primer CPH-06 generated 100% polymorphism.Polymorphism analysis was done for 2cultivars of Quill (off-white, Dark brown) and RAPD amplification within these resulted in a total of 55 amplified products of which 15 were monomorphic and 40 were polymorphic. Primer CHP-10 generated maximum number (6) of polymorphic bands while CHP-06 produced the minimum number (1) of polymorphic bands. The highest level of polymorphism (100) was detected by primer CHP-10 whereas primer CHP-01 detected the lowest level of polymorphism (33%) among this group.

Genetic polymorphism among chrysanthemum varieties: Data of RAPD markers scanned from the 21 genotypes with 10 reproducible primers was used to generate similarity coefficients. A maximum similarity value of 0.95 was observed between off white quill and dark brown quill.

The cluster tree analysis (Figure 2) showed that the chrysanthemum genotypes were broadly divided into 3 main groups representing Single, Semi double and double. Three main groups were branched at similarity value of 0.59. First majorcluster consisted of two sub-clusters in which a high genetic similarity was observed between off white quill and dark brown quill (0.95). Dark purple regular incurved merged into this sub-group at different similarity coefficient value of 0.69. The second subgroup consisting of yellow irregular and purple irregular showed a similarity coefficient of 0.72. The second major cluster included double type chrysanthemum showed genetic similarity of yellow anemone with dark pink anemone at a coefficient value of 0.77 and light pink anemone showed similarity 0.77 with these two species. Yellow reflex was found to be closed to purple intermediate at the value of 0.79 which merged with the above cluster at similarity value of 0.76. Yellow regular incurved merged with the above two species at a similarity coefficient 0.75. Purple reflex merged into this sub-group at different similarity coefficient value of 0.72. The third major cluster was comprised of three subgroups. Two genotypes of semi double chrysanthemum namely pink and maroon semi double showed closeness at a similarity value of 0.92.

Table 1. Chrysanthemum Cultivars used to study phylogenetic relationship by RAPD markers.

Sr#	Chrysanthemum Cultivars	Groups based on Flowering
	Pompon chrysanthemum	
01	White pompon	
02	Mustard pompon	
	Korean	Single
03	White Korean	
	Spoon shaped	
04	maroon	
05	purple	
	Semi double	
06	Purple	Semi
07	Pink	double
08	Marron	
	Anemone	
09	Yellow	
10	Light pink	
11	Dark pink	
	Reflex	
12	Purple reflex	
13	Yellow reflex	
	Intermediate incurved	
14	Brown	
15	Purple	Double
	Irregular incurved	
16	Yellow	
17	Purple	
	Regular incurved	
18	Dark purple	
19	Yellow	
	Quill	
20	Off-white	
21	Dark brown	

The purple semi double was merged in this cluster at a similarity coefficient of 0.86. The second sub groups included maroon spoon and purple spoon showed a similarity coefficient 0.83. In the third subgroup two genotypes of single chrysanthemum group namely white pompon and mustard pompon which were close to each other and showed a similarity value of 0.94. The white Korean merged in this subgroup at a similarity value of 0.79.

Table 2. Polymorphism generated in 21 chrysanthemum varieties using RAPD markers.

S. No.	Primer code	Primer Sequence	Total bands	Polymorphic bands	% of Polymorphism
01	CHP-01	CAAACGTCGG	9	9	100
02	CHP-02	AGCGTGTCGT	12	10	83
03	CHP-03	CGGCCCCGGT	11	10	90
04	CHP-04	GATGCAGGAT	6	5	83
05	CHP-05	GAGGGTGGAG	10	8	80
06	CHP-06	AGCCAGCGAA	10	6	60
07	CHP-07	GTTTCGCTCC	7	6	85
08	CHP-08	CTCACCGTCC	7	3	42
09	CHP-09	GAGAGCCAAC	8	8	100
10	CHP-10	CATCCGTGCT	7	6	85
		Total	**87**	**71**	**81.6**

Figure1: **A)** Single Chrysanthemum plant height, **B)** Leaf arrangement and shape, **C)** Flower color and size, **D)** Semi double Chrysanthemum plant height, **F)** Leaf arrangement and shape; **F)** Flower color and size; **G)** Double chrysanthemum plant height; **H)** Leaf arrangement and shape; **I)** Flower color and size

