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Detection of genetic variability among chrysanthemum radiomutants using RAPD markers

Sudhir Kumar[1], K. V. Prasad[2,*] and M. L. Choudhary[3]

[1]Department of Biotechnology, Bundelkhand University, Jhansi 284 128, India
[2]Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi 110 012, India
[3]Horticulture Commissioner, Krishi Bhawan, New Delhi 110 001, India

Eleven radiomutants from two chrysanthemum cultivars Ajay and Thai Chen Queen were characterized by RAPD to understand the extent of diversity and relatedness. Out of 40 random primers screened, 21 gave reproducible polymorphic bands. PCR product of radiomutant genome revealed a total of 156 bands, out of which 118 were found to be polymorphic. Cluster analysis of the radiomutants indicated that they fell into three major groups. Yellow and Bright Orange mutants derived from cv. Thai Chen Queen have been placed in a separate group, indicating their high genetic diversity from the rest of the mutants and parents. The study revealed that RAPD molecular markers can be used to assess polymorphism among the radiomutants and can be a useful tool to supplement the distinctness, uniformity and stability analysis for plant variety protection in future.

Keywords: Chrysanthemum, genetic variability, polymorphism, radiomutants, RAPD markers.

CHRYSANTHEMUM is the second largest cut-flower after rose among the ornamental plants traded in the global flower market. It is cultivated both as a cut-flower and as a potted plant (pot mums). The commonly grown chrysanthemums are hexaploid complex with average number of 54 chromosomes[1]. It is propagated vegetatively as it has a strong sporophytic self-incompatibility system as shown by all members of Asteraceae family[2].

Mutation breeding by radiation, an agricultural application of nuclear technology has been widely utilized to upgrade the well-adapted plant varieties by one or two major traits and also develop new varieties with improved agricultural characteristics. Although most cultivated chrysanthemum cultivars are polyploids with high genetic heterogeneity, mutants with altered flower colour, shape, floret size and shape are often recovered. Altered flower colours with chimeric tissue can be easily induced by radiation and can be isolated using *in vitro* tools. Identification and characterization of cultivars is extremely important in horticultural crops in order to protect the plant breeders' rights. Earlier, new varieties were identified based on horticultural and physiological parameters. New cultivars of chrysanthemum are developed from a single progenitor either spontaneously (sports) or by radiation-induced mutation[3]. Since the effect of mutation in ornamentals is clearly visible, selection for changed flower colour, shape, and size is possible in the M1 generation itself because most of the ornamental crops are vegetatively propagated. Novelty visible in any form is of high value in ornamental crops and hence mutation breeding played a key role in the improvement of ornamental crops in general and chrysanthemum in particular.

Williams *et al.*[4] developed DNA fingerprinting using RAPD markers during 1990. Since then, chrysanthemum cultivars and other closely related family members of Asteraceae have been characterized based on RAPD[1,5,6] and DAF analysis in other parts of the world. Genetic variation between two genetically diverse tissues and three chimeral cell types in leaf has also been demonstrated earlier using the RAPD technique[1].

*For correspondence. (e-mail: kvprasad66@rediffmail.com)

Figure 1. Novelty generated through mutation breeding in chrysanthemum spray cv. Ajay (different flower shape, floret shape, flower colour).

Figure 2. Novelty generated through mutation breeding in chrysanthemum standard cv. Thai Chen Queen (different flower shape, floret shape, flower colour).

The main advantage of the RAPD method lies in its rapidity, applicability to any organism without prior knowledge of nucleotide sequence and its ability to detect mutations. RAPD method has also been used earlier to study genetic variability in radiomutants from the lady group of chrysanthemum[7]. Studies have been carried out on molecular variation in chrysanthemum based on RAPD markers[8] elsewhere in the world. However, there is no information available in India on genetic diversity studies of mutant cultivars of chrysanthemum evolved through mutation breeding. Although mutation breeding efforts are not new to IARI, which evolved six rose varieties earlier through mutation breeding[9], we have embarked on mutation breeding in chrysanthemum four years ago. We have

