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Molecular characterization of chrysanthemum (*Dendranthema grandiflora*) genotypes using ISSR and SSR markers

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ABSTRACT

An investigation was carried out to distinguish genotypes and to assess the genetic relatedness among 30 selected chrysanthemum (*Dendranthema grandiflora* Tzvelev) genotypes using ISSR and SSR markers. Ten ISSR primers were used to generate polymorphism which resulted in 175 amplicons with 100% polymorphism showing significant differences among the 30 chrysanthemum genotypes. Molecular analysis of 50 loci using 17 SSR primer pairs resulted in 80.4% mean polymorphism. Using dendrogram, three distinct groups were found based on SSR and ISSR indicating significant differences among the genotypes. The results showed that, there was a large genetic diversity exists among the chrysanthemum genotypes which helps in the selection of suitable genotypes for breeding. The results suggested that both ISSR and SSR were found to be effective for characterization studies in chrysanthemum.

Key words: Chrysanthemum, Genetic diversity, ISSR, Molecular characterization, SSR

Chrysanthemum (*Dendranthema grandiflora* Tzvelev) (family Asteraceae) is one of the leading commercial flower crops, which is important both as cut flower and potted plant in the international market. The numerous species involved in the development of present chrysanthemum were mentioned by various workers as *Chrysanthemum sinense, C. indicum, C. japonicum, C. ornatum, C. satsumense* and *C. boreale*.

In order to develop an efficient breeding program, it is necessary to characterize and understand the genetic relationships of the various accessions available in the gene bank. Molecular markers have a number of perceived advantages over the morphological markers for the assessment of genetic diversity. ISSR and SRAP markers have been shown to be effective for DNA fingerprinting, genetic diversity analysis and germplasm evaluation since they can identify many informative loci. ISSR and SRAP markers clearly indicated the genetic relationship among 29 populations of different chrysanthemum species (Shao *et al.* 2010). Recently, development of microsatellite (SSR) markers has been an increasing trend in crop genetic studies because of the applicability of these markers in breeding programs. SSR markers are highly polymorphic, co-dominant and have got high reproducibility. SSR markers have got potential application for the cultivar classification, studies on genetic diversity and molecular breeding of chrysanthemums (Zhang *et al.* 2013). The germplasm accessions collected from different locations are being characterized and evaluated for a set of internationally recognized descriptors by the breeder, which include stable botanical (qualitative) characters and horticultural traits. The present study was initiated to complement the morphological characterization, with the objective of identification of new diverse sources with specific traits that will enhance the use of germplasm in breeding programs.

MATERIALS AND METHODS

A total of 30 chrysanthemum genotypes were examined (Table 1) which are being maintained at Division of Ornamental Crops, ICAR-IIHR, Hesaraghatta Lake Post, Bengaluru, Karnataka.

The CTAB method of DNA extraction as described by Doyle and Doyle (1990) was followed with some minor modifications. The total genomic DNA was isolated through CTAB method with minor modifications. The DNA quality was checked by electrophoresis using agarose gel (0.8%).

A total of 50 UBC ISSR were screened initially for testing their capability to amplify the target genomic regions. Each PCR amplification was performed in a final volume of 10 μl reaction mixture containing 80 ηg of template DNA,

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Table 1 Chrysanthemum genotypes with their geographical origin, flower type and flower colour

