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Research Article

Genetic diversity revealed in commercial varieties of Chrysanthemum (*Dendranthemagrandiflora*) using RAPD markers

P.Lalitha Kameswari[1], G.Anuradha[2] , M. Pratap[3]and Hameedunnisabegum[4]
[1]Floricultural Research Station, Rajendranagar, Hyderabad
[2]Institute of Biotechnology, ANGRAU, Rajendranagar, Hyderabad
[3]College of Horticulture, Rajendranagar, Hyderabad
[4]Vegetable Research Station, Dr. YSR Horticultural University, Hyderabad
Email: lalitha_email@yahoo.com

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Abstract
Molecular characterization using RAPD analysis was carried out in 37 chrysanthemum genotypes.With 27 RAPD primers, total of 278 amplified fragments were scored.Out of which 271 were found to be polymorphic (97.4%). The genetic similarity ranged from a coefficient of 0.174 to 0.600 with an average similarity coefficient of 0.387 indicating a moderate diversity among the group of genotypes studied. In the present study, Ratlam Selection and Snow Cem were found to be the most genetically similar (0.60) followed by Akitha and Shintome with 55.3%. Cluster analysis based on UPGMA method led to the classification of genotypes intofour major clusters and seven minor clusters with one genotype each in cluster V (Arka Ravi), VI (Meera), VII(Asha), VIII(Silper), IX(Autumn Joy), X (Lilith) and cluster XI (Chandrika).

Key words: Chrysanthemum, genotypes, RAPD markers, genetic divergence

Introduction

Chrysanthemum (*Dendranthemagrandiflora*) is one of the leading commercial flower cropgrown throughout the world. In India, it occupies a prime position as a piouscut flower for all traditional uses and also as an ornamental flower for the garden. Because of its multifarious traditional uses, the crop has its own commercial value and of late good number of varieties has been released. In Andhra Pradesh chrysanthemum is grown in an area of 3198 ha with an annual production of 36,777 MT (NHB, 2010). Its wide popularity is due to its wide variation exhibited with respect to its growth, habit, size, shape and colour of bloom.The modern, large, double and exquisitely flowered cultivars owe their origin to relatively small, single and non-attractive types. This great transformation is the result of centuries of natural cross pollination, spontaneous and artificial hybridization coupled with mutation, chromosomal differentiation and polyploidy (Nazeer and Khashoo, 1982). Genetic diversity is being used as source of genes utilizes in crop improvement for the production of high yielding varieties and hybrids. In chrysanthemumthe emergence of new cultivars is closely connected with the problem of identifying and distinguishing the variation among the cultivars so developed.With the advent of molecular biology techniques, DNA based markers played a significant role in identification and characterization of germplasm.Among these, RAPD marker technology is one of the best available DNA – based tools for scoring variations between cultivars within species. It is convenient in performance and does not require any information about the DNA sequence to be amplified (Weder, 2002). Owing to the facts, the present investigation was conducted with 37 commercial varieties of chrysanthemum using 27 RAPD markers.

Material and Methods

The material used for the study comprised of 37 genotypes of chrysanthemum (Table3) collected from germplasm block of chrysanthemum maintained at Floricultural Research Station, Rajendranagar, Hyderabad.The top shoot cuttings of all the genotypes were planted in lines on raised nursery beds for rooting. After thirty five days, the rooted cuttings were transplanted in the main field. Each genotype was grown in a plot of 2 X 2 m consisting of 36 plants spaced at 30 x 30 cm.Recommended package of practices were followed uniformly for successful raising of genotypes.

Total genomic DNA was extracted from fresh, young leaves of chrysanthemum using modified CTAB method (CetylTrimethyl Ammonium Bromide) (Murray and Thompson, 1980). Purity of extracted DNA was assessed electrophoretically on 0.8% agarose gel stained with ethidium bromide in comparison with standard DNA ladders and the concentration and quality of DNA was also estimated spectrophotometrically by using Nano Drop spectrophotometer at 260/280 nm. The template DNA samples were diluted to make the working solutions of 5ng/µl for PCR analysis.

