Electronic Journal of Plant Breeding, 6(1): 167-175 (Mar 2015)
ISSN 0975-928X

Research Article

Detection of genetic variability in Chrysanthemum (*Dendranthema grandiflora* T.) using ISSR primers

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(Received: 18 July 2014; Accepted:25 Mar 2015)

Abstract
The genetic diversity of 37 genotypes of Chrysanthemum was characterized using inter simple sequence repeats (ISSR) technique. A total of 46 primers were screened, of which 10 polymorphic and informative patterns were selected to determine genetic relationships. Among 114 amplified DNA fragments obtained, 107 bands were polymorphic, accounting for 93.86% polymorphism. The percentage of polymorphism exhibited by different ISSR primers ranged from 71.43% (ISSR-825) to 100% (ISSR-808, 810, 812, 840 and 842). The similarity coefficient values ranged from 0.275 to 0.775 with a mean similarity matrix of 0.525. Among the 37 genotypes studied, the closest relationship was scored between Geetanjali and Red Stone with similarity level of 77.5% while, the most distantly related genotypes were Autumn Joy and Flirtation with the lowest similarity index of 0.275. The UPGMA dendrogram revealed that the chrysanthemum genotypes were grouped into ten clusters.

Key words:
Chrysanthemum, genotypes, ISSR markers, genetic divergence

Introduction

Chrysanthemum is one of the oldest cultivated garden flower crop which plays a significant role in the culture and life of people. There is hardly any other flower crop which has such diverse and beautiful range of colour, shapes, form, size and height as that of chrysanthemum. It belongs to the family Asteraceae and as it is the national flower of Japan, it is commonly known as "Glory of the East". It is mainly grown for cut flower and loose flower for garland making, general decoration, hair adornments and religious function. Most extensive work has been done for developing novelties in chrysanthemum through induced mutation using physical and chemical mutagens (Broetjes and Van Harten, 1978), although extensive tissue culture and transgenic techniques have also been employed (Shinoyama *et al*, 2006). India has a particularly impressive share as it has commercially released 46 mutant cultivars in chrysanthemum alone from the year 1990 to 2004 (Chopra, 2005).

Identification of varieties or breeding lines is very important in ornamental crops, and is particularly interesting in chrysanthemum when in many cases the origin of varieties is unknown. The germplasm resources of chrysanthemum have so far been characterized entirely on the basis of morphological traits, biochemical and physiological characters (Anderson *et al.*, 2001); Datta *et al.*, 2009 ; Liu *et al.* 2008 ;Yin Dongmei *et al.*, 2010). These approaches have, however, not been able to accomplish the desired goals. The precise cataloguing of germplasm resources including cultivars by molecular DNA markers has lately gained lot of attention (Guena *et al*., 2003) for molecular breeding. DNA molecular marker technology, which are based on sequence variation of specific genomic regions, provide powerful tools for cultivar identification andgenetic diversity in various crops with the advantages of time-saving, less labor-consumption and more efficiency (Garg *et al*., 2006). Over the last 15 years, polymerase chain reaction technology has led to the development of many techniques of which Inter Simple Sequence Repeats (ISSR) is one among. ISSR markers seem to produce more reliable and reproducible bands than RAPDs because of the longer length of their primer and the higher annealing temperature (Quian *et al*. 2001). Hence, the present investigation was aimed to apply ISSR analysis for the detection of genetic polymorphism among 37 *chrysanthemum* genotypes.

Material and Methods

Plant material: The experimental material comprised of 37 genotypes of chrysanthemum maintained at germplasm block of Floricultural Research Station, Rajendranagar, Hyderabad, India. All the genotypes were maintained in the nursery for rooting of the top shoot cuttings. After thirty five

days, the rooted cuttings were transplanted in the main field. Each genotype was grown in a single row plot of 9 m consisting of 30 plants spaced at 30 x 30 cm. Recommended package of practices were followed uniformly for successful raising of genotypes.

Isolation of DNA**:** Fresh and young leaves of chrysanthemum were used for the extraction of total genomic DNA using modified CTAB method (Cetyl Trimethyl Ammonium Bromide) (Murray and Thompson, 1980). Purity of extracted 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 using Nano Drop spectrophotometer at 260/280 nm. The template DNA samples were diluted with TE buffer of pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA) to make the working solution of 5 ng/µl for ISSR diversity analysis.