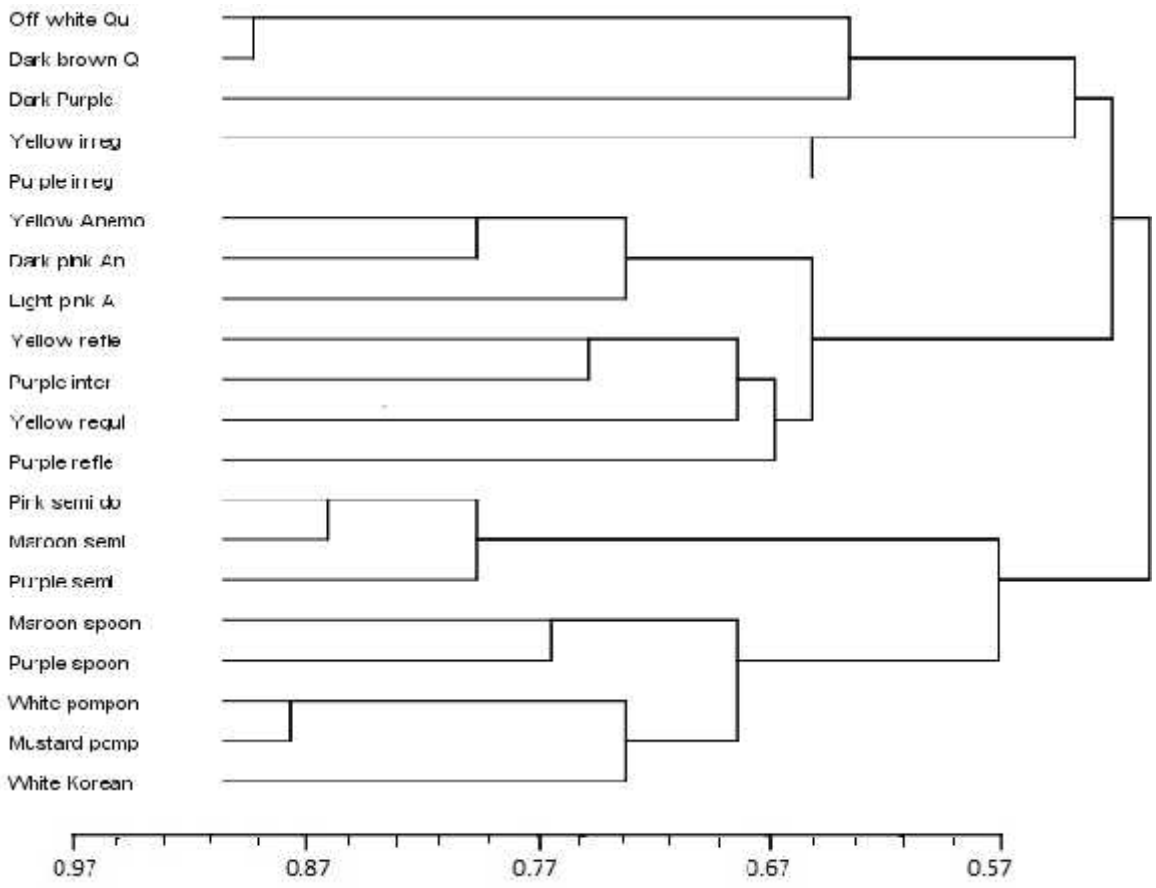

Figure 2. Phylogenetic tree illustrating the genetic relationship among 21 Chrysanthemum genotypes, generated by UPGMA cluster tree analysis

