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Genetic diversity analysis of chrysanthemum (*Chrysanthemum grandiflorum*) cultivars using RAPD markers

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ABSTRACT

Identification and characterization of new cultivars is essential to meet DUS testing, address IPR issues and their utilization and conservation. Traditionally, morphological markers were used for germplasm characterization as being simple and irreplaceable. A total of 57 RAPD primers were screened and out of these 22 primers which gave sufficient amplification were selected for the study. Out of 207 bands generated, 175 were polymorphic with a polymorphism percentage of 84.54%. The polymorphism percentage ranged between 60% (OPA04) to 100% (OPA17, OPB-06, OPX14, OPX-19). The primer OPA08 generated least numbers of bands (4) and OPA06 generated maximum number of bands (17). The Rp values ranged between 0.82 to 8.18 for RAPD primers OPY-4 and OPX16, respectively with a mean value of 3.94 and the correlation between Rp and number of cultivars identified by each primer was fairly high (0.96). The primers OPX16, OPY6 and OPA04 distinguished higher number of cultivars of 49, 46 and 37 respectively. The UPGMA dendrogram indicated that the cultivars Maghi Orange and Maghi Yellow were sharing the maximum similarity (89%) and were close to Maghi White at a similarity level of 86%. The other cultivars sharing higher similarity levels were Flirt with Yellow Gold, Pink Cloud with Korean Small and Sadbhawna with Jubilee. RAPD proved to be useful for the characterization of the genotypes for their efficient utilization, management and IPR protection.

Key words: Chrysanthemum, Cluster analysis, Genetic diversity, Polymorphism, PCA, RAPD markers

Crop improvement is a never ending challenge to meet the needs of the changing scenario and more so in ornamentals due to the demand for novelty besides other attributes. It is reported that chrysanthemum is being bred in China and Japan for nearly 3 000 years. In modern times, no single cultivar can sustain in market for a longer period, hence arises the need of development of novel cultivars with quality traits and therefore a large number of cultivars are being bred every year with desired characteristics and aesthetic values. Hence, accurate identification and characterization of such cultivars is essential to meet DUS testing, address IPR issues and their utilization and conservation in any crop improvement programme. Traditionally, morphological markers were used for germplasm characterization as being simple and irreplaceable. But one of the major drawbacks with these markers is their environment dependent expression, hence, they might not be appropriate for accurate analysis. Although isozyme markers are useful to characterize genetic diversity (Fiebich and Henning 1992, Roxas *et al.* 1993), and to identify the hybrids (Roxas *et al.* 1993), the paucity of isozyme loci restricts their usefulness in breeding (Helentjaris *et al.* 1986). Morphological and cytological markers are not as useful for breeding analysis as molecular markers (Roxas *et al.* 1993). Molecular markers detect more variation than morphological and biochemical markers, and are stage independent, not affected by environment and simply inherited. Thus, DNA based molecular markers are becoming increasingly important for characterisation and diversity analysis of the plant varieties and germplasm, detection of redundancies of gene bank collections and in monitoring genetic changes during germplasm conservation. The use of molecular markers to pre-select groups of related varieties has advantage over the use of morphological characters normally used for DUS testing. Molecular markers give more reliable information for germplasm characterization and diversity analysis. Development of PCR has allowed the introduction of Randomly Amplified Polymorphic DNA (RAPD) approach for molecular analysis of genomes (Williams *et al.* 1990). Among the molecular markers, RAPD is the most widely exploited largely due to the fact that results are obtained quickly and are fairly inexpensive. The other major advantage of this approach lies in exploration of large genomic portions without prior

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sequence information and requires small quantity of DNA. In this technique, a single primer is used to anneal to template DNA at two different sites on opposite strands. If these sites are within an amplifiable distance of each other, a discrete DNA product is generated. RAPD markers have been widely utilized for plant germplasm characterization (Wilde *et al.* 1992, Wolff *et al.* 1995). Though vast diversity exists in chrysanthemum, there are only a few reports on the use of molecular markers for diversity analysis in chrysanthemum. So keeping this in view, the present investigations were undertaken to characterize the chrysanthemum (*Chrysanthemum grandiflorum*) cultivars with RAPD markers.

MATERIALS AND METHODS

The experimental material comprised of 75 genotypes of chrysanthemum is presented in Table 1. The present investigations were carried out at the Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi and diversity analysis using molecular markers was done at National Bureau of Plant Genetic Resources, New Delhi during 2008-09.