The RAPD reaction mixture consisted of 5 ng of template DNA, 1x PCR buffer (10 mMTris pH 9.0, 50 mMKCl, 1.5 mM $MgCl_2$), 100 M of each of the four dNTPs, 0.4 M of RAPD primer and 0.3 Units of *Taq*DNA polymerase (Bangalore Genei, India) in a reaction volume of 10 µl. PCR amplifications were performed in a Gene Amp 9700 thermal cycler (Perkin Elmer Applied Biosystems) with initial denaturation at 94^{o}C for 3 minutesfollowed by 45 cycles of 1 min at 92^{o}C, annealing temperature of 37^{o}C for 30 sec and primer extension at 72^{o}C for 2 min and final extension at 72^{o}C for 7 min. The PCR amplified products were separated on 1.0% agarose gel in 1x TAE buffer by electrophoresis at 100 V for 3 h and visualized with ethidium bromide staining. The gel images were recorded using the Alpha Innotech Fluorchem gel documentation system and the sizes of amplification products were determined by comparison to *Eco* RI and *Hind* III double digest (Bangalore Genei, India) as molecular weight standard. The reproducibility of the amplification was confirmed by repeating each experiment three times.

The banding pattern obtained from RAPDs in each genotype was scored as '1' to all the bands that could be reproducible and detected in the gel and '0' for the absence of band for each primer. Jaccard's similarity coefficient (J) was used to calculate similarity between pairs of genotypes. Where, J = nx,y / (nt - nz), nx,y is the number of bands common to genotype A and genotype B; nt the total number of bands presenting all samples and nz the number of bands not present in A and B but found in other samples.Cluster analysis was performed on molecularsimilarity matrices using the Unweighted Pair Group Method using Arithmetic means (UPGMA) algorithm, from which dendrograms depicting similarity among genotypes were drawn and plotted using NTSYS-pc. 2.1 Software (Rohlf, 2000).

Results and Discussion

RAPD markers have the potential to measure variations accurately utilizing the entire genome and can produce reliable data for authentic characterization of genetic diversity. But it requires a properly standardized protocol, replication of amplification reaction and conservative criterion of band selection (Faseela and Salkutty, 2007). So in the present study standardization of various experimental steps was done in order to overcome the problem of sensitivity in the RAPD technique.

Thirty seven genotypes of chrysanthemum were scanned by 27 selected RAPD primers (Table 1) which gave better profile among 124 primers initially screened. The number of DNA fragments amplified per primer ranged from 7 (OPE-15, OPG- 9, OPG-16, OPH-13, OPH-20) to 17 (OPK-19) with a mean value of 10.3 bands per primer. A total of 278 amplified fragments were scored out of which 271 were found to be polymorphic (97.4%). The number of scored polymorphic bands ranged from 6 (OPE-15) to 17 (OPK-19) in different primers with an average of 10.11 polymorphic fragments per primer. The amplicon sizes ranged from 350 bp to 3500 bp.All the primers except OPE-14, OPE-15, OPE-18, OPF-3, OPF-5 and OPI-18 gave highest polymorphism (100%)**.**Kumar *et al*.,(2006) also noticed a high degree of polymorphism in chrysanthemum cultivars using RAPD markers.The high polymorphism observed in the present study confirms that much diversity exists within this germplasm. The total number of amplified fragments generated per primer had no correlation with proportion of polymorphic bands. Similar pattern was observed by Williams *et al.*, (1993).Wolff (1996) reported that the choice of the primers may be an important factor in obtaining a rapid discrimination between samples.Bhat*et al* (1995) suggested that the number of polymorphisms might be more important than the number of primers for the generation of stable phenogram and it would vary with plant material used for investigation and sequences that are amplified.

Banding profiles obtained with 27 primers for 37 genotypes of chrysanthemum were analysed on the basis of presence (1) or absence (0) of the band. Only distinct, reproducible polymorphic bands were considered for the genetic analysis.Jaccard's similarity coefficients among these genotypes were calculated to establish the genetic relationships. Genetic similarity based on Jaccard's coefficient revealed considerable level of diversity among the genotypes under the study. The genetic similarity among 37 chrysanthemum genotypes ranged from a coefficient of 0.174 to 0.600 with an average similarity coefficient of 0.387 among the group of genotypes .

In the present study, Ratlam Selection and Snow Cem were found to be most genetically similar (60.0%) followed by Akitha and Shintome with 55.3% and Terry and Salora with 55.1%. On contrary, Aparajitha and Lilith were found to be the least genetically similar (17.4%). The genotypes Punjab Anuradha is having similarity coefficient value of 0.482 with Punjab Gold indicating the existence of high genetic similarity among these cultivars because they were selected from the same geographical region.

The genetic relationships of chrysanthemum genotypes were further evaluated by UPGMA cluster analysis, on RAPD data set, using a minimum variance algorithm (Fig 1).It resulted in 11 clusters at a genetic similarity coefficient of 0.23 with Chandrika and Aparajitha at the extreme ends of the dendrogram.The reason for the

separation of these genotypes as individual clusters may be due to their different genetic backgrounds.All the 37 genotypes were grouped into four major clusters of which three genotypes weregrouped in cluster I, five genotypes in cluster II, 19 genotypes in cluster III, three genotypes in cluster IV and seven minor clusters with one genotype each in cluster V(Arka Ravi), VI(Meera), VII(Asha), VIII(Silper), IX(Autumn Joy), X(Lilith) and cluster XI (Chandrika).

