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Molecular characterization of chrysanthemum by using RAPD and ISSR markers

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Abstract
Chrysanthemum (Compositae) is the second largest cut-flower after rose among the ornamental plants traded in the global flower market. It is propagated vegetatively as it has a strong sporophytic self-incompatibility system as shown by all members of Asteraceae family. Chrysanthemum grandiflora is a hybrid species which is the result of repeated cycles of inter specific crossing among elemental species bear blooms with two types of florets arranged on a flattened axis called capitulium. The outer florets are called ray floret and inner florets as disc floret. The ray florets have only female parts while disc floret may have both male and female parts and a tubular made up of five united petals. Being a cross pollinated crop, a lot of variation is present in chrysanthemum varieties. Twelve random amplified polymorphic DNAs (RAPDs) and eighteen and inter simple sequence repeats (ISSRs) markers were used for the evaluation of genetic variation and to assess polymorphism of cultivated chrysanthemum tested the genetic stability of chrysanthemum. The results indicate that the rate of polymorphism showed significant differences as compared to other existing varieties. The average number of total polymorphic bands were 3.78 for RAPD and 2.94 for ISSR markers. The size of RAPD and ISSR amplified fragments varied from 100bp – 2000 bp. Therefore RAPD allow the rapid detection of DNA polymorphisms, from many individuals or pooled samples. The advantages of RAPD lies in its simplicity, rapidity, requirement for only a small quantity of DNA and the ability to generate numerous polymorphisms. Thus it is considered a powerful technique for genetic analysis ISSR technique independently or in combination with other markers is used for genetic characterization of different plant species as an alternative reliable technique to study genetic diversity based on the presence of micro-satellites throughout genome.

Keywords: Chrysanthemum, variety identification, RAPD markers, ISSR markers

Introduction
Chrysanthemum is the second largest cut-flower after rose among the ornamental plants traded in the global flower market it occupies a place of pride both as commercial flower crop and as a popular exhibition flower. 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.

Chrysanthemum derived from Greek word “Chryos” means golden and “anthos” means flower. It is propagated vegetatively as it has a strong sporophytic self-incompatibility system as shown by all members of Asteraceae family. This diversity is combined with a wide variation in growth habit, vase life and amenability to various growth regulatory practices has made this flower popular. *Chrysanthemum grandiflora* is a hybrid species which is the result of repeated cycles of inter specific crossing among elemental species bear blooms with two types of florets arranged on a flattened axis called capitulium. The outer florets are called ray floret and inner florets as disc floret. The ray florets have only female parts while disc floret may have both male and female parts and a tubular made up of five united petals. Being a cross pollinated crop, a lot of variation is present in chrysanthemum varieties. Since chrysanthemum is a very popular flower, large numbers of varieties are released every year catering to consumer tastes. These varieties have not been characterized systematically. Confusion exists particularly in respect to the naming of varieties. For this there is an urgent need to systematically characterize the available chrysanthemum varieties. Molecular markers are excellent tools for analysis of genetic diversity and relationship as they are not dependent on environment and based on DNA fingerprinting. Polymerase chain reaction (PCR) based molecular makers like random amplified polymorphic DNA (RAPD) has been extensively used in DNA finger printing. Identification of varieties or breeding lines is very important in agricultural species, and is particularly interesting in *Chrysanthemum* when in many cases the origin of varieties is unknown. ISSR marker is a useful technique for the rapid and easy assessment of genetic variation among the variants. Random amplified polymorphic DNAs

(RAPDs) and inter simple sequence repeats (ISSRs) markers were used to elucidate the genetic diversity and relationships of the *C. indicum* polyploid complex. One of them is the PCR based technique RAPD that has been widely used for plant germplasm characterisation (Wolff *et al*., 1995). In this study, PCR-based marker assays including random amplified polymorphic DNAs (RAPDs) and intersimple sequence repeats (ISSRs) were employed to assess the genetic diversity and relationships of the *C. indicum* complex. RAPD provides a simple, PCR based molecular tool for the evaluation of genetic variation, while ISSR generates a large number of markers by using SSR repeat-anchored primers that target multiple microsatellite loci distributed throughout the genome (Awasthi *et al*., 2004) [1]. Genetic improvement of chrysanthemum is hampered mainly as a result of its genome complexity, high level of heterozygosity and the occurrence of both inbreeding depression and self-incompatibility (Li and Chen, 2007) [8].