Genotypes	Source of collection	Flower type	Flower colour
Anmol	NBRI, Lucknow	Decorative	Pink
Ajay	NBRI, Lucknow	Pompon	Pink
Garden Beauty	PAU, Ludhiana	Double Korean	Salmon
Birbal Sahni	NBRI, Lucknow	Pompon	White
Arka Ravi	IIHR, Bengaluru	Korean	Bronze
Sunil	NBRI, Lucknow	Double Korean	Purple
Flirt	PAU, Ludhiana	Quilled	Purple
Fitonia	NBRI, Lucknow	Anemone	Yellow
NBRI Little Hemant	NBRI, Lucknow	Pompon	Bronze
NBRI Little Darling	NBRI, Lucknow	Pompon	Purple
Jayanti	NBRI, Lucknow	Decorative	Yellow
Co-Semi Double	TNAU, Coimbatore	Reflexing	Yellow
Co-1	TNAU, Coimbatore	Double Korean	Yellow
Statesman	NBRI, Lucknow	Anemone	Yellow
Winter Queen	PAU, Ludhiana	Quilled	Red
Kargil	NBRI, Lucknow	Spoon	White
Chandini	NBRI, Lucknow	Semi-Double	Yellow
Arka Nilima	IIHR, Bengaluru	Decorative	Salmon
Arka Yellow Gold	IIHR, Bengaluru	Decorative	Yellow
Arka Red Gold	IIHR, Bengaluru	Double Korean	Greyish Orange
Arka Ushakiran	IIHR, Bengaluru	Semi – Double	Yellow
Red Stone	NBRI, Lucknow	Single Korean	Red
Arka Kirti	IIHR, Bengaluru	Double Korean	White
Coffee	NBRI, Lucknow	Pompon	Bronze
Pusa Anmol	IARI, New Delhi	Double Korean	Salmon
Arka Pankaj	IIHR, Bengaluru	Decorative	Pink
Appu	NBRI, Lucknow	Anemone	Red
Nayantara	NBRI, Lucknow	Anemone	Yellow
Vijaykiran	NBRI, Lucknow	Double Korean	Yellow
Shukla	NBRI, Lucknow	Double Korean	White

1x buffer, 1 mM dNTPs, 1 U of Taq DNA polymerase, 0.3 pM primer with the following thermal conditions: initial denaturation at 94ºC for 4 min, followed by 35 cycles; each cycle consisting of denaturation at 94ºC for 1 min, specific annealing for each primer at 49º to 56.1ºC for 45 seconds, primer extension at 72ºC for 1 minute, a final extension of 8 minutes at 72ºC and hold at 4ºC. The annealing temperature for each primer is presented in Table 2. Amplification products were separated by electrophoresis on 1.4% (w/v) agarose gel in 0.5x TBE (1- tris-borate-ethylene diamine tetra acetic acid) buffer (pH 8.0) at a constant voltage (50 V). Then gel was visualized under UV light by using Gel Documentation System. The molecular weights were estimated using a 100 bp DNA ladder.

A total of 23 SSR primer pairs (Li *et al*. 2013) were screened initially to select primers that can generate informative fragments. Each PCR amplification was performed in a final volume of 20 µl reaction mixture containing 75 ηg of template DNA, 2x buffer, 0.25 mM dNTPs, 1.25 mM of $MgCl_2$, 1 U of Taq DNA polymerase, 0.03 pM each of forward and reverse primer with the following conditions: initial denaturation at 94ºC for 4 min, followed by 35 cycles; each cycle consisting of denaturation at 94ºC for 1 min, primer annealing at 55ºC for 45 seconds and primer extension at 72ºC for 1 min and a final extension of 10 min at 72ºC and hold at 4ºC. Amplification products were separated by electrophoresis on 3% (w/v) agarose gel in 0.5x TBE buffer (pH 8.0) at a constant voltage (50 V). Then gel was visualized under UV light by using Gel Documentation System. The molecular weights were estimated using a 100 bp DNA ladder.

Only reproducible and unambiguous ISSR and SSR fragments were scored for presence (1) or absence (0). Data were compiled in a binary data matrix using MS Excel. The binary data obtained by scoring the ISSR and SSR profiles with different primers individually as well as collectively were subjected to the construction of distance matrix through Squared Euclidina Distance method using STATISTICA software (Version 4.5) which provided the estimation of all pair wise differences in the amplification product. The similarity values were used for cluster analysis in accordance with unweighted pair-group with arithmetic averages (UPGMA) method. The different genetic diversity parameters for ISSR and SSR were estimated through the software crop DNA fingerprinting using the pool of binary data scored.