Total genomic DNA was extracted from five gm of young leaf tissues using CTAB method as given by Saghai-Maroof *et al.* (1984). The DNA was purified and further quantified by using DyNA Quant Fluorimeter. Each part of DNA sample was diluted with 10:1 TE solution to yield a working concentration of 5 ng/ml.

The different components used for amplification reaction conditions were 10X PCR buffer with $MgCl_2$ (2 mM), $MgCl_2$ (25 mM), dNTP mixture (25 mM), Taq DNA polymerase (5U/µl), Primer (5µM) and Genomic DNA (10 ng/µl).The final volume was made to 25µl by adding different components as 10 X PCR buffer with $MgCl_2$ (2.5 µl), $MgCl_2$ (1.0 µl), dNTP (0.2 µl), Taq DNA polymerase (1U), Primer Mixture (0.5 µl), Genomic DNA (5.0 µl) and remaining sterile distilled water. Fifty seven RAPD primers were screened for amplification and the ones that gave better resolution were chosen for the study (Table 2). The PCR amplification was carried out in a DNA thermocycler with initial denaturation at 94°C for 4 minutes, 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 72°C for 1.30 min, primer extension at 72 °C for 1.30 min and final extension at 72°C for 4 min. Annealing temperature (AT) of each primer was standardized using gradient PCR in thermo cycler. The temperatures ranging from ± 5°C of melting temperature (T^m) were tested and the temperature resulting satisfactory amplicons was used as AT in the PCR amplification. PCR amplified products were separated on a 1.5% agarose gel. Electrophoresis was carried out at 80V for 3 hours. The resolved amplification products were visualized under UV transilluminator or on a gel documentation system. The gel was photographed using a gel documentation system (Chemi Genius).

The amplification products were scored across the lanes comparing their respective molecular weights. Each band was treated as one RAPD allele. Scoring of bands was done from gel photographs. Presence of band was scored as '1', absence as '0' and missing data as '3'.

Table 1 List of *Chrysanthemum* varieties included in the study

Cultivars	Cultivars
Kanchil	Pusa Anmol
Gulmohr	Yellow Bangla
Shyamal	Sharad Mala
Sadwin Yellow	Star White
White Andaman	TERI
Aparjita	Poornima White
Sadbhawna	Waters May
Flirt	Beauty
Neelima	Jubilee
White Prolific	Maghi Orange
Ravikiran	Maghi White
Birbal Sahni	Maghi Yellow
Shukla	Kalvin Orange
Yellow Charm	Diana
Pink Cloud	Pankaj
Kajole	Kalvin Pink
Gaity	Sonali Tara
Geetanjali	Mother Teressa
Star Pink	Pinked White
Korean Small	Gajra
Yellow Star	Santa Dine
Ajay	Red Shringar
Meghavi	Shwet Shringar
Yellow Gold	White Anemone
Lalpari	Mahatma Gandhi
Red Gold	Raja Orange
Vasantika	Tokyo Soldier
Kundan	Korean Small
Kargil	Kalvin Yellow
Shanti	Greenish White
Taichen Queen	Ajay
Star Yellow	Texas Gold
Snowball	Yellow Reflex
Presiden Viger	Golden Yellow
Jayanti	Red D spoon
Dolly Orange	Annual Chrysanthemum
Liliput	Annual Chrysanthemum
FDL	

The degree of similarity between genotypes was estimated for molecular marker by applying the Jaccards (1908) similarity coefficient. Jaccard's similarity coefficient S_{ij}= a/(a+b+c), where S_{ij} is the similarity between two individuals *i* and *j*, a is the number of bands present in both *i* and *j*, b is the number of bands present in *i* and absent in *j* and c is the number of bands present in *j* and absent in *i*. The genetic associations between accessions were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by Unweighted Pair Group Method for Arithmatic Mean (UPGMA) and a dendrogram was

Table 2 List of RAPD primers selected for PCR amplification

Primer code	Primer sequence (5'-3')
OPA 02	TGCCGAGCTG
OPA 03	AGTCAGCCAC
OPA 04	AATCGGGCTG
OPA 05	AGGGGTCTTG
OPA 08	GTGACGTAGG
OPA 16	AGCCAGCGAA
OPA 17	GACCGCTTGT
OPA 18	AGGTGACCGT
OPA 19	CAAACGTCGG
OPB01	GTTTCGCTCC
OPB 04	GGACTGGAGT
OPB 06	TGCTCTGCCC
OPX 01	CTGGGCAGCA
OPX 04	CCGCTACCGA
OPX 16	CTCTGTTCGG
OPX 17	GACACGGACC
OPX 19	TGGCAAGGCA
OPX 20	CCCAGCTAGA
OPY 04	GGCTGCAATG
OPY 06	AAGGCTCACC
OPY 08	AGGCAGAGCA
OPY 17	GACGTGGTGA

generated. The computations were performed using the program NTSYS-PC Version 2.1 (Rholf 1993).