Material and Methods

Commercial varieties were selected to produce variants. The selected varieties were cultivated in the Horticulture Farm, Rajasthan College of Agriculture, Udaipur.

DNA isolation and quantification

The DNA was extracted from fresh young leaves using cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). 5 g of leaf tissue material was ground in liquid nitrogen using a mortar pestle. The ground powder was transferred to 15 ml pre-warmed (65 ℃) DNA extraction buffer consisting of (100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2 % CTAB and 2 µl/ml β-mercaptoethanol). The homogenate was incubated for 1 hr at 65 ℃ with an equal volume of chloroform: Isoamyl alcohol (24:1) mixture and centrifuged at 5000 rpm for 10 minutes. Then DNA was precipitated from the aqueous phase by mixing with double the volume of chilled isopropanol. DNA-CTAB complex was precipitated as a fibrous network. It was lifted by pasteur pipette and transferred to washing solution. 500µl of 70% alcohol was added to pellet and was kept for 20 minutes with gentle agitation. The pellet was collected by centrifugation at 8000 rpm for 10 minutes at 20 ℃. The tubes were inverted and dried on a paper towel. The pellet was dried overnight after covering with parafilm with tiny pores. The pellet was resuspended in 500 µl of TE buffer by keeping over night at room temperature without agitation. DNA quantification were performed by observing it at 260 nm and 280 nm wavelengths using a spectrophotometer (uv5704 ss) on 0.8% agarose gel at 50 V for 1 hour and compared with a known amount of DNA. The resuspended DNA was then diluted in TE buffer to 500ng/ul concentration for use in polymerase chain reaction (PCR).

RAPD analysis

Polymerase chain reaction (PCR) with single RAPD primer was carried out in a final volume of 20 µl containing 1 X Assay Buffer (Merck Specialities, Bangalore), 1 units of Taq DNA polymerase, 200 µM each of dNTPs, 1.5 mM of $MgCl_2$, 500 µM /reaction of random primer's and 50 ng of tempelate DNA. Amplification was performed in PCR machine (Thermocycler) programmed for a preliminary 3 min denaturation step at 92 ^{0}C, followed by 45 cycles of denaturation at 92 ^{0}C for 1 min, annealing at required temperature 40 ^{0}C for 1 min, finally at 72 ^{0}C for 10 min for amplification. Amplication products were separated alongside amolecular weight marker (100bp and 1000bp ladder) by 1% agarose gel, which was prepared in 1 X TAE buffer gel was having Etbr at working concentration of 0.5 µg/ml of gel, 3 µl of 10 X loading dye (1 X final concentration) consisting of 0.4% bromophenol blue, 0.4% xylene cyanol and 50% glycerol in water, sterile water to make 100 ml was added to the PCR amplification products for visualization of gel run. PCR products (20µl) were loaded in gel. The Amplified products were electrophoresed for 3-4 hrs at 50 V with cooling. After separation the gel was viewed under UV transilluminator and photographed with the help of gel documentation system (Bio Rad Gel DOC).

ISSR analysis

Polymerase chain reaction (PCR) with single ISSR primer was carried out in a final volume of 20 µl containing 1 X Assay Buffer (Merck Specialities, Bangalore), 1 units of Taq DNA polymerase, 200 µM each of dNTPs, 1.5 mM of $MgCl_2$, 500 µM /reaction of random primer's and 50 ng of tempelate DNA. Amplification was performed in PCR machine (Thermocycler) programmed for a preliminary 5 min denaturation step at 94 ^{0}C, followed by 40 cycles of denaturation at 94 ^{0}C for 40 sec, annealing at required temperature 52 ^{0}C for 45 sec, finally at 72 ^{0}C for 10 min for amplification. Amplication products were separated alongside amolecular weight marker (100bp and 1000bp ladder) by 1% agarose gel, which was prepared in 1 X TAE buffer gel was having Etbr at working concentration of 0.5 µg/ml of gel, 3 µl of 10 X loading dye (1 X final concentration) consisting of 0.4% bromophenol blue, 0.4% xylene cyanol and 50% glycerol in water, sterile water to make 100 ml was added to the PCR amplification products for visualization of gel run. PCR products (20µl) were loaded in gel. The Amplified products were electrophoresed for 3-4 hrs at 50 V with cooling. After separation the gel was viewed under UV transilluminator and photographed with the help of gel documentation system (Bio Rad Gel DOC).

