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A Comparative Analysis of Genetic Diversity in *Chrysanthemum* (*Dendranthema grandiflora* Tzvelec) Cultivars based on RAPD and ISSR Markers

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A B S T R A C T

Two molecular techniques, RAPD and ISSR were used to study the genetic relatedness in 37 genotypes of chrysanthemum. With 27 RAPDs and 10 ISSRs, a total of 271 and 107 polymorphic bands were generated accounting to 97.4% and 93.86% polymorphism respectively. Both kinds of markers could able to distinguish all the genotypes. Based on RAPD, ISSR and combination of RAPD and ISSR markers, dendrograms were constructed according to Jaccard's Coefficient of similarity. Though the results obtained from cluster analysis based on RAPD and ISSR data sets were different, the genotypes Snow Cem and Ratlam Selection; Akitha and Shintome as well were clustered in one group in both the clusters indicating the efficiency of two systems. This was also reflected in the correlation coefficient calculated based on similarity matrices of RAPD and ISSR by using Mantel test. Although the value of correlation coefficient between RAPD and ISSR markers was significant as r = 0.3906, indicating that there is a faint concordance between RAPDs and ISSRs. This inferred that the two sets of markers explore genetic variation differently among the chrysanthemum genotypes. The data generated in the present investigation provide information useful not only for selection programmes for further improvement of chrysanthemum, but also for the establishment of relationship among genotypes worldwide.

Introduction

Chrysanthemum (*Dendranthema grandiflora* Tzvelec), a herbaceous perennial flowering plant has been much loved by the people world-wide as a cut flower. It is one of the oldest cultivated flower crop which plays a significant role in the culture and life of people. Today *Chrysanthemum*s can be found with most of the colours of the spectrum and the pot-mum production has become the most profitable form of commercial *Chrysanthemum* growing. Economy of space, time, material, etc. has made this style of growing very promising and an excellent range of colour, form, long lasting quality of blooms and ease in handling make them most popular. Because of its multifarious traditional uses, the crop has its own commercial value and a good number of varieties have been released.

The commonly grown *Chrysanthemum*s are hexaploids with average number of 54 chromosomes (Wolff, 1996). 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 intentional hybridization coupled with mutation, chromosomal differentiation and polyploidy (Nazeer and Khashoo, 1982). Most extensive work has been done for developing novel *Chrysanthemum* genotypes through induced mutation using physical and chemical mutagens (Broetjes and Van Harten, 1978).

Since most of the ornamental plant improvement programs concentrate on aesthetic qualities such as flower and plant characteristics, the genetic base of the modern cultivars is becoming more and more narrow. Coupled with global marketing and adoption of these cultivars worldwide, many heir loom varieties of these crops are being replaced by modern narrow genetic base cultivars, resulting in continuous loss of our traditional cultivars. Hence, characterization of germplasm is essential to provide information on the traits of accessions assuring the maximum utilization of the germplasm collection for the benefit of end user. The assessment of diversity based on morphological parameters has been often constrained by lack of precise data on distinguishable morphological characters and their weakness of environmental influence. With the advent of molecular biology techniques, DNA based markers played a significant role in the identification and characterization of germplasm. The first study on the identification of *Chrysanthemum*s with the application of RAPD markers was carried out by Wolff and Peters-Van Rijn (1993).Considering the potentials of the DNA marker based genetic diversity analysis, the present study aimed to evaluate the usefulness of molecular markers *viz*. RAPD and ISSRs, in assessing and analysing the nature and the extent of genetic diversity among the genotypes of *Chrysanthemum*.

Materials and Methods

Plant Material

The plant material used for the study consisted of 37 genotypes of *Chrysanthemum* listed in Table 1, collected from germplasm block of *Chrysanthemum* belonging to Floricultural Research Station, Rajendranagar, Hyderabad.

DNA Isolation

Total genomic DNA was extracted from fresh, young leaves of *Chrysanthemum* following the standard CTAB method (Cetyl Trimethyl Ammonium Bromide) with minor modifications (Murray and Thompson, 1980). RNA was removed by digesting with RNaseA (10 mmol dm^{-3}). Purity of 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 nm. The template DNA samples were diluted to make the working solutions of 5ng/µl for PCR analysis.