Primer Resolving Power (Prevost and Wilkinson 1999) was used to identify the primers that distinguish the accessions most efficiently. Resolving power (*Rp*) of primer is defined as:

$Rp=\sum lb$, where *lb*= band informativeness, that takes the values of $(Ib_i) = 1-(2|0.5 - p|)$, where, *p* is the proportion of accessions containing the band.

RESULTS AND DISCUSSION

RAPD polymorphism

A total of 57 RAPD primers were screened and out of these 22 primers which gave sufficient amplification were selected for the study (Table 2) after excluding the primers which gave monomorphism or those whose banding pattern was difficult to score. A total of 22 RAPD primers generated 207 bands out of which 175 were polymorphic with a polymorphism percentage of 84.54%. The polymorphism percentage across all the RAPD primers ranged between 60% (OPA04) to 100% (OPA17, OPB-06, OPX14, OPX-19). The primer OPA08 generated least numbers of bands (4) of which 3 were polymorphic with 75% polymorphism. The primer OPA6 generated maximum number of bands (17) out of which 14 were polymorphic with 82.35% polymorphism. The mean values of all the RAPD primers

Table 3 'Generated Fragments' Statistics and cultivars identified by RAPD primers

RAPD Primer	No. of bands	No. of Polymorphic bands	% polymorphism	Rp	PIC=2 *pi*qi	Gene Div.	No. of cultivars identified
OPA 02	8	5	62.5	3.43	0.37	0.68	7
OPA 03	9	6	66.67	1.5	0.24	0.54	5
OPA 04	12	12	100	5.71	0.29	0.88	37
OPA 05	6	4	66.67	1.82	0.3	0.72	1
OPA 08	4	3	75	1.82	0.36	0.69	0
OPA 16	14	13	92.86	6.93	0.35	0.84	35
OPA 17	7	7	100	3	0.36	0.69	10
OPA 18	10	9	90	6.1	0.33	0.81	36
OPA 19	10	8	80	3.35	0.26	0.69	10
OPB 01	6	5	83.33	2.71	0.43	0.69	5
OPB 04	15	12	80	4.75	0.28	0.68	32
OPB 06	8	8	100	3.55	0.38	0.81	10
OPX 04	10	8	80	3.29	0.28	0.75	8
OPX 14	6	6	100	3.63	0.43	0.83	16
OPX 16	14	13	92.86	8.18	0.39	0.82	49
OPX 17	11	9	81.82	4.25	0.37	0.64	22
OPX 19	9	9	100	4.82	0.38	0.77	24
OPX 20	11	10	90.91	4.73	0.23	0.89	19
OPY 04	5	3	60	0.82	0.12	0.44	1
OPY 06	17	14	82.35	7.64	0.34	0.69	46
OPY 08	7	6	85.71	3.61	0.38	0.79	14
OPY 17	8	5	62.5	1.14	0.22	0.58	1
Total	207	175	84.54				
MIN	4.00	3.00	60.00	0.82	0.12	0.44	0.00
MAX	17.00	14.00	100.00	8.18	0.43	0.89	49.00
Mean	9.41	7.95	83.33	3.94	0.32	0.72	17.64