Data analysis

Data were recorded as presence (1) or absence (0) of band products from the photographic examination. Each amplification fragment was named by the source of the primer, the primer number and its approximate size in base pairs. Bands with similar mobility to those detected in negative control, if any, were not scored. The average of similarity matrices was used to generate a tree by UPGMA (Unweighted Pair-Group Method Arithmetic Average).

Results

RAPD Analysis

A total of Thirty two random decamer primers were used in this study. Out of which 14 primers produced amplification showed variable degree of polymorphism ranging from 50 to 100 per cent. Average polymorphism was found to be 79.45 per cent. Fourteen primers on thirty six chrysanthemum genotypes generated a total of 61 bands out of which only 53 were polymorphic bands (Table 1). The average number of bands per primer was found to be 4.35. The average numbers of polymorphic bands per primer were 3.78.Overall polymorphism was found to be 86.88 per cent. Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard's Coefficient Analysis. The similarity coefficient matrix generated for the primers was subjected to algorithm UPGMA (Unweighted Pair Group Method with Arithmetic averages) and dendrogram was

generated using NTSYS-pc 2.02 programme (Rohlf, 1997) (Fig. 1).The RAPD data were used to obtain a similarity matrix. The similarity coefficient for different genotypes was in the range of 0.44 to 0.98. The maximum similarity coefficient (0.98) was observed in T_2 whereas, the minimum similarity coefficient (0.44) was found in T_{24}. The dendrogram generated on the basis of Jaccard's Similarity Coefficient, clearly indicated seven main clusters (Fig 1). According to molecular dendrogram only PAU D-1 and PAU B-43 are genetically similar at similarity coefficient 0.98. Sadbhavana was unclustered which means are genetically diverse from rest of the genotypes.

ISSR Analysis

Thirty four selected Inter Simple Sequence Repeat (ISSR) primers were used in 36 different genotypes of chrysanthemum for detecting polymorphism. Out of 34 primers screened, 18 primers produced amplification. Out of these 4 primers showed 100 per cent polymorphism. Thirty four primers on thirty six chrysanthemum genotypes generated a total of 77 bands out of which only 53 were polymorphic bands (Table 4.2). The average number of bands per primer was found to be 4.27. The average numbers of polymorphic bands per primer were 2.94. Overall polymorphism was found to be 68.83 per cent. A dendrogram (Fig.2) was constructed using similarity matrix value as determined from ISSR data for 36 genotypes using UPGMA (Unweighted Pair Group Method with Arithmetic averages) subprogram of NTSYSpc programme. The dendrogram constructed depicted the relationship among 36 genotypes of chrysanthemum used on the basis of Jaccard's Similarity Coefficient. The similarity coefficient range from 0.55 to 0.94 clearly indicated seven main clusters. The maximum similarity coefficient (0.94) was observed between T_{14} and T_{18}, followed by T_{36} showed similarity of 0.93. The minimum similarity coefficient (0.55) was observed in T_{34}. According to molecular dendrogram genotype Agnishikha and Jaya also Punjab Anuradha and Agnidev are genetically similar at similarity coefficient 0.94. Silk Brockade and Shyamal Small were unclustered which means are genetically diverse from rest of the genotypes.

RAPD and ISSR combined analysis

Combined dendrogram (RAPD and ISSR) using similarity matrix value as determined from RAPD and ISSR data for 36 genotypes using UPGMA (Unweighted Pair Group Method with Arithmetic averages) subprogram of NTSYSpc programme (Fig.3) consists of ten clusters and result showed that genotype T_1 (PAU D-1) which was closely similar to T_2 (PAU B-43) at similarity coefficient of 0.94. Genotype T_{24} (Silk Brockade) was more divergent than other genotypes.