RAPD PCR – amplification

Total 124 primers (Operon, USA) were screened out of which 27 decamers belonging to OPE, OPH, OPI, OPF, OPG, OPK, OPJ, OPL and OPM series were selected for PCR amplification of genomic DNA of *Chrysanthemum* genotypes. List of primers used in the study was furnished in Table 2.

The RAPD reaction mixture consisted of 5 ng of template DNA, 1x PCR buffer (10 mMTris pH 9.0, 50 mMKCl, 1.5 mM MgCl2), 100 mM of each of the four dNTPs, 0.4 mM of RAPD primer and 0.3 Units of *Taq*DNA polymerase (Bangalore Genei, India) in a

reaction volume of 10 µl. Amplifications were carried out in a Gene Amp 9700 thermal cycler (Perkin Elmer Applied Biosystems) with initial denaturation at 94^0 C for 3 minutes followed by 45 cycles of1 min at 92^0 C, annealing temperature of 37^0C for 30 sec and primer extension at 72^0C for 2 min and final extension at 72^0C for 7 min. The PCR amplified products were separated electrophoretically on 1.0% agarose gels. 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 two times.

ISSR PCR – amplification

A set of 46 UBC primers (UBC primer set No. 9, University of British Columbia, Canada) were screened out of which 10 primers were used for ISSR amplification (Table-3).The PCR reaction was carried out in a total volume of 10 µl containing 1.0 µl of 5 ng template DNA, 1.0 µl of 1x PCR buffer (10 mMTris pH 9.0, 50 mMKCl, 1.5 mM MgCl2), 0.2 µl of 25 mM $MgCl_2$, 0.6 µl of 200 mM of each of the four dNTPs, 1.0 µl of 0.4 mM ISSR primer and 0.2 µl of 0.6 U*Taq* DNA polymerase (Bangalore Genei, India).

PCR amplifications were performed in a Gene Amp 9700 thermal cycler (Perkin Elmer Applied Biosystems) with initial denaturation at 94^0 C for 4 minutes followed by 35 cycles of30 sec at 92^0 C, 1 min at annealing temperature of 45^0C (+/- 5^0C) for 1 min and primer extension at 72^0C for 1 min and final extension at 72^0C for 7 min. The amplified products were resolved on 1.7% gel and documented in a gel documentation system (Alpha Innotech Flourchem).

Data Analysis

Ambiguous bands that could not be easily distinguished were not scored (Williams *et al.*, 1990). A clear band was scored as '1' 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; the total number of bands present in all samples and nz the number of bands not present in A and B but found in other samples. Cluster analysis was performed on molecular similarity 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 Analysis

A total of 278 amplified fragments were scored with 27 selected RAPD primers, out of which 271 were found to be polymorphic (97.4%)(Table-2). 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. The amplification products obtained with primer OPE-18 are illustrated in Fig 1.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%)**.**The high polymorphism observed in the present study confirms much diversity existing 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). Genetic similarity based on Jaccard's coefficient

revealed considerable level of diversity among the genotypes under the study. The similarity index varied from 0.174 to 0.600 with an average of 0.387 among the group of genotypes.

The genotypes 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%).All the remaining ones exhibited diverse intermediate levels of similarity. The derived UPGMA dendrogram resulted in eleven clusters at a genetic similarity coefficient of 0.23 with Chandrika and Aparajitha at the extreme ends of the dendrogram (Fig 2).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 were grouped in cluster I, five genotypes in cluster II, nineteen 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).

ISSR Analysis

Among forty six ISSR primers used in the preliminary analysis, only ten primers generated the scorable PCR products. A total of 114 bands were produced, of which 107 bands were polymorphic, accounting for 93.86% polymorphism (Table-3).