The cluster I included the three yellow coloured genotypes Aparajitha, Basanthi and Local button, while the other genotypes of this class *i.e*., Punjab Gold, Yellow Gold, CO-3, Punjab Anuradha and Rekha which includes decorative and double Korean types were included in the cluster II.Sharing of genetic similarity, among the genotypes originating from widely divergent locations was also evident as Punjab genotypes were grouped together with one genotype from Coimbatore in cluster II. This suggested a distinct genetic identity among plant selections of the geographically diverse genotypes. It might be due to sharing a similar gene pool before their geographical separation (Jaiswal and Amin, 1992).

As shown in the dendrogram, the Clusters V, VI, VII, VIII, IX, X and XI had one genotype each with Arka Ravi, Meera, Asha, Silper, Autumn Joy, Lilith and Chandrika respectively. Among these solitary clusters, Chandrika and Lilith were found to be more diverse with low similarity index value of 0.23. Similar pattern of moderate genetic diversity with RAPD markers were observed in 15 commercial varieties of chrysanthemum (Martin *et al.*, 2002) in which level of similarity was in all the cases less than 80%. A high genetic distance among the different chrysanthemum cultivars showed a possibility of introgressing new and novel genes from the chrysanthemum gene pool.

Principal Component Analysis was carried out based on the Jaccard's similarity matrix (Table 2)to visualize the genetic relatedness among the chrysanthemum genotypes. The First component accounts for 38.64% of all changes and second component accounted for 3.35% of principal changes. In this manner, totally 5 principal components accounted for 50.68% of changes. The description of the data was done using three dimensional pictorial graph and the same is represented in Fig. 2. From the graph, it is evident that the chrysanthemum genotypes were dispersed on the PC plot, which is a reflection of moderate genetic base. However, as depicted in figure some of the genotypes appear to be overlapping with each other depicting high similarity among themselve. It was clear from the analysis that the results obtained from the dendrogram were repeated in the PCA, which strengthened the ability and accuracy of the RAPD analysis applied to chrysanthemum genotypes in the present study.

To conclude, the results suggest the need of further molecular characterization of chrysanthemum gene pool with large number of markers in order to get an insight into molecular complexity involved in the gene pool as well as to derive an accurate phylogenetic relationship existing among them. It also indicated that RAPD markers are useful not only for varietal identification and detection of duplicate entries. These results can be used in future chrysanthemum breeding programmes to design crosses that maximize genetic variability.

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Table 1. Details of polymorphism and number of bands generated with RAPD primers in chrysanthemum

S. No.	RAPD primer	Nucleotide sequence(5'-3')	Number of bands	Total no. of polymorphic bands	Percentage polymorphism (1%)	Size of amplified product(bp)
1	OPE-14	TGCGGCTGAG	10	9	90.00	450-3300
2	OPE-15	ACGCACAACC	7	6	85.71	830-2000
3	OPE-16	GGTGACTGTG	11	11	100.0	420-2600
4	OPE-18	GGACTGCAGA	11	10	90.90	600-3300
5	OPE-19	ACGGCGTATG	12	12	100.0	440-3000
6	OPF-3	CCTGATCACC	9	8	88.89	500-2200
7	OPF-5	CCGAATTCCC	8	7	87.50	750-3000
8	OPF-19	CCTCTAGACC	10	10	100.0	450-2500
9	OPG-9	CTGACGTCAC	7	7	100.0	750-2000
10	OPG-16	AGCGTCCTCC	7	7	100.0	800-1600
11	OPG-19	GTCAGGGCAA	11	11	100.0	350-2027
12	OPH-13	GACGCCACAC	7	7	100.0	600-3000
13	OPH-16	TCTCAGCTGG	11	11	100.0	350-1900
14	OPH-17	AAGCAGCAAG	11	11	100.0	400-2000
15	OPH-20	CACCGTTCTG	7	7	100.0	420-2000
16	OPI-6	AAGGCGGCAG	14	14	100.0	560-2000
17	OPI-18	TGCCCAGCCT	12	10	83.33	600-3500
18	OPI-19	AATGCGGGAG	12	12	100.0	450-2200
19	OPI-20	AAAGTGCGGG	11	11	100.0	450-2500
20	OPJ-14	CACCCGGATC	13	13	100.0	350-2000
21	OPJ-15	TGTAGCAGGG	13	13	100.0	564-3000
22	OPM-10	TCTGGCGCAC	9	9	100.0	500-1900
23	OPK-8	GAACACTGGG	11	11	100.0	750-2200
24	OPK-18	CCTAGTCGAG	9	9	100.0	550-3300
25	OPK-19	CACAGGCGGA	17	17	100.0	450-2500
26	OPL-1	GGCATGACCT	8	8	100.0	450-2200
27	OPL-18	ACCACCCACC	10	10	100.0	600-2000