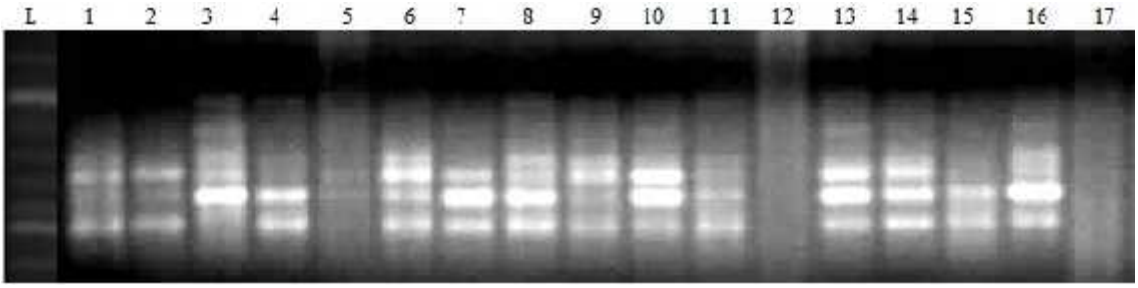

Figure.3. RAPD fragment pattern of 21 chrysanthemum varieties by primer 10. [Lane 1, Marker; Lane 2, white pompon; Lane 3, Mustard Pompon; Lane 4, White Korean; Lane 5;Maroon spoon shaped; Lane 6, Purple spoon shaped; Lane 7, Purple semi double; Lane 8, Pink semi double; Lane 9, Maroon semi double; Lane 10, Yellow Anemone; Lane 11, Light pink Anemone; Lane 12, Dark pink Anemone; Lane 13, Purple reflex; Lane 14, Yellow reflex; Lane 15, Brown intermediate incurved; Lane 16, Purple intermediate incurved; Lane 17, Yellow irregular incurved; Lane 18, Purple irregular incurved; Lane 19 Dark purple regular incurved; Lane 20, Yellow regular incurved; Lane 21, Off-white Quill; Lane 22, Dark brown Quill].

DISCUSSION

Experiments with chrysanthemum cultivars have shown the potential of RAPD markers as a fast, reproducible and useful method for distinguishing among different cultivars and clustering genotypes in the chrysanthemum species.These primers could also be used as marker assisted selection in the chrysanthemum breeding programs. New variants in chrysanthemum are generally formed through vegetative propagation but

spontaneous breeding or artificially induced 'sports' can also contribute to develop new variants (Chatti *et al*., 2007).A number of different molecular markers (Aparajita and Rout, 2010) especially RAPDs have been used to detect the variants in ornamental plants (Barik *et al*., 2006; Rout and Mohapatra, 2006) including different species of chrysanthemum (Seherawat *et al*., 2002). RAPDs being fast, easily generated, reliable and reproducible were also used to study marker assisted selection and to analyze molecular variation in chrysanthemum hybrid combinations including parents and off springs (Chanug *et al*., 2000).

The high level of polymorphism (81%) obtained in this study indicates greater genetic diversity between the selected genotypes which include genotypes that belong to 3 major categories i.e. single, semi double and double chrysanthemum.The total obtained amplified products (87) using 10 RAPD primers in the present study is quite consistent with the results reported byWolff, 1993; Wolff *et al*., 1995). They reported 67 polymorphic bands were generated by using 8 RAPD primers while working on two cultivar of chrysanthemum namely Nero and Wonder.Similar results were also obtained by using RAPD fingerprinting. Average polymorphism across all 21 chrysanthemum genotypes was 81.6%.

RAPD amplification within 2 Pompon cultivars (White pompon, Mustard pompon) resulted in a total of 90 bands. Out of these, 16 were monomorphic and 74 were polymorphic. Ruminska *et al*., (2008) also studied 79 polymorphic fragments produced by 8 primers among 10 radio-mutants of chrysanthemum cultivars.The number of DNA fragments amplified ranged from 6-12 depending upon the primer and the DNA sample. High similarity coefficient value of 0.94 was found between two cultivars namely off white quill and dark brown quill similar to the results obtained by Huang *et al*., (2001) that male parent and offspring were more similar with similarity coefficient 0.68.

The results from the present study clearly demonstrate that the production of different polymorphisms were restricted to a specific variant or varieties. In some cases, few light bands with lower intensity appeared in the variants. The differences at the DNA level as shown in the present study were in agreement with Malaure *et al*., (1991 b) and Wolff and Peters-Van (1993).The high similarity (0.95) between the cultivars is probably due to their common origin by mutation.

In conclusion, PCR technology can be deployed to distinguish the variants established through artificial sporting, spontaneous breeding and chimeras. RAPDs used in the present study revealed variations among the variants and hence proved that these can be helpful if used in combination with traditional morphological markers for the identification of variants among Chrysanthemum species.

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