Table.1 List of genotypes used for molecular studies in *Chrysanthemum*

S.No.	Name of the genotype	S.No.	Name of the genotype
1	Aparajitha	20	Autumn Joy
2	Punjab Gold	21	Anjali
3	CO-3	22	Akitha
4	Raichur	23	PAU-B-107
5	Silper	24	Farr
6	Yellow Gold	25	Jaya
7	Punjab Anuradha	26	Harvest House
8	Rekha	27	Lilith
9	Chandrika	28	Asha
10	Snow Cem	29	Pusa Centenary
11	Meera	30	Salora
12	Shaffoli	31	IIHR-13
13	Terry	32	Basanthi
14	Shintome	33	Red Stone
15	Arka Ravi	34	Geetanjali
16	Ratlam Selection	35	Red Gold
17	Neelima	36	Kalyani Mauve
18	Flirtation	37	Local Button
19	Mother Teresa		

Table.2 Details of RAPD primers used in *Chrysanthemum*

S. No.	RAPD primer	Nucleotide sequence(5'-3')	Number of bands	Total no. of polymorphic bands	Percentage polymorphism (100%)	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

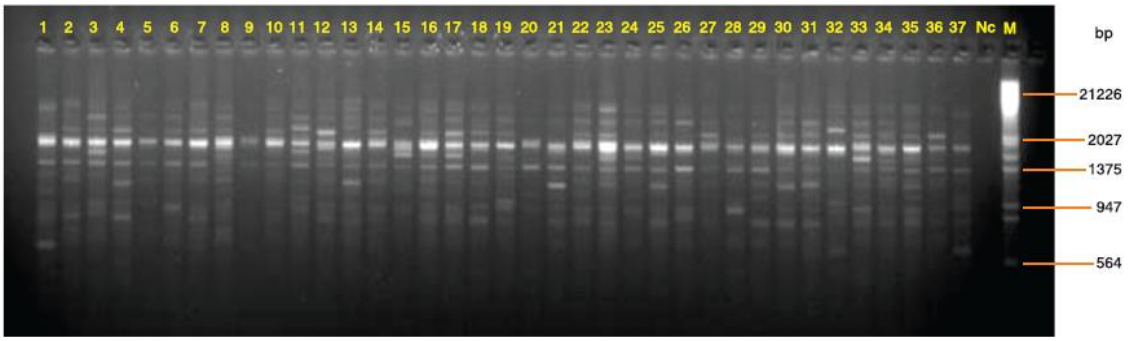
Source: Operon Technologies, INC. 1000 Atlantic Avenue Suite 108, Alameda, CA

Table.3 Details of ISSR primers and amplified bands of all the DNA samples as obtained from thirty seven genotypes of *Chrysanthemum*

S. No	Primer	Annealing temperature (^{0}C)	DNA repeats	No. of total bands	Polymorphic bands(%)	Size range of amplified product(bp)
1	808	52^{0}C	$(AG)_8C$	15	15(100%)	250-1550
2	810	50^{0}C	$(GA)_8T$	8	8(100%)	220-1800
3	812	50^{0}C	$(GA)_8A$	9	9(100%)	400-1400
4	825	50^{0}C	$(AC)_8T$	7	5(71.43%)	400-1400
5	836	53^{0}C	$(AG)_8YA$	11	10(90.91%)	250-2000
6	840	53^{0}C	$(GA)_8YT$	12	12(100%)	300-2000
7	842	55^{0}C	$(GA)_8YG$	12	12(100%)	420-1900
8	846	53^{0}C	$(CA)_8RT$	12	11(91.67%)	400-2000
9	855	53^{0}C	$(AC)_8YT$	13	11(84.62%)	250-1600
10	857	55^{0}C	$(AC)_8YG$	15	14(93.33%)	220-1550
				114	107	

Note : Y = Pyrimidine (C or T) and R = Purine (A or G)

Fig.1 RAPD profile of 37 genotypes of *Chrysanthemum* with primer OPE-18



Amplicon size ranged from 600bp to 3300bp. M- marker, EcoR1- Hind III double digest of ג DNA; NC- negative control (no DNA), 1-37 represent the genotypes. Refer table 1 for identity code of these cultivars

Fig.2 Dendrogram generated using UPGMA analysis showing the genetic relationship among *Chrysanthemum* genotypes using RAPD data