Discussion

RAPD Analysis

DNA analysis using randomly amplified polymorphism DNA (RAPD) technique offers a simple and economic means for rapid identification of large number of accessions. The information obtained through germplasm characterization using RAPD is extensively used for the identification of germplasm, screening of duplicates, assessing genetic diversity and monitoring the genetic stability of conserved germplasm (Kumar *et al*., 2001) [7]. RAPD markers have the advantage of detecting polymorphism simply and quickly (Demeke *et al.* 1996) [4]. Chatterjee *et al.* (2005) [3] studied that RAPD is a simple, easy and inexpensive way to compare the genetic relationship and pattern of variation among the gene pool in chrysanthemum crop. Out of 32 primers screened, 14 primers produced amplification. Average polymorphism was found to be 79.45 per cent. Similar results were reported by Kumar *et al.* (2006) [6], they reported that twenty one randomly amplified polymorphic DNA (RAPD) marker in chrysanthemum produced total of 156 clear bands, of which 118 bands were polymorphic and average polymorphism was found to be 71.76 per cent. Fourteen primers on thirty six chrysanthemum genotypes generated a total of 61 bands out of which only 53 were polymorphic bands. The average number of bands per primer was found to be 4.35. The average numbers of polymorphic bands per primer were 3.78. Overall polymorphism was found to be 86.88 per cent. Similar results were observed by Chatterjee *et al.* (2005) [3] in chrysanthemum genotypes. The RAPD data were used to obtain a similarity matrix. The maximum similarity coefficient (0.98) was observed in T_2 whereas, the minimum similarity coefficient (0.44) was found in T_{24}. The results obtained were in conformity with the report by Mukherjee *et al.* (2013) [9] selected 40 accession of chrysanthemum to study genetic diversity using RAPD with a similarity coefficient ranged from 0.20 to 0.68. Dendrogram was constructed using similarity matrix value as determined from RAPD data depicted the relationship among the genotypes of chrysanthemum. The dendrogram generated on the basis of Jaccard's Similarity Coefficient, clearly indicated seven main clusters (Fig 1). Mukherjee *et al.* (2013) [9] also studied the genetic diversity of 40 elite varieties of chrysanthemum characterized by means RAPD marker. The result suggested the level of genetic diversity.

ISSR Analysis

Out of 34 primers screened, 18 primers produced amplification. Out of these 4 primers showed 100 per cent polymorphism. Thirty four primers on thirty six chrysanthemum genotypes generated a total of 77 bands out of which only 53 were polymorphic bands. The average number of bands per primer was found to be 4.27. The average numbers of polymorphic bands per primer were 2.94. Overall polymorphism was found to be 68.83 per cent. Similar results were observed by Shao *et al*. (2010) [11] in chrysanthemum and obtained 81.87% polymorphism. Baliyan *et al.* (2014) [2] studied higher level of informativeness by the ISSR markers indicated the capability of microsatellites to quantify genetic variability in chrysanthemum species. The similarity coefficient range from 0.55 to 0.94 clearly indicated seven main clusters. The maximum similarity coefficient (0.94) was observed between T_{14} and T_{18}, followed by T_{36} showed similarity of 0.93. The minimum similarity coefficient (0.55) was observed in T_{34}. The results obtained were in conformity with the report by Reddy *et al*. (2002) [12] in plant breeding. The similarity coefficients of the 36 chrysanthemum genotypes based on RAPD and ISSR markers ranged from 0.53 to 0.94. The maximum similarity coefficient (0.94) was observed between T_2 and T_1 and minimum (0.53) between T_{24} and T_3. Namita *et al*. (2013) [10] also reported genetic diversity in marigold using ISSR markers.

RAPD and ISSR combined analysis

The present work used a combination of RAPD and ISSR markers to determine the further genetic affinities between chrysanthemum genotypes at the DNA level, the results indicated a close correspondence between the similarity matrices of both RAPD and ISSR individually or combined,

hence both the marker systems could be effectively used in determination of genetic relationship among chrysanthemum species. Mukherjee *et al.* (2013) [9] studied genetic diversity in elite chrysanthemum varieties using RAPD and ISSR markers. Among the 20 primers used, 14 showed polymorphism. RAPD and ISSR data were analyzed separately as well as in combination to determine relationship between the genetic lines. ISSR data was more useful in assertion of genetic diversity. Namita *et al*. (2013) [10] studied genetic diversity in marigold using both RAPD and ISSR markers. Correlation between genetic distances obtained through RAPD and ISSR markers was relatively high and indicating that both techniques are efficient for evaluating genetic diversity in the genotypes of marigold and RAPDs yielded more polymorphisms. Besides this Joshi *et al*. (2009) [5] investigated and characterized genetic variation of 22 isolates of tea plant from five different regions in Southern India and showed the combination of RAPD and ISSR analysis could be a useful method for assessing genetic variation and could be helpful for breeders to plan crosses for positive traits.