chosen two varieties of chrysanthemum, one belonging to the spray group (Ajay) and the other to a standard group (Thai Chen Queen). The significance of the cultivar Ajay is that it is a thermo and photo-insensitive variety and produces three flower flushes as against one in the rest of the cultivars. Creation of variability in this cultivar has more commercial significance because it provides a wider choice of colours to farmers of the northern plains. Similarly, the cultivar Thai Chen Queen is a light orange standard cut-flower type, which is suited for growing in open and greenhouses for cut-flower purpose. Initially we embarked on three doses of radiation (10, 20 and 30 Gray (Gy) γ rays: 1 Gy = 100 rads) and later on included one more dose (15 Gy) based on the response of the terminal cuttings. The hormetic influence of lower doses was evident at 10 and 15 Gy, where we have noticed perceptible improvement in growth and flowering attributes (data not presented). The pink coloured spray chrysanthemum cultivar Ajay was irradiated with 15 Gy gamma rays during 2001 and promising radiomutants comprising colour and flower form variants were isolated (Figure 1). We succeeded in isolating one of the promising chimeras possessing yellowish pink-colour through petal regeneration and successfully established them under field conditions. We have successfully isolated and multiplied other promising mutants like Brick Red mutant, Fluted mutant, Cup-shaped mutant, which need to be evaluated (Figure 1). Similarly, we have obtained a wide array of promising mutants like Bright Orange, Bright Yellow, Orange Pink and Lemon Yellow from a standard cut-flower cultivar Thai Chen Queen (Figure 2). There are also reports of creation of solid mutants of chrysanthemums by gamma radiation[10] from other groups.

The main objective of the present study is to explore the possibility of differentiating promising mutants and understanding the genetic diversity among radiomutants using RAPD.

Fresh leaves from 11 radiomutants (Table 1) and two parents were collected from adult plants grown in a greenhouse of the Division of Floriculture and Landscaping, Indian Agricultural Research Institute (IARI), New Delhi, India. DNA was extracted from one gram of fresh leaves ground in liquid nitrogen. Extraction of genomic DNA was performed according to Doyle and Doyle[11], with partial modification using CTAB. Genomic DNA was precipitated by isopropanol and finally washed with 70% alcohol and dissolved in 500 µl of Tris EDTA. RNAase 5 µl solution (10 mg/ml) treatment was given to remove RNA from the samples. It was purified by phenol : chloroform : isoamyl alcohol (25 : 24 : 1). Finally the dried sample was dissolved in TE (pH 8.0). DNA was quantified by two methods using UV-spectrophotometer and comparative study on agarose gel. Yield of genomic DNA was found to be 250 µg per gram of leaf.

PCR amplifications were performed in a thermal cycler (Applied Biosystems 9600) by loading 25 µl of reaction mixture having 1 unit *Taq* DNA polymerase (Bangalore Genei, Bangalore), 25 ng of initial genomic DNA, 100 µM of each dNTPs and 2.5 µl of amplification buffer. The amplification buffer comprised of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl and 0.1% triton X-100. Ten base long arbitrary primers (0.4 µM; Operon Technologies Inc. Almada, USA) were used. A temperature cycle

Table 1. Chrysanthemum cultivars used in the study

Parent	Original colour	Range of mutants
Ajay	Pink	Red, Pink, Yellow, Fluted
Thai Chen Queen	Orange	Orange, Yellow, Small Yellow, Spoon Yellow, Bright Orange, Pink and Purple

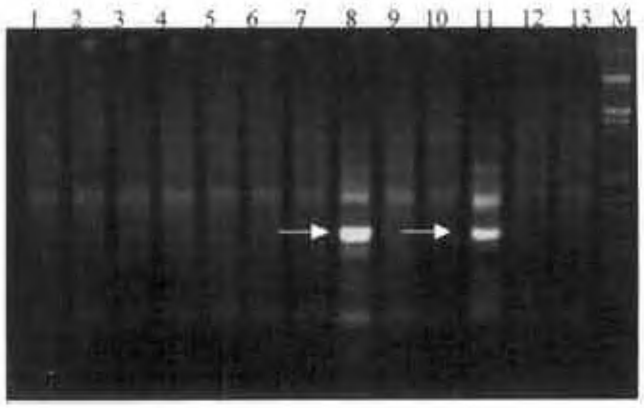

Figure 3. Amplification of parent and mutant cultivars of chrysanthemum using primer OPB 11. Lanes 1–5, Cultivar Ajay and its mutants; lanes 6–13, Cultivar Thai Chen Queen and its mutants. 1, Ajay; 2, Red; 3, Pink Cups; 4, Yellow; 5, Fluted; 6, Thai Chen Queen; 7, Orange; 8, Yellow; 9, Small Yellow; 10, Spoon Yellow; 11, Bright Orange; 12, Pink and 13, Purple. M, DNA molecular weight marker λ DNA digested with *Eco*RI/*Hin*dIII.