Table 2. Principal Component Analysis showing the contribution of 27 RAPD primers among 37 chrysanthemum genotypes

S. No.	Genotypes	Eigen value	Percentage variation explained	Cumulative variation (%)
1.	Aparajitha	14.30	38.64	38.64
2.	Punjab Gold	1.24	3.35	41.99
3.	CO-3	1.12	3.03	45.02
4.	Raichur	1.07	2.90	47.92
5.	Silper	1.02	2.76	50.68
6.	Yellow Gold	0.94	2.54	53.22
7.	Punjab Anuradha	0.85	2.29	55.51
8.	Rekha	0.82	2.22	57.73
9.	Chandrika	0.78	2.11	59.85
10.	Snow Cem	0.76	2.06	61.91
11.	Meera	0.75	2.03	63.94
12.	Shaffoli	0.73	1.96	65.90
13.	Terry	0.72	1.95	67.85
14.	Shintome	0.69	1.86	69.71
15.	Arka Ravi	0.67	1.82	71.53
16.	Ratlam Selection	0.64	1.72	73.25
17.	Neelima	0.63	1.70	74.95
18.	Flirtation	0.63	1.70	76.65
19.	Mother Teresa	0.61	1.64	78.30
20.	Autumn Joy	0.58	1.57	79.87
21.	Anjali	0.56	1.52	81.38
22.	Akitha	0.55	1.48	82.86
23.	PAU-B-107	0.51	1.39	84.25
24.	Farr	0.51	1.38	85.63
25.	Jaya	0.50	1.35	86.98
26.	Harvest House	0.48	1.30	88.28
27.	Lilith	0.47	1.27	89.55
28.	Asha	0.46	1.24	90.79
29.	Pusa Centenary	0.45	1.22	92.01
30.	Salora	0.42	1.14	93.15
31.	IIHR-13	0.41	1.09	94.24
32.	Basanthi	0.39	1.06	95.30
33.	Red Stone	0.39	1.04	96.35
34.	Geetanjali	0.37	0.99	97.34
35.	Red Gold	0.35	0.95	98.29
36.	Kalyani Mauve	0.34	0.92	99.21
37.	Local Button	0.29	0.79	100.0

Fig. 1. Dendrogram generated using UPGMA analysis showing the genetic relationship among 37 chrysanthemum genotypes using 27 RAPD primers