Combined dendrogram (RAPD and ISSR) using similarity matrix value as determined from RAPD and ISSR data for 36 genotypes using UPGMA (Unweighted Pair Group Method with Arithmetic averages) subprogram of NTSYSpc programme (Fig. 3) consists of ten clusters. Cluster A included T_1 and T_2 genotypes at similarity coefficient of 0.74. These two genotypes are more closely related genotypes to each other. From the results based molecular studies it is resulted that 36 chrysanthemum genotypes were classified more precisely in 7 clusters through RAPD markers, 7 clusters through ISSR markers and when the data was combined they were classified into 10 clusters. This indicated that molecular markers are least affected by environmental fluctuations and can be more precisely used in assessing genetic variability and identification of genotypes. Therefore the dendrograms obtained through RAPD and ISSR showed classification of genotypes into 7 clusters individually and 10 clusters jointly reflecting some relationship**.** The results further indicated that both of the marker systems RAPD and ISSR can be precisely and effectively used in determination of genetic variability among chrysanthemum genotypes. Therefore these can assist in further breeding programmes by characterizing the variable genotype.

Table 1: Polymorphism information of RAPD primers analyzed

Sr. No.	Primers code	Total No. of bands (a)	Total No. of polymorphic bands(b)	Polymorphism % (b/a × 100)
1.	OPA05	1	0	0
2.	OPA07	7	6	85.7
3.	OPA08	5	4	80
4.	OPA09	7	7	100
5.	OPA10	6	6	100
6.	OPA11	2	1	50
7.	OPA14	NA	NA	NA
8.	OPA15	2	2	100
9.	OPA16	3	3	100
10.	OPB02	NA	NA	NA
11.	OPB04	NA	NA	NA
12.	OPB05	NA	NA	NA
13.	OPB06	NA	NA	NA
14.	OPB07	NA	NA	NA
15.	OPB08	3	3	100
16.	OPB10	8	8	100
17.	OPB11	NA	NA	NA
18.	OPC01	NA	NA	NA
19.	OPC05	NA	NA	NA
20.	OPC06	NA	NA	NA
21.	A01	NA	NA	NA
22.	A04	NA	NA	NA
23.	B01	NA	NA	NA
24.	B03	NA	NA	NA
25.	C02	NA	NA	NA
26.	D02	5	5	100
27.	G04	NA	NA	NA
28.	I 18	5	4	80
29	J05	NA	NA	NA
30.	M17	NA	NA	NA
31.	P09	4	2	50
32.	P16	3	2	66.6
	Total	61	53	1112.3
	Average	4.35	3.78	86.88

NA - Not amplified

Table 2: Polymorphism information of ISSR primers analyzed

Sr. No.	Primers code	Total No. of bands (a)	Total No. of polymorphic bands (b	Polymorphism %(b/a × 100)
1.	ISSR-1	NA	NA	NA
2.	ISSR-2	NA	NA	NA
3.	ISSR-3	NA	NA	NA
4.	ISSR-4	4	1	25

5.	ISSR-5	6	5	83.3
6.	ISSR-6	5	5	100
7.	ISSR-7	NA	NA	NA
8.	ISSR-8	4	4	100
9.	ISSR- 9	NA	NA	NA
10.	ISSR-3-	NA	NA	NA
11.	ISSR-4-	4	3	75
12.	ISSR-5-	NA	NA	NA
13.	ISSR-33	6	3	50
14.	ISSR-35	3	1	33.3
15.	ISSR-42	6	4	66.6
16.	ISSR-43	2	0	0
17.	ISSR-44	3	1	33.3
18.	ISSR-45	6	4	66.6
19.	ISSR-46	5	3	60
20.	ISSR-52	NA	NA	NA
21.	ISSR-55	NA	NA	NA
22.	ISSR-57	4	3	75
23.	ISSR-58	NA	NA	NA
24.	ISSR-60	3	2	66.6
25.	ISSR-61	NA	NA	NA
26.	ISSR-64	2	1	50
27.	ISSR-65	6	5	83.3
28.	ISSR-67	NA	NA	NA
29	ISSR-Y5	NA	NA	NA
30.	ISSR- Y10	3	3	100
31.	ISSR-Y11	NA	NA	NA
32.	IG-03	NA	NA	NA
33.	IG-11	NA	NA	NA
34.	IG-12	5	5	100
	Total	77	53	1168
	Average	4.27	2.94	68.83