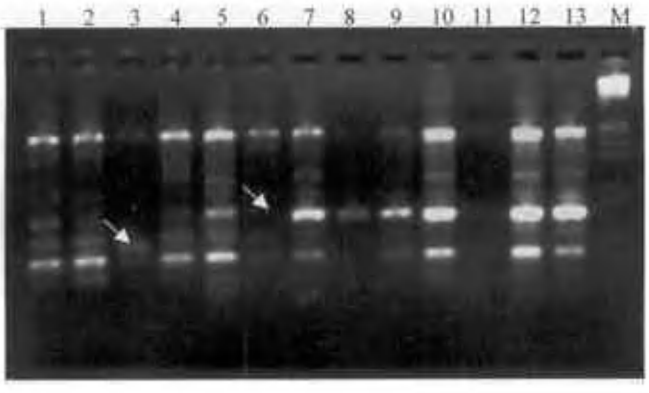

Figure 4. Amplification of parent and mutant cultivars of chrysanthemum using primer OPS 2. Lanes 1–5, Cultivar Ajay and its mutants; lanes 6–13, Cultivar Thai Chen Queen and its mutants. 1, Ajay; 2, Red; 3, Pink Cups; 4, Yellow; 5, Fluted; 6, Thai Chen Queen; 7, Orange; 8, Yellow; 9, Small Yellow; 10, Spoon Yellow; 11, Bright Orange; 12, Pink and 13, Purple. M, DNA molecular weight marker λ DNA digested with *Eco*RI/*Hin*dIII.

of 3 min at 94°C (initial melting), 40 cycles of (1 min, 94°C; melting)/(1 min, 37°C; annealing)/(1 min, 72°C; elongation) – (8 min, 72°C; final elongation) was used for amplification. The PCR-amplification products were fractionated by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining and UV illumination. The experiments were repeated twice to confirm consistency of the results obtained.

Table 2. Percentage polymorphism in parent and mutant cultivars of chrysanthemum with reproducible random primers

Primer	Total no. of bands	No. of polymorphic bands	Percentage polymorphism
OPK 3	9	9	100
OPK 9	7	4	57
OPK20	5	4	80
OPH10	7	6	85
OPH18	7	4	57
OPA2	4	2	50
OPA5	12	10	83
OPA20	6	5	83
OPR2	5	1	20
OPR15	4	3	75
OPR16	4	2	50
OPT13	6	3	50
OPT17	11	10	90
OPB1	10	7	70
OPB2	10	8	80
OPB4	11	9	81
OPB5	10	8	80
OPB14	5	3	60
OPB20	11	9	81
OPS2	8	8	100
OPS4	4	3	75
Total	156	118	

Each accession was scored for presence (1) and for absence (0) of each polymorphic band. RAPD bands within accessions were scored as missing if they were poorly resolved on the gel or if the template DNA did not amplify well. Genetic distance was calculated on the basis of Jaccard's coefficient method[12]. A dendrogram was constructed using the TREE procedure by the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) computer program version 1.70 (SAS Inc., 1994) based on Jaccard's similarity coefficient using Unweighted Pair Group Method using Arithmetic Average Method (UPGMA).

Wolff and Van Rijn[13] also noticed a high degree of polymorphism in chrysanthemum cultivars using RAPD markers as in the present study. Out of forty primers screened, twenty-one were selected on the basis of robustness of amplification, reproducibility, scorability of banding patterns and were employed for diversity analysis. The percentage polymorphism varied from 20 (OPR 2) to 100 (OPS 2, OPK 3) (Table 2).