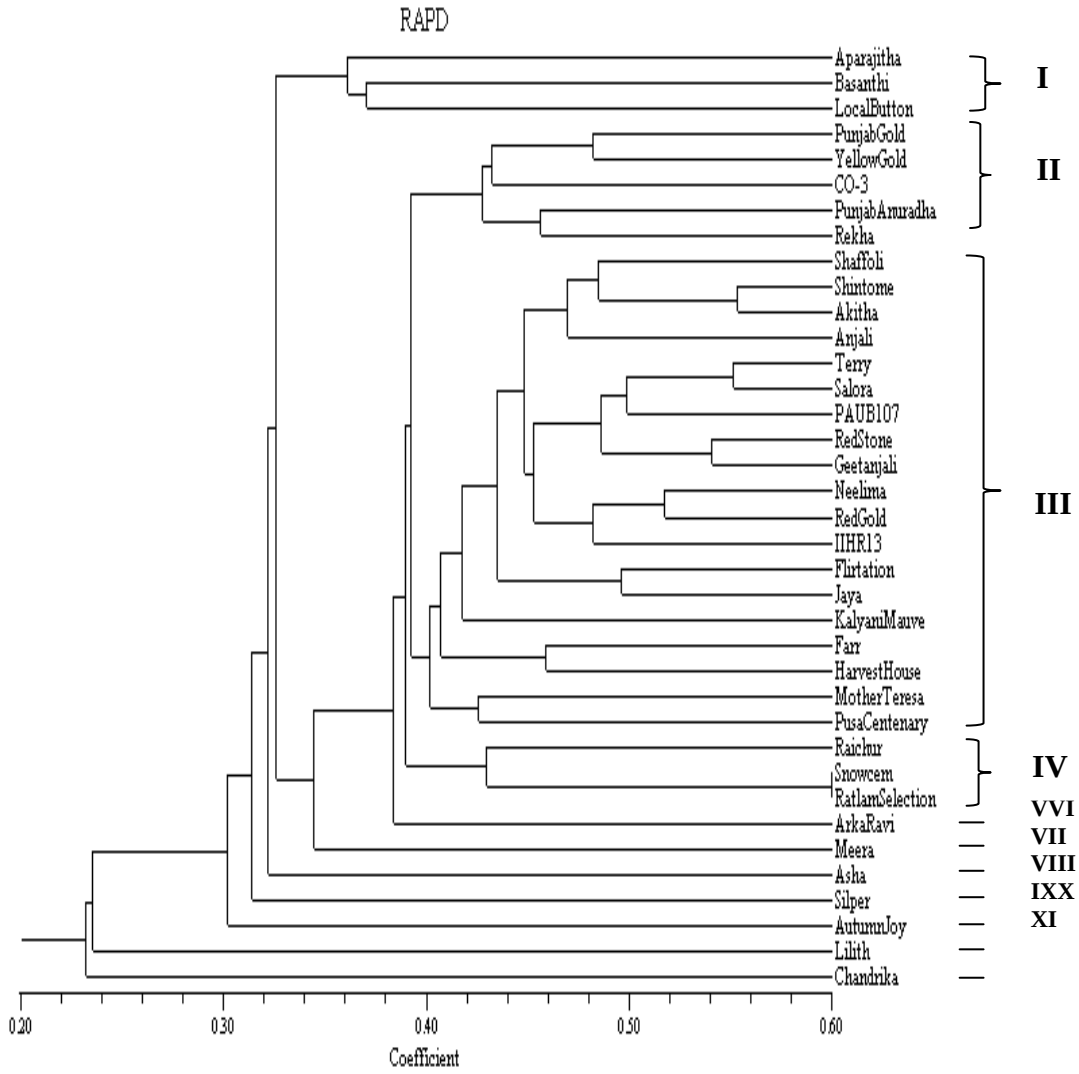


Fig.3 Dendrogram generated using UPGMA analysis showing the genetic relationship among *Chrysanthemum* genotypes using ISSR data