RESULTS AND DISCUSSION

ISSR polymorphism

ISSR profiles were generated from the selected ten primers that were consistent, unambiguous and repeatable. A total of 175 ISSR markers of size ranging from 100 to 1 900 bp were used for DNA profiling and to estimate genetic diversity among 30 genotypes of chrysanthemum (Table 2). This was comparable with the reports of Baliyan *et al*. (2014) in chrysanthemum using ISSR technique wherein more than 150 amplified products generated for distinguishing genotypes. Out of 175 amplification products recorded, all were polymorphic. The marker index was high for all the selected primers as they generated cent percent polymorphism. The high level of polymorphism may also be

Table 2 Details of ISSR primers used for molecular characterization of 30 chrysanthemum genotypes

Primer code	Primer sequence (51- 31)	Annealing temperature (ºC)	Total bands produced	Polymorphic bands (No.)	Polymorphism (%)
UBC 823	TCT CTC TCT CTC TCT CC	56.1	18	18	100
UBC 843	CTC TCT CTC TCT CTC TRA	50	19	19	100
UBC 845	CTC TCT CTC TCT CTC TRG	50	18	18	100
UBC 846	CAC ACA CAC ACA CAC ART	51	20	20	100
UBC 847	CAC ACA CAC ACA CAC ARC	55	20	20	100
UBC 849	GTG TGT GTG TGT GTG TYA	50	17	17	100
UBC 855	ACA CAC ACA CAC ACA CYT	50	20	20	100
UBC 856	ACA CAC ACA CAC ACA CYA	55	17	17	100
UBC 857	ACA CAC ACA CAC ACA CYG	51.6	11	11	100
UBC 890	VHV GTG TGT GTG TGT GT	49	15	15	100
Total			175	175	100
Average			17.5	17.5	100

due to the high potential of primers to detect polymorphism (Huang *et al*. 2012).

Assessment of genetic diversity based on ISSR data

The investigation was undertaken to assess the genetic diversity and relatedness in chrysanthemum genotypes using ISSR markers. The ISSR data were used to make pair wise comparisons of the genotypes based on amplification patterns. ISSR analysis revealed that the genetic distance was maximum between NBRI Little Darling and Arka Nilima with the dissimilar matrix value of 74, while Anmol and Ajay differed by the value of 28.

Chrysanthemum maintains relatively high genetic diversity (Fang *et al*. 2012). This could be attributed to the mating systems, processes of germplasm exchange, ecotype selection and successive cross breeding including the genotypes from different regions. The ISSR markers are potential of identifying more number of informative loci per primers (Shao *et al*. 2010), and detecting sufficient polymorphism (Khaing *et al*. 2013) in chrysanthemum. One of the contributory factors to the high degree of polymorphism may be on account of its mating systems and outcrossing species with wide and continuous distributions tend to have high level of genetic diversity in chrysanthemum (Fang *et al*. 2012).

Cluster analysis of the chrysanthemum genotypes employing UPGMA method led to the grouping of the accessions into three distinct major groups (A, B and C) (Fig 1). There was no clear cut difference among the genotypes based on their geographical locations and morphological traits. In the major groups ‘A’ and ‘B’, 14 genotypes and 15 genotypes, respectively, from different geographic regions were grouped together. This is because of individual genotypic differences. The results are in accordance with Fang *et al*. (2012) in chrysanthemum. The single genotype Co- Semi double from TNAU, Coimbatore was included in the group ‘C’. This result is comparable with the clustering of 29 *Ceynodon radiates* accessions where the group A included only 1 accession (Huang *et al*., 2012). Similar results were also obtained by Buldewo *et al*. (2012) while studying genetic diversity in *Anthurium andraeanum* cultivars in which the cultivar Scorpio remained as a single cultivar in a clade of its own with ISSR analysis.