Most of the primers failed to amplify cvs Yellow and Bright Orange mutants of Thai Chen Queen (out of 40 primers tested 15 amplified both the mutants). However, PCR amplification with primer OPB 11 clearly revealed that in mutants Yellow (lane 8) and Bright Orange (lane 11), a highly specific band (1.9 kb) is present in comparison to other mutants indicating that they are highly polymorphic when compared to other mutants (indicated by arrow in Figure 3). A band of 1.5 kb was noticed in mutant cultivar Pink Cups (lane 5) from cv. Ajay when the genomic DNA was amplified with primer OPS2, indicating that the mutant Pink Cups is polymorphic when compared to other mutants (indicated by arrow in Figure 4). Similarly, a polymorphic band of 1.87 kb is absent in parent cultivar Thai Chen Queen (lane 6) but present in its mutants (in-

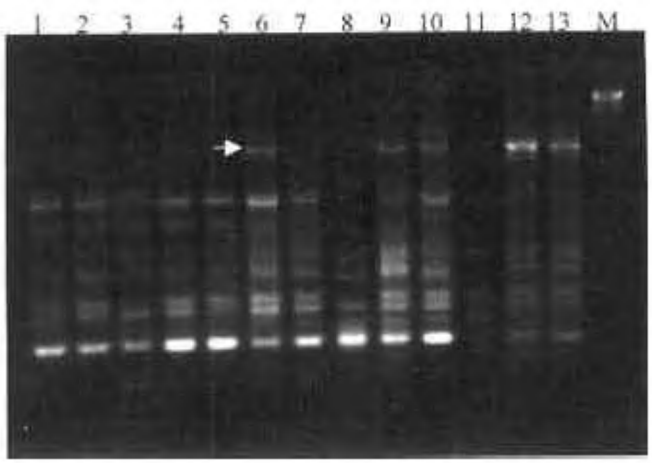

Figure 5. Amplification of parent and mutant cultivars of chrysanthemum using primer OPB 4. Lanes 1–5, Cultivar Ajay and its mutants; lanes 6–13, Cultivar Thai Chen Queen and its mutants. 1, Ajay; 2, Red; 3, Pink Cups; 4, Yellow; 5, Fluted; 6, Thai Chen Queen; 7, Orange; 8, Yellow; 9, Small Yellow; 10, Spoon Yellow; 11, Bright Orange; 12, Pink and 13, Purple. M, DNA molecular weight marker λ DNA digested with *Eco*RI/*Hin*dIII.

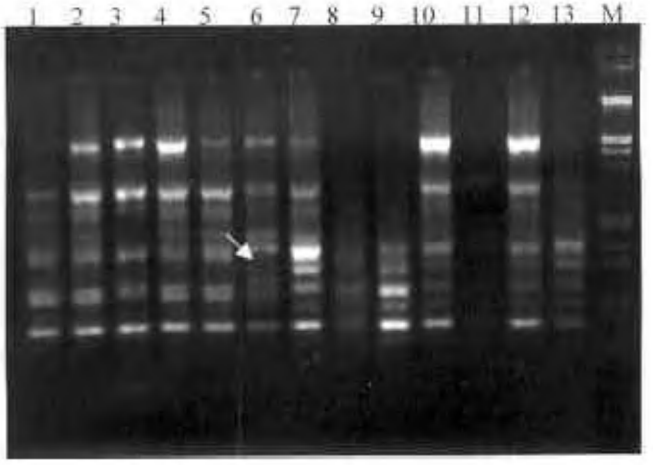

Figure 6. Amplification of parent and mutant cultivars of chrysanthemum using primer OPB 1. Lanes 1–5, Cultivar Ajay and its mutants; lanes 6–13, Cultivar Thai Chen Queen and its mutants. 1, Ajay; 2, Red; 3, Pink Cups; 4, Yellow; 5, Fluted; 6, Thai Chen Queen; 7, Orange; 8, Yellow; 9, Small Yellow; 10, Spoon Yellow; 11, Bright Orange; 12, Pink and 13, Purple. M, DNA molecular weight marker λ DNA digested with *Eco*RI/*Hin*dIII.