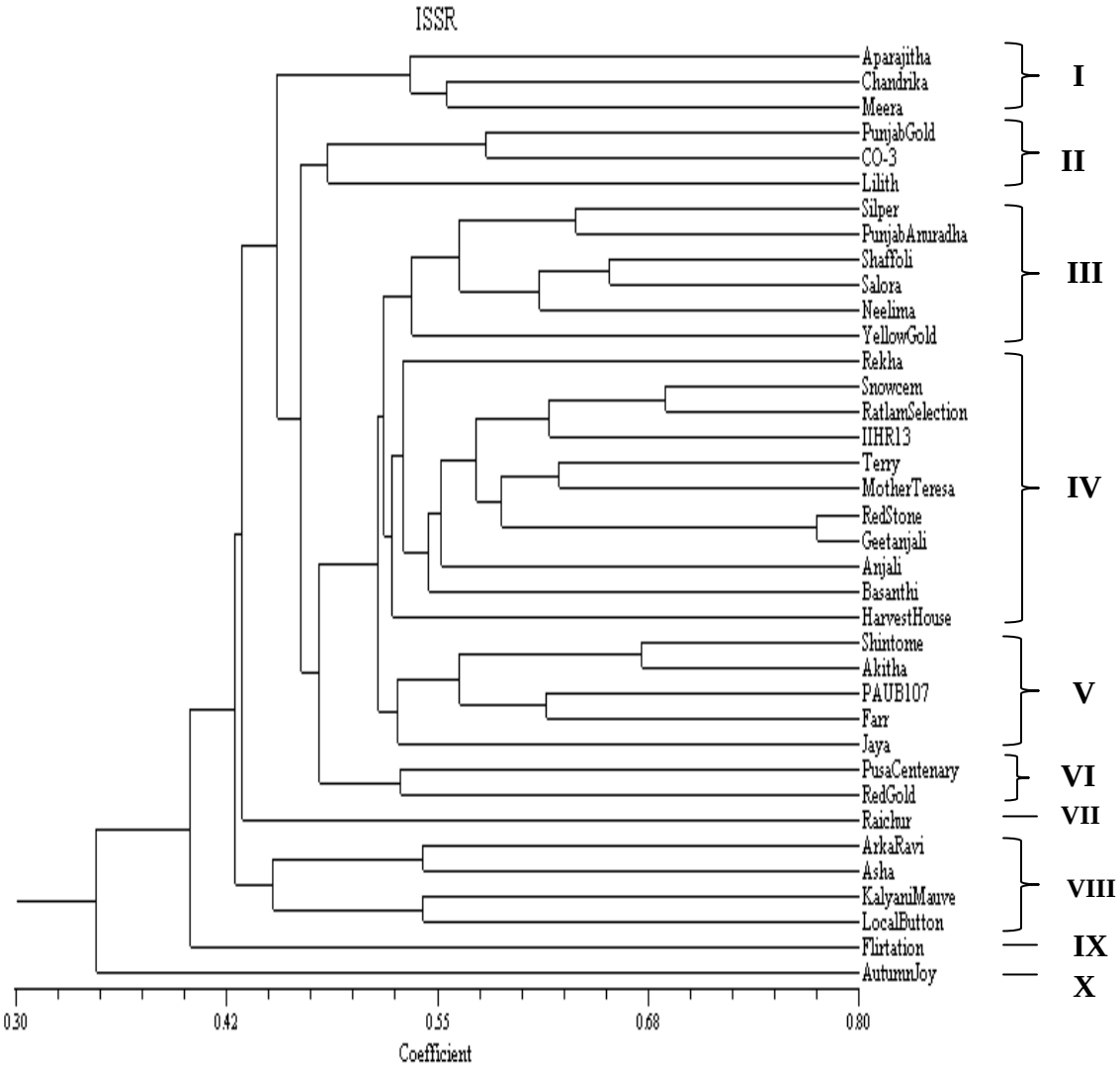
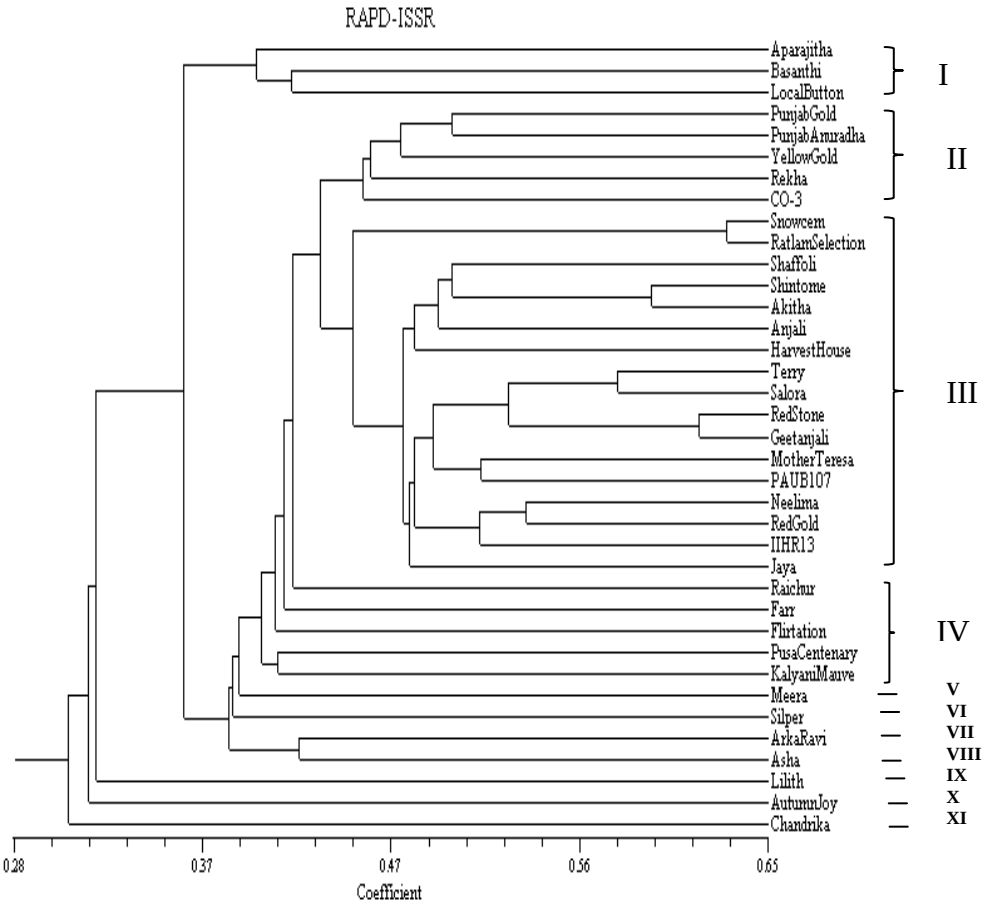