ISSR genetic diversity does not necessarily match morphological differences. However, in the present investigation, to certain extent, clustering of genotypes in the sub-clusters seemed to correspond with few phenotypic traits. Anmol and Ajay with same flower colour (pink) and both having short plant height formed sister relationships within cluster A1. Within cluster A4, two yellow flowered genotypes (Arka Yellow Gold and Jayanti) formed the sister relationships. The genotype Arka Pankaj remained as a single cultivar in a separate cluster within the major ‘B’ which is the only one pink colour genotype in the group ‘B’. Similar clustering on phenotypic traits/colour done by Buldewo *et al*. (2012) based on spathe colour in anthurium. Palai and Rout (2011) used ISSRs for clustering of chrysanthemum genotypes based on flower colour. In the major group ‘B’, the genotypes with incurving flower type (Co-1, Shukla, Red Stone, Vijaykiran, Arka Nilima, Arka Red Gold, Arka Kirti, Pusa Anmol, Arka Ushakiran and Pankaj) were grouped together with few exceptions. Dai *et al*. (2012) classified chrysanthemum germplasm based on the inflorescence using ISSR markers. The economic uses of different genotypes were also found correlated with different clusters within the major groups. Two cut flowered genotypes (Arka Ravi and Flirt) formed sister relationships in cluster A2. Similarly, two pot culture genotypes (Jayanti and NBRI Little Darling) were grouped together within cluster A4. The two pot culture genotypes (NBRI Little Hemant and Fitonia) formed the cluster within A5. The cluster B1 was again divided into two sub-clusters based on their economic uses, in which three loose flower genotypes (Co-1, Shukla and Coffee) were grouped together and four garden display genotypes (Red Stone, Nayantara, Appu and Vijaykiran) formed another group. The six loose flower genotypes Statesman,