NA - Not amplified

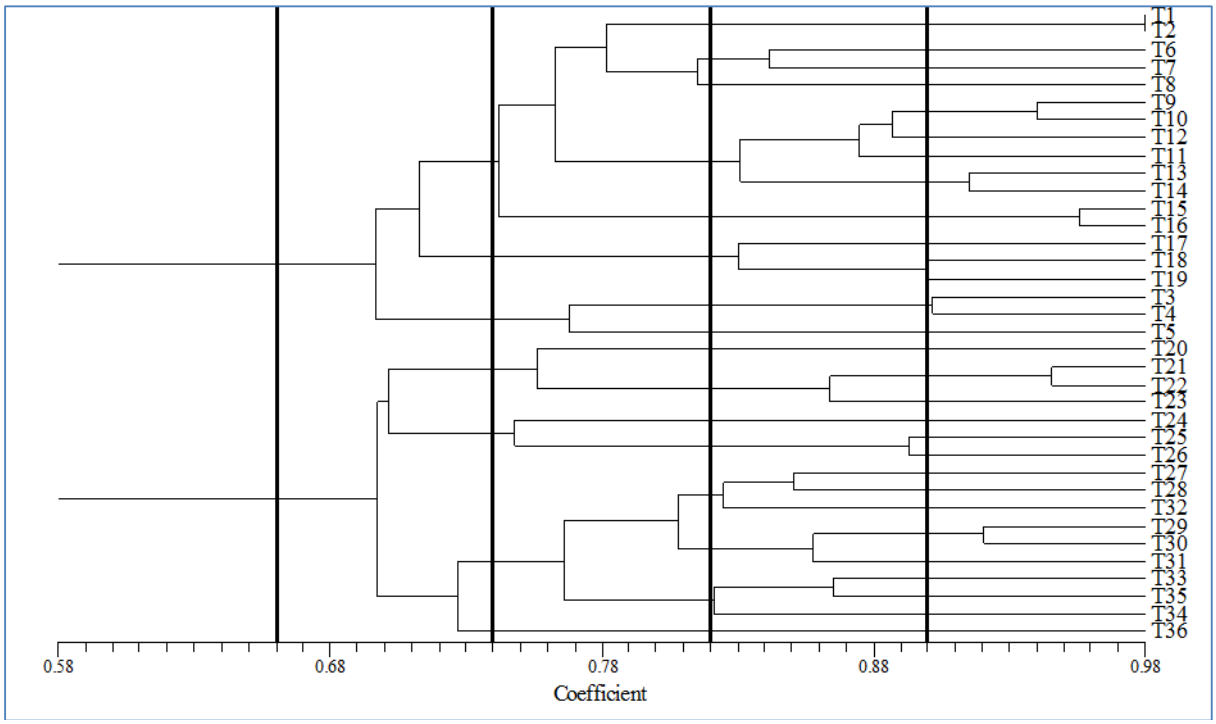

Fig. 1: Dendrogram Generated for 36 chrysanthemum genotypes using UPGMA Cluster Analysis based on RAPD marker