Fig.4 Dendrogram generated using UPGMA analysis showing the genetic relationship among *Chrysanthemum* genotypes using RAPD + ISSR data



The number of bands varied from 7(ISSR-825) to 15(ISSR-808 and ISSR-857) depending on the primer with a mean value of 11.4 bands per primer. The amplicon sizes obtained with the ISSR primers ranged from 220bp to 2000bp. The percentage of polymorphism exhibited by ISSR primers ranged from71.43% (ISSR-825) to 100% (ISSR-808, 810, 812, 840 and 842).The genetic similarity matrix ranged from 0.275 to 0.775 with an average of 0.525. The lowest similarity index (0.275) was scored between Autumn Joy and Flirtation that seem to be most divergent cultivars. The genotypes Geetanjali and Red Stone exhibiting the highest similarity index value of 0.775, are the most similar cultivars. The derived UPGMA dendrogram (Fig 3) exhibits ten clusters and the pattern revealed that, cluster IV was the largest one consisting of 11 genotypes followed by cluster III with six genotypes. Cluster V included five genotypes, cluster VIII with four genotypes, cluster I and II with three genotypes each, cluster VI with two genotypes and cluster VII, IX and X with one genotype each.

RAPD and ISSR Polymorphism

The RAPD data was combined with ISSRs in order to precise the relationships between the cultivars studied and a total of 378 polymorphic bands were generated. The similarity coefficients ranged from 0.243 to 0.629 with a mean similarity index of 0.436. The genotypes Snow Cem and Ratlam

Selection were almost similar with highest similarity index of 0.629 registered between them. The genotypes Autumn Joy and Basanthi showed lowest similarity index of 0.243. The dendrogram generated (Fig 4) illustrates the divergence among the genotypes. The grouping from combined analysis was similar in composition to that of RAPD. The results obtained from cluster analysis based on RAPD and ISSR data sets were different which was also reflected in the correlation coefficient value of r = 0.3906 by using Mantel test. This inferred that the two sets of markers explore genetic variation differently. According to the results, both the marker techniques could satisfactorily detect the genetic variation and thereby demonstrates the usefulness of these markers for further use in germplasm characterization of cultivars, including legal issues like assessing infringements on plant breeders rights.

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