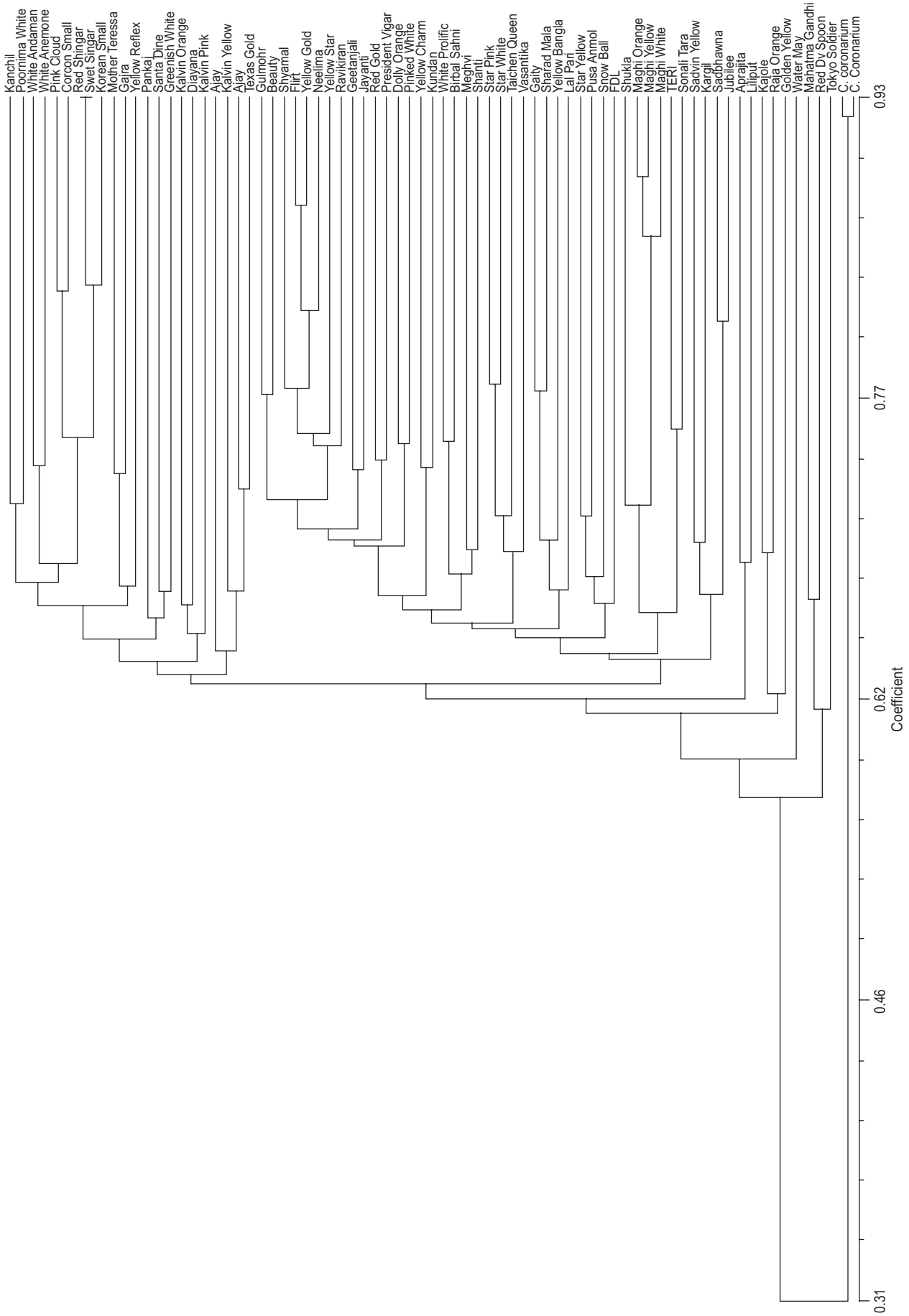

Fig 1 Dendrogram by RAPD markers

were 9.41 for number of bands generated, 7.95 for number of polymorphic bands and 83.33 for percent polymorphism. The band statistics generated by RAPD is presented in Table 3. The Rp values ranged between 0.82 to 8.18 for RAPD primers OPY-4 and OPX16, respectively with a mean value of 3.94 for all the primers. The correlations between Rp and number of cultivars identified by each primer were fairly high (0.96) for RAPD markers. The minimum (0.12) and maximum (0.43) values for Polymorphic Information Content (PIC) were obtained for RAPD primers namely, OPY-04 and OPB-1 respectively with a mean value of 0.32 across all the primers. The estimates for number of cultivars identified by RAPD primers ranged between 0 (OPA08) to 49 (OPX16). Other RAPD primers, which distinguished reasonably higher number of cultivars were OPY6 (46) followed by OPA04 (37), OPA18 (36), OPA16 (35) and OPX19 (24). RAPD markers have been already used to study the genetic variability in alstroemeria (Anastassopoulos and Keil 1996), chrysanthemum (Wolff and Peters-Van Rijn 1993), rose (Torres *et al.* 1993), lily (Yamagishi 1995); to determine the genetic relationships in alstroemeria (Dubouzet *et al.* 1998), *viola* (Ko *et al.* 1998) and rose (Jan *et al*. 1999). The differentiation of chrysanthemum cultivars and diversity analysis using molecular techniques are also reported by Martin *et al.* (2002), Sehrawat *et al.* (2003), Chatterjee *et al.* (2005, 2006), Lee *et al.* (2005), Mukherjee *et al.* 2013). No single RAPD primer was able to distinguish all the cultivars though produced polymorphic bands. However, the cultivars were distinguishable with the combinations of polymorphic bands generated by various primers in roses (Panwar *et al.* 2009) and marigold (Namita *et al.* 2013). Chrysanthemum varieties possess high level of genetic variability, which is logical for a strictly outcrossing plant species, as stated by Wolff & Peters-van Rijn (1993) in a study on other varieties. The high level of polymorphism obtained in marigold genotypes was because of presence of mating system of strict out crossing which resulted in high level of genetic variability in the gene pool. The representative banding pattern as observed with Primer OPX 16 is given in Fig 1.