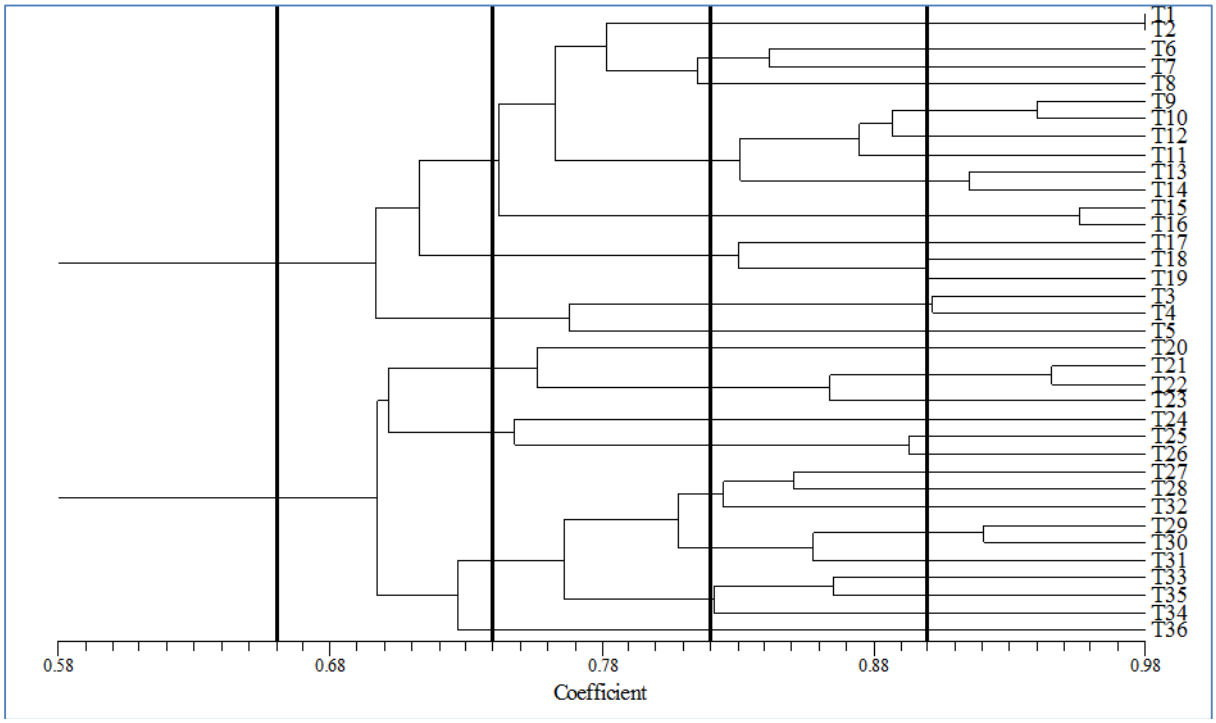

Fig. 2: Dendrogram Generated for 36 chrysanthemum genotypes using UPGMA Cluster Analysis based on ISSR marker

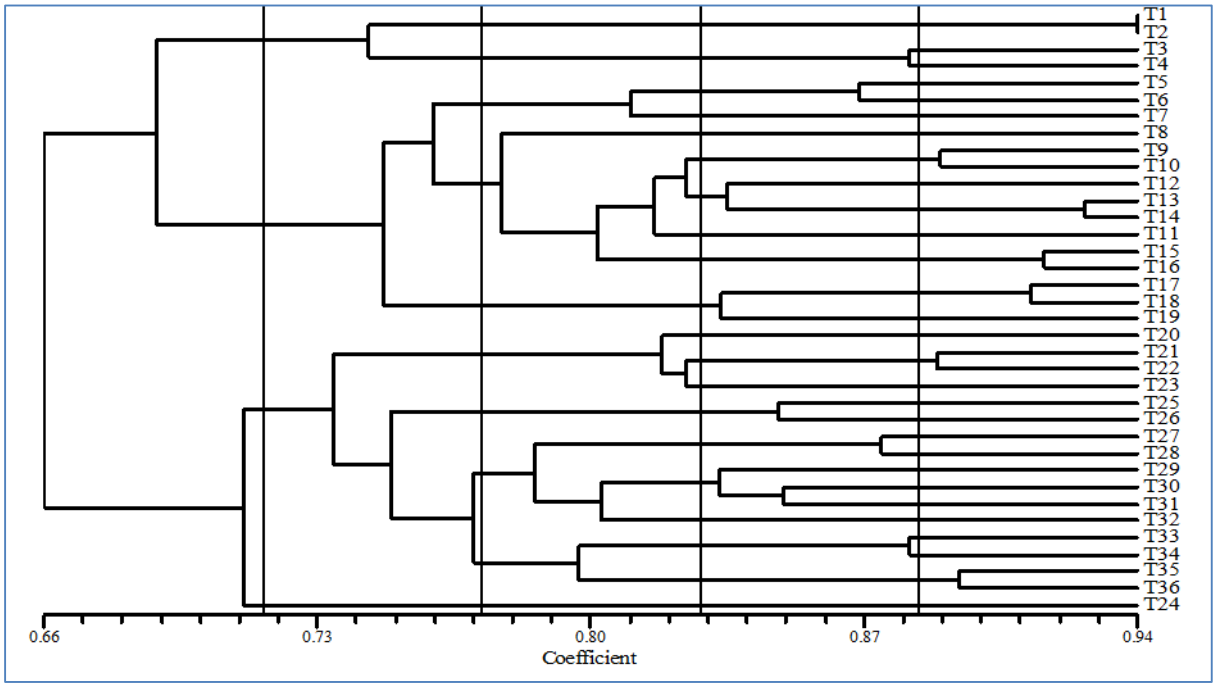

Fig. 3: Dendrogram Generated for 36 chrysanthemum genotypes using UPGMA Cluster Analysis based on RAPD and ISSR marker

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