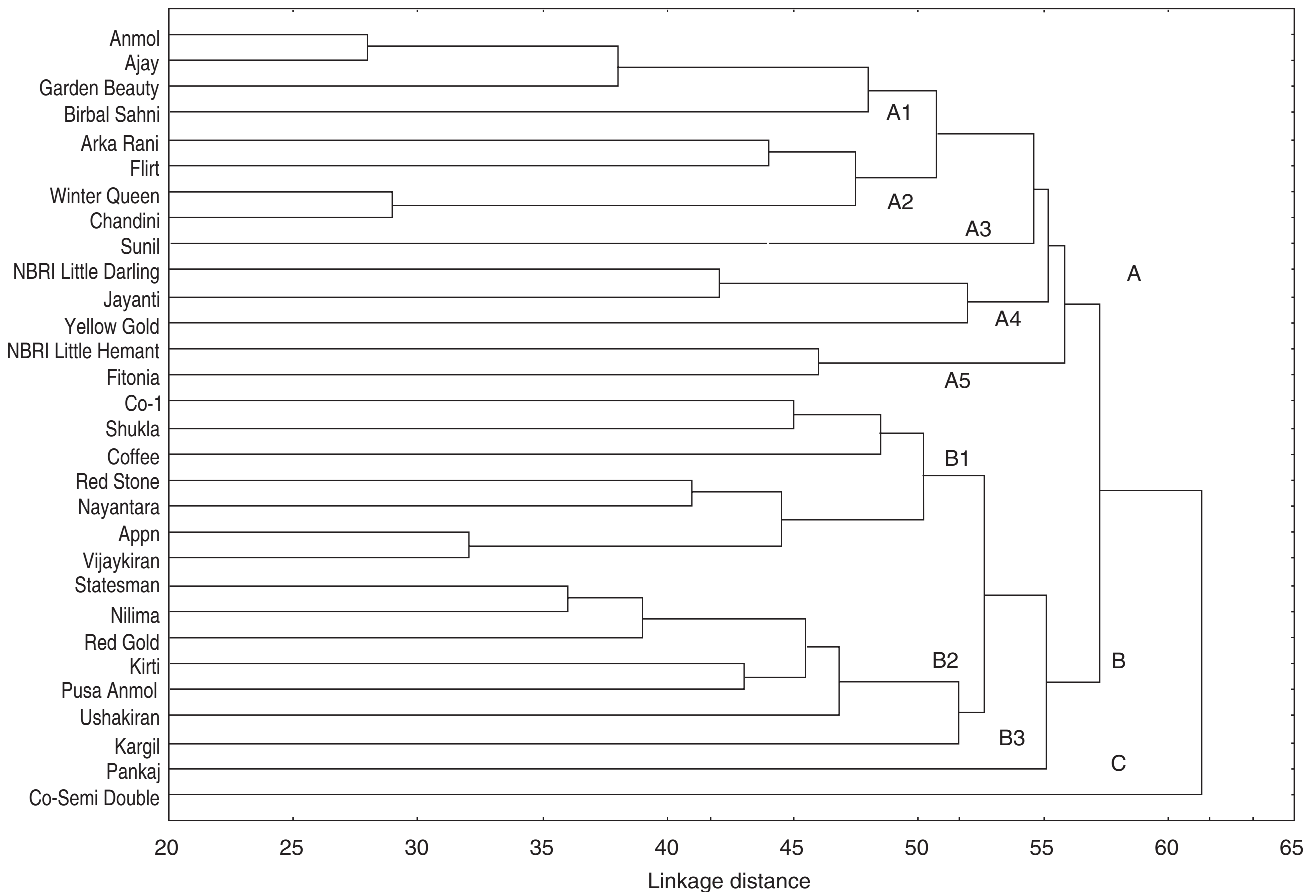

Fig 1 Dendrogram showing genetic relationship among 30 chrysanthemum genotypes based on ISSR data

Arka Nilima, Arka Red Gold, Arka Kirti, Pusa Anmol and Ushakiran were grouped together under the cluster B2. The genotype Kargil remained separately in B2 which is the only one genotype with spoon type of flower and spreading growth habit among the selected 30 genotypes. Minano *et al*. (2009) clustered chrysanthemum cultivars which were consistent with their inflorescence type and kind of culture.

SSR polymorphism

SSR profiles were generated using identified polymorphic SSR primer pairs to distinguish genotypes and to assess the genetic relatedness among the selected chrysanthemum genotypes. The selected primer pairs (Table 3) generated 51 alleles with an average of 3 alleles per locus. This result is comparable with the findings on SSR alleles representing several loci on the genome were obtained by Zhang *et al*. (2013) in chrysanthemum. The investigation demonstrated relatively high level of polymorphism i.e. out of 51 alleles, 41 were polymorphic showing 80.4% of polymorphism. The possibility for high level of polymorphism might be that chrysanthemum genotypes under study were derived from cross-pollination and propagated vegetatively and thus they might have a high level of heterozygosity. Gong and Deng (2010) also found 67.7% of polymorphism among seven gerbera cultivars. Similarly Zhang *et al*. (2013) detected 74.3% polymorphism among 40 chrysanthemum cultivars. The number of alleles amplified using 14 polymorphic SSR primers ranged from 2 to 6 which are similar to the reports of Gong and Deng (2010) in gerbera, though the number of polymorphic SSR loci (67) and samples (16) of the two studies are different.

Assessment of genetic diversity based on SSR data

The SSR data were used to make pair wise comparisons of the genotypes based on amplification patterns. The genetic dissimilarity value ranged from 3 to 19 suggesting a narrow genetic base within the selected chrysanthemum genotypes (data not shown). The result is in agreement with Rusanov *et al*. (2005) in which SSR data revealed high genetic similarity in large group of 31 rose genotypes. Low genetic variation was revealed by Wang *et al*. (2014) using SSRs. The low genetic diversity in chrysanthemum may be due to close relationship among the genotypes and also because of vegetative propagation.

Clustering was done based on the dissimilarity matrix values through UPGMA method, which grouped 30 chrysanthemum genotypes into three major groups (A, B and C) (Fig 2). Similar clustering performed by Wang and Chuang (2013), Wang *et al*. (2014) and Zhang *et al*. (2013) using SSRs in different ornamental crops.