RAPD based genetic diversity and clustering pattern of chrysanthemum cultivars

The UPGMA dendrogram based on Jaccard's Coefficients were generated for RAPD analysis. The outlier annual chrysanthemum accessions remained fairly distant from all other cultivars, which is obvious due to their genetic dissimilarity with other cultivars. The UPGMA dendrogram indicated that the outliers remained separate at a dissimilarity level of 70%. The cultivars Maghi Orange and Maghi Yellow were sharing the maximum similarity (89%) and were close to Maghi White at a similarity level of 86%. The other cultivars sharing higher similarity levels were Flirt with Yellow Gold, Pink Cloud with Korean Small and Sadbhawna with Jubilee. The cultivars Red D Spoon, Yellow Reflex and Tokyo Soldier grouped together

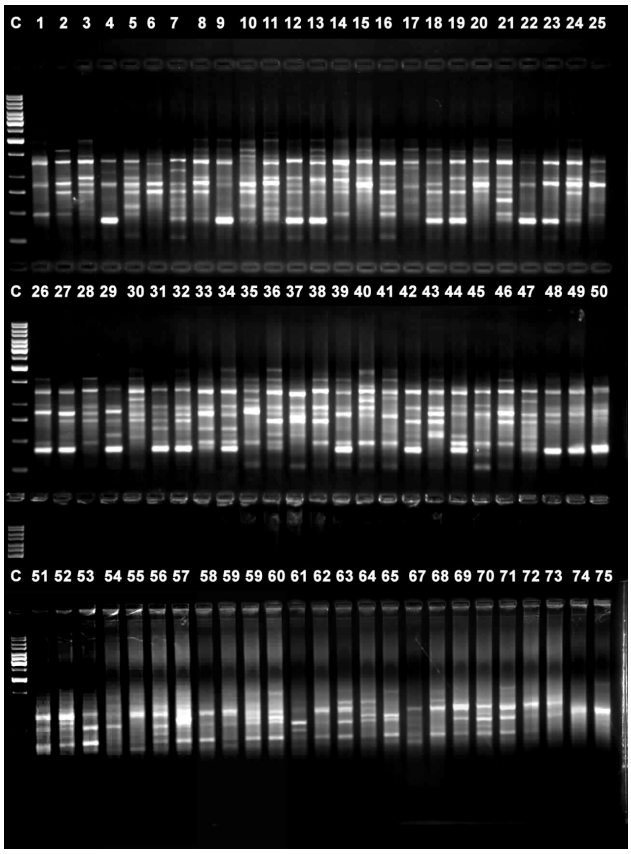

Fig 2 PCR amplification by RAPD primer OPX 16

and showed maximum dissimilarity of approximately 45% with other cultivars. The foregoing results and discussion indicated that RAPD (Random Amplified Polymorphic DNA) markers are found to be very useful in studying the genetic variability of different species, including ornamentals. In conclusion, the data obtained confirmed that the RAPD technique offers a rapid and relatively inexpensive way to resolve many highly discriminatory bands, useful for the characterization of the genotypes utilized in breeding programmes and for the identification of cultivars for plant patent protection.

The systematic characterization is the backbone of chrysanthemum germplasm management and its conservation, IPR protection, DUS testing, discriminate accessions, monitoring genetic integrity during long term conservation and for assessment of diversity. Awareness and need for IPR protection and large number of varieties submitted for registration have caused an increase in the size of reference collections of varieties, required to be grown and tested to ensure that candidate variety is distinct. The genotypes were found to be diverse based on RAPD markers and can be further utilized in crop improvement programmes. The high level of genetic variability among the genotypes would be useful for selecting parents in the development of new varieties. Moreover, the present study can be a major input into conservation biology and this information is helpful in many breeding programmes.

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