Table 3. Similarity matrix of parent and mutant chrysanthemum cultivars based on Jaccard's coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1.00												
2	0.81	1.00											
3	0.70	0.70	1.00										
4	0.76	0.80	0.79	1.00									
5	0.76	0.82	0.73	0.85	1.00								
6	0.61	0.61	0.59	0.60	0.66	1.00							
7	0.53	0.56	0.54	0.56	0.61	0.81	1.00						
8	0.43	0.44	0.52	0.49	0.48	0.55	0.53	1.00					
9	0.51	0.54	0.56	0.57	0.62	0.74	0.77	0.63	1.00				
10	0.57	0.59	0.56	0.62	0.65	0.78	0.81	0.53	0.78	1.00			
11	0.48	0.50	0.57	0.54	0.52	0.58	0.58	0.61	0.58	0.51	1.00		
12	0.59	0.62	0.56	0.65	0.69	0.76	0.78	0.53	0.80	0.86	0.46	1.00	
13	0.61	0.63	0.57	0.66	0.71	0.77	0.77	0.55	0.82	0.85	0.50	0.96	1.00

1, Ajay; 2, Red Ajay; 3, Pink Cups; 4, Yellow; 5, Fluted; 6, Thai Chen Queen; 7, Orange; 8, Yellow; 9, Small Yellow; 10, Spoon Yellow; 11, Bright Orange; 12, Pink and 13, Purple.

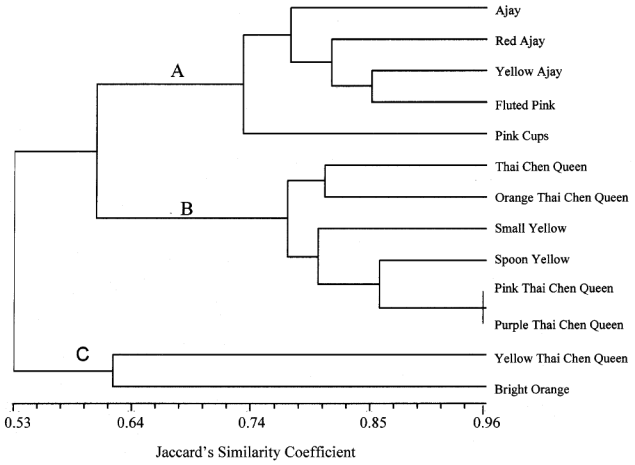

Figure 7. Dendrogram of parent and mutant chrysanthemum cultivars.

dicated by arrow in the Figure 4), when RAPD marker OPS 2 was used. Amplification product with primer OPS 5 indicated a highly distinct and polymorphic band (4.1 kb) present only in Orange mutant of Thai Chen Queen, which can be used as a specific marker (data not shown). A highly polymorphic band (4.9 kb) is present (indicated by arrow in Figure 5) in the parent cultivar Thai Chen Queen (lane 6) and its mutants like Small Yellow (lane 9), Spoon Yellow (lane 10), Pink (lane 12) and Purple (Lane 13), which can be used to differentiate among the parent and its mutants when the RAPD marker OPB 4 was used.

In the parent cultivar Thai Chen Queen (lane 6) a specific band of 1.42 kb is absent (shown by arrow in Figure 6), indicating that this cultivar is highly polymorphic compared to its mutants. Similarly, in cv. Ajay (lane 1), Yellow Thai Chen Queen (lane 8), Small Yellow (lane 9), Bright Orange (lane 11), Purple Thai Chen Queen (Lane 13), a specific band (5.1 kb) is absent indicating that these cultivars are distinct from other cultivars when the RAPD marker OPB 1 was used.

Twenty-one informative RAPD primer combinations generated a total of 156 reproducible amplification fragments across all radiomutants and parent cultivars, among which 118 bands were polymorphic (Table 2). The number of amplified RAPD bands varied from a minimum of 3 to a maximum of 14. Genetic distance between all thirteen cultivars varied from 0.43 to 0.96, as revealed by Jaccard's similarity coefficient matrix (Table 3).