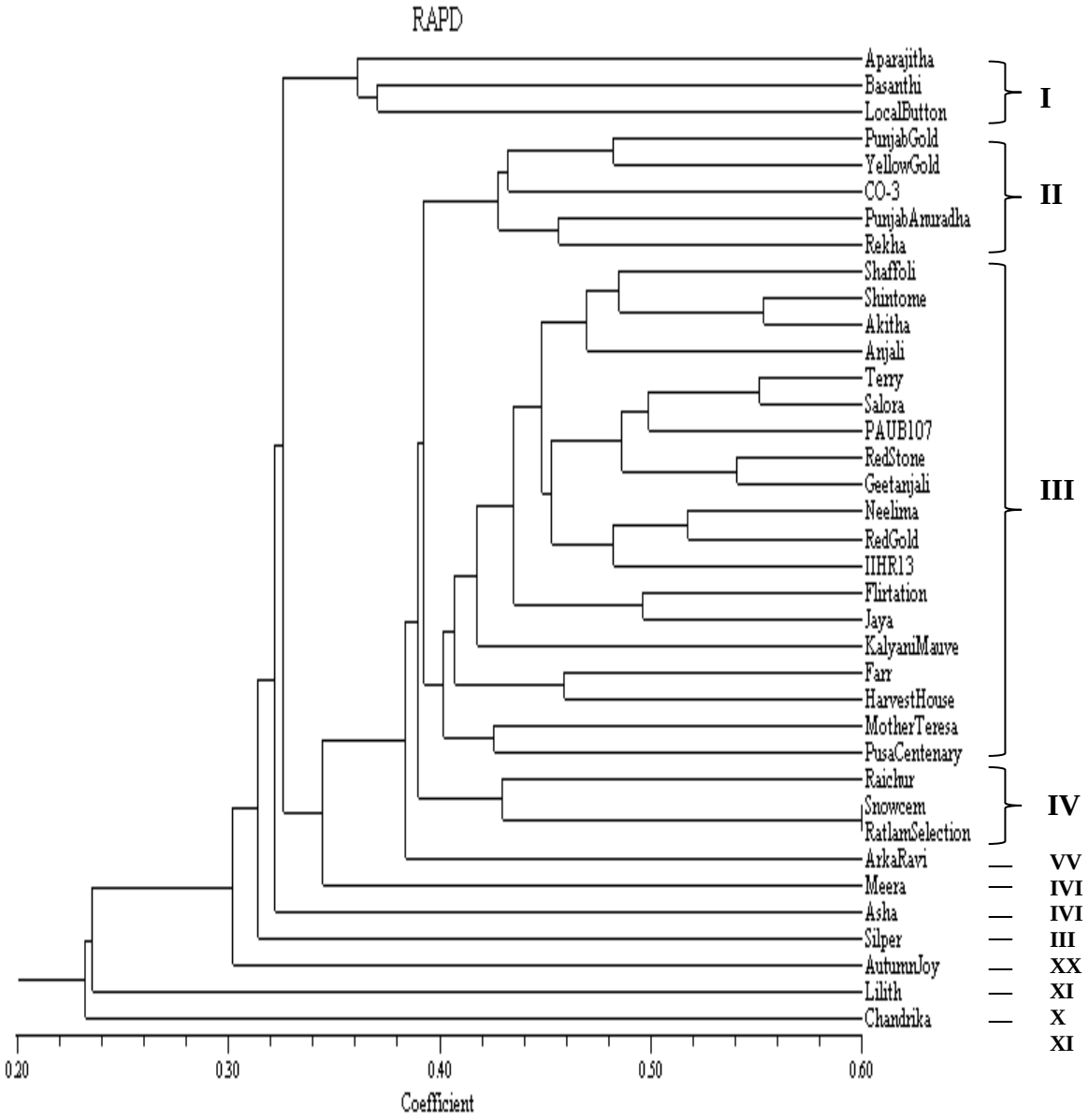
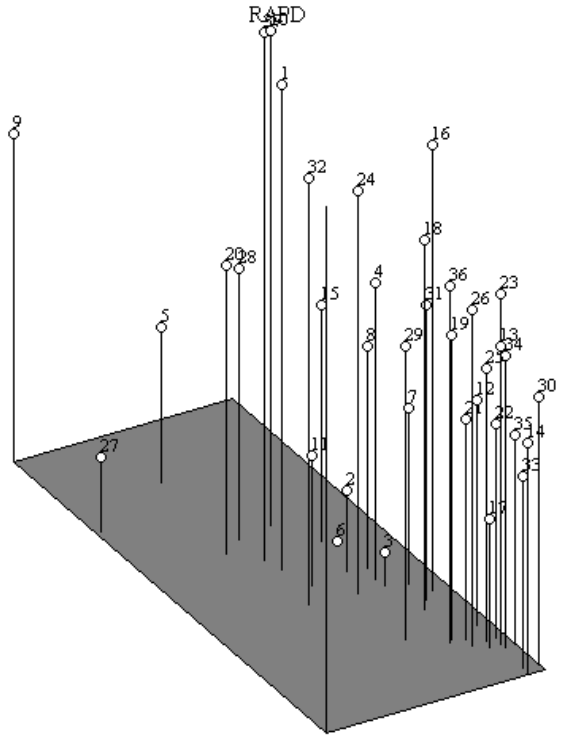


Fig. 2.The relative position of 37 chrysanthemum genotypes based on 27 RAPD markers



Name of the genotypes:

1. Aparajitha
2. Punjab Gold
3. CO-3
4. Raichur
5. Silper
6. Yellow Gold
7. Punjab Anuradha
8. Rekha
9. Chandrika
10. Snow Cem
11. Meera
12. Shaffoli
13. Terry
14. Shintome
15. Arka Ravi
16. Ratlam Selection
17. Neelima
18. Flirtation
19. Mother Teresa
20. Autumn Joy
21. Anjali
22. Akitha
23. PAU-B-107
24. Farr
25. Jaya
26. Harvest House
27. Lilith
28.Asha
29.Pusa Centenary
30.Salora
31. IIHR-13
32.Basanthi
33. Red Stone
34.Geetanjali
35. Red Gold
36.Kalyani Mauve
37.Local Button