In general, the groupings were not in consistent with their geographical locations and morphological traits. The

Table 3 Details of SSR primers used for molecular characterization of 30 chrysanthemum genotypes

Primer code	Core sequence of microsatellite	Microsatellite primer sequence (5'-3')	Size range of alleles (bp)	Total bands produced	Polymorphic bands (No.)	Polymorphism (%)
YW A 2-1	(AG)18	F: 5'TTTTCCATACCAACACTCC 3' R: 5' TTCCACAATCTTCCCACTC3'	86-136	2	1	50
YW A04	(AG)28	F: 5' ACCCTTACGCTTACTCATT 3' R: 5' TCGTCACCATTCTCTCCTG 3'	300-419	1	0	0
YW A08	(AG)6(TG)3 (GA)12GG (GA)15	F: 5'CTACACAAGTTAGGCGAGAT 3' R: 5' ACACAGTCCCTAAAATCC 3'	229-304	5	5	100
YW A 12	(AG)25	F: 5' CTGTCAGTTAGCCGTTTTCG 3' R: 5' CCTCATTTGTAAGGTGTGTG 3'	138- 195	4	3	75
YW A 15-1	(AC)15TA (AG)30	F: 5'TGGAGCTGAGAACCCTG 3' R: 5'TACCGCCGAACATACGA 3'	121- 313	4	3	75
YW A 25	(GA)25	F: 5'TATTGTTCTGACTGTCCC 3' R: 5'TATCTGGCAGTTCATTACGC 3'	87-109	2	2	100
YW A 33	(GA)12GG (GA)15	F: 5' ACACAAGTTAGGCGAGATAC 3' R: 5' CACACAGTCCCTAAAATCC 3'	117- 192	3	2	66.7
YW B07	(CA)8(CT)14	F: 5' CTCCTGCTTCCCTCTCCTCC 3' R: 5' GACGGGTAGAAAGGTGGTGC3'	252- 316	3	2	66.7
YW B08	(TC)40… (TG)5… (GC)7	F: 5' GATTGGTGAGATGTTGCC 3' R: 5' AGACGGAACGCACATAAG 3'	224- 314	3	3	100
YW B10	(TC)25	F: 5' ACTAACCCACCATTCCAC 3' R: 5' CAAATCCACCAAACCAAC 3'	118- 251	1	0	0
YW B 13-1	(TC)22	F: 5' TCAGTGAGTGGAGGGTTT 3' R: 5' TTGTATTCTCGGTAAGGATG 3'	143- 240	6	6	100
YW B 15-1	(TC)16	F: 5' GCATCAATCACCCTCTTTC 3' R: 5' TATGGCGTCACCGTTGTCT 3'	107- 164	2	1	50
YW B 17-1	(CT)24	F: 5' CTTTCCGATTTTCCCTCCT 3' R: 5' GTGTGTAGGTTTGCCATAG 3'	90- 157	2	1	50
YW B 18	(TC)6G(CT)21	F: 5' GGGCGTTTTTCCTTGTATT 3' R: 5' CACAAACACGATCTAAACC 3'	95- 268	5	5	100
YW B 21	(CAT)10	F: 5' TACCGCCGAACTTACGAAAC 3' R: 5' GATAAACGGAGGATGGTGTG 3'	161- 319	4	4	100
YW C 12	(TGA)6	F: 5' GCTCATTCCTCACATCT 3' R: 5' ATAAGGCTGAAGACGAG 3'	140- 151	1	0	0
YW C 15	(TGA)6AGATG ACGTGGCA (GAT)5	F: 5' GCCGAAGAGTAAACAGAG 3' R: 5' CGAACACGACACAAATCC 3'	172- 210	3	3	100
Total				51	41	80.4
Average				3	2.4	

genotypes from different regions were grouped together in the major groups 'A' and 'B'. This contrary results reported by Zhang *et al*. (2013) among chrysanthemum genotypes using SSRs. This indicates that the genotypes from different regions can be genetically similar. This could also be attributed to intensive vegetative multiplication and cultivation followed by selection of best performing plants and development of supposedly new clones from the same genotype (Rusanov *et al*. 2005). The major group 'C' included only one genotype Pusa Anmol developed at ICAR-