The dendrogram constructed using the UPGMA method differentiated two parents and eleven mutants into three major clusters A, B and C (Figure 7). Cluster A consists of four radiomutants (Red, Pink Cups, Yellow and Fluted) derived from parent cultivar Ajay. Cluster B consists of parent cultivar Thai Chen Queen and five (Orange, Small Yellow, Spoon Yellow, Pink and Purple) of the seven radiomutants. Two radiomutants Yellow and Bright Orange from Thai Chen Queen have been placed in an entirely different cluster C, indicating high genetic diversity from their parent and other radiomutants. Similar results were interpreted by Ruminska[7] after RAPD analysis of the radiomutants of the lady group (*Dendranthema grandiflora* Tzvelev). Earlier, Wolff[1] also used RAPD markers to identify chrysanthemums and studied the stability of DNA fingerprint patterns. He distinguished chrysanthemum cultivars which do not belong to a single group using two or three primers. Contrary to our findings, Wolff *et al.*[6] suggested that there is no possibility at the DNA level to distinguish mutant family cultivars derived vegetatively from one original cultivar, which could be due to less number of RAPD markers used.

Cluster analysis separated radiomutants into different groups but genetic distance observed between them was low, except two mutants of Thai Chen Queen (Yellow and Bright Orange). This is in accordance with high values of similarity indices revealed for the closely related accessions of chrysanthemum[14]. The artificially induced mutants showed polymorphic bands, which distinguished them and suggested greater changes in the genome than those

in spontaneous mutants. The changes could have concerned point mutations in genes as well as greater chromosomal aberration[1,15].

Our study clearly indicated that RAPD markers could be effectively used for genetic diversity studies among radiomutants of Indian origin at genomic level. The results obtained suggested that by using RAPD molecular markers the newly evolved chrysanthemum cultivars can be easily differentiated from their parents. This would be a useful tool in identifying and protecting them from possible infringements in future.

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Seed germination behaviour of some medicinal plants of Lahaul and Spiti cold desert (Himachal Pradesh): implications for conservation and cultivation

Rajender Kumar Sharma, Shashi Sharma* and Shanti S. Sharma

Department of Biosciences, Himachal Pradesh University, Shimla 171 005, India

Seed germination/dormancy status of seven plant species of reasonably high medicinal value from the cold desert region of Lahaul and Spiti (Himachal Pradesh, India), namely *Podophyllum hexandrum*, *Hyoscyamus niger*, *Inula racemosa*, *Bunium persicum*, *Carum carvi*, *Saussurea costus* and *Rheum australe* was determined. These species are under pressure due to overexploitation from their natural habitats. Seeds of *P. hexandrum*, *H. niger*, *I. racemosa* and *B. persicum* were completely dormant at harvest. The efficacy of chilling, acid scarification, KNO_3 and GA_3 treatments for germination improvement was tested. The most effective treatments in different species were – *P. hexandrum*: $H_2SO_4/10^{-3}$ M GA_3; *H. niger*: 10^{-3} M GA_3; *I. racemosa*: chilling; *B. persicum*: chilling; *C. carvi*: chilling; *S. costus*: chilling; *R. australe*: 10^{-3} M GA_3. The presence of chemical inhibitors in dormant seeds, assessed as the degree of inhibition of seed germination of *Triticum aestivum* and *Brassica juncea* was indicated in *B. persicum* and *C. carvi*. The seedlings derived from seeds exposed to the various treatments performed well when grown in a glasshouse. The data have implications for conservation and cultivation of the species studied.

Keywords: Cold desert, conservation, cultivation, medicinal plants, seed germination.

THE demand for medicinal plants has increased globally due to the resurgence of interest in and acceptance of herbal medicine. Most of the demand is being met through collection of large quantities of medicinal plants and plant parts from wild populations. The methods of extraction employed are almost invariably crude and unsystematic. As a consequence, the rates of exploitation may exceed those of local natural regeneration. Also, the natural habitats are fast depleting[1,2]. The Indian Himalayan Region (IHR) is a rich reservoir of biological diversity in the world. Lahaul and Spiti (lat. 31°44′57″–32°59′57″N and long. 76°46′29″–78°41′34″E) constitute parts of cold desert in Himachal Pradesh (India) within the IHR[3]. This region, home to many a high-value medicinal herbs, has a rich local tribal tradition of herbal medicine. Interestingly, the high altitude populations of medicinal plants such as

*For correspondence. (e-mail: shashi_sh22@yahoo.com)