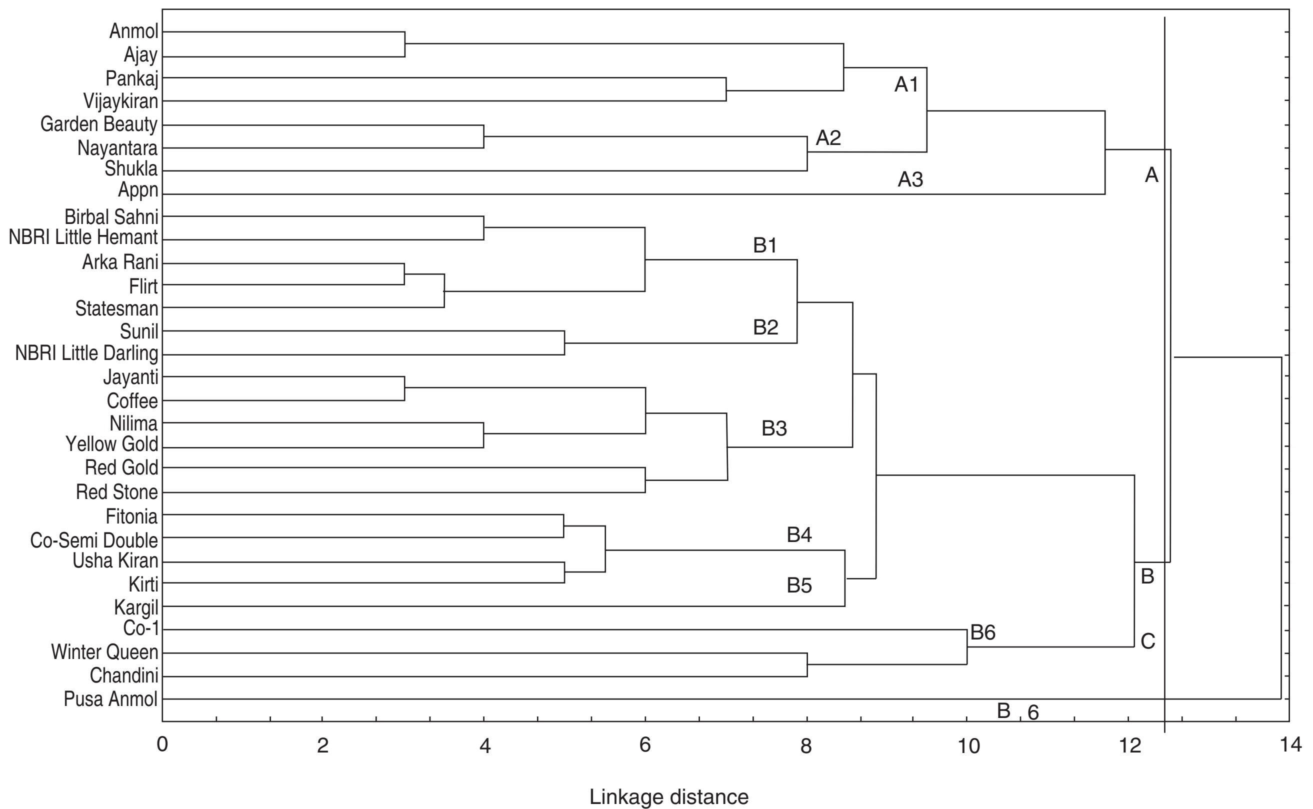

Fig 2 Dendrogram showing genetic relationship among 30 chrysanthemum genotypes based on SSR data

IARI, Pusa, New Delhi, which is the only one genotype from this region which showed the most difference from the other 30 chrysanthemum genotypes. Wang and Chuang *et al*. (2013) obtained the similar clustering with only one cultivar Southern Blush in anthurium using SSRs.

One of the striking results obtained from this study is that the cultivars Anmol and Ajay formed sister relationships with both the ISSR and SSR data. Pusa Anmol clustered with Arka Kirti with ISSR data, but remained as a single cultivar in a cluster of its own with SSR analysis. Similarly, Co-semi double was clustered with Fitonia with SSR data but remained as a single cultivar with ISSR. The ISSR and SSR markers were found to be effective for characterization and genetic diversity studies in chrysanthemum.

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