PCR amplification**:** For ISSR analysis, a total of 46 UBC primers (UBC primer set No. 9, University of British Columbia, Canada) were screened out of which 10 primers that produced clear and unambiguous bands were used. The PCR reaction was carried out in a total volume of 10 µl containing 5 ng of template DNA, 1x PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 25 mM $MgCl_2$, 200 mM of each of the four dNTPs, 0.4 mM ISSR primer and 0.6 Units *Taq* DNA polymerase (Bangalore Genei, India). PCR amplifications were performed in a Gene Amp 9700 thermal cycler (Perkin Elmer Applied Biosystems) and the conditions were programmed for initial denaturation at 94ºC for 4 min, 35 cycles of 30 sec denaturation at 92ºC, 1 min annealing at 50-55ºC, 1 min extension at 72ºC, and final extension for 7 min at 72ºC. The annealing temperature for PCR amplification was maintained based on the specificity of the primer pair used. The amplified products were subjected to gel electrophoresis using 1.7% agarose (Bangalore Genei, India) gels buffered with 1x TAE at 100 V for 3 h and visualized with ethidium bromide staining. 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.

Data analysis**:** The data obtained by scoring the ISSR profiles with different primers were subjected to the construction of similarity matrix using Jaccard's similarity coefficient (J). The similarity values were used for cluster analysis. Sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group method with arithmetic averages (UPGMA) method, from which dendrograms depicting similarity among genotypes were drawn and plotted using NTSYS-pc. 2.1 Software (Rohlf, 2000).

Results and Discussion

Among the 46 ISSR primers used in the preliminary analysis, only ten primers produced the polymorphic bandsamong 37 genotypes of chrysanthemum (Table 1). These ISSR primers produced bright, distinguishable, scorable and unambiguous bands. A total of 114 bands were generated across 37 genotypes, of which 107 bands were polymorphic, accounting for 93.86% polymorphism. The PCR amplification with primers ISSR -855 and ISSR -857 clearly revealed polymorphic bands among the cultivars (Fig 1). The number of DNA loci amplified varied from seven (ISSR-825) to fifteen (ISSR-808 and ISSR-857) depending on the primer used with a mean value of 11.4 loci per primer. The amplicon sizes obtained with the ISSR primers ranged from 220bp to 2000bp. The percentage of polymorphism exhibited by ISSR primers ranged from 71.43% (ISSR-825) to 100% (ISSR-808, 810, 812, 840 and 842). Though, the numbers of bands produced were more with the poly AG primers (15) and with the poly AC primers (15), the primers that were based on the poly GA and poly AG motif produced more polymorphism than the primers based on any other motifs used in the present investigation. This is in accordance with the findings of Shwetha Gupta *et al*., (2008) among different genotypes of *Jatropa curcus.*

The similarity coefficient based on 10 ISSR markers ranged from 0.275 to 0.775 with a mean similarity matrix of 0.525 showing moderate to high diversity among the genotypes. Miao Heng-bin *et al*., (2008) have also reported the same range of similarity coefficient (0.282-0.757) while studying the genetic diversity in 25 cultivars of *Chrysanthemum morifolium* using ISSR primers. The most distantly related genotypes were Autumn Joy and Flirtation with the lowest similarity index of 0.275 and the most closely related genotypes were Geetanjali and Red Stone with highest similarity index value of 0.775 although they exhibit considerable morphological variation with yellow and red coloured flowers respectively.

The 37 genotypes were clustered into ten clusters as shown in figure 2 at 0.35 similarity coefficient value, on the basis of their similarity calculated as proportion of shared alleles. Cluster 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. The first cluster (cluster I) contained three genotypes Aparajitha, Chandrika and Meera sharing more common alleles with 53% similarity. In cluster II, the genotypes Punjab Gold and CO-3 were found to be more closer with 58% similarity though they originated from widely divergent locations. This might be due to sharing of similar gene pool before their geographical separation (Jaiswal and Amin, 1992).

The local collection Raichur forms a distinct genotype under cluster VII as reflected by its unique nature of extended duration of flowering. The cluster IX (Flirtation) and cluster X (Autumn Joy) are having one genotype each which forms a separate entity from rest of the genotypes. Principal Component Analysis carried out based on the Jaccard's similarity matrix revealed that the first component accounts for 48.89% of all changes and second component accounted for 3.42% of principal changes (Table 2). In this manner, totally 4 principal components accounted for 57.98% of changes.

The description of the data was done using three dimensional pictorial graph and was represented in Fig.3. From the graph, it was evident that the chrysanthemum genotypes were dispersed on the PC plot, which was a reflection of its genetic base. However, as depicted in figure some of the genotypes appear to be overlapping with each other depicting high similarity in these genotypes. It was clear from the analysis that the results obtained from PCA were in agreement with the dendrogram generated by UPGMA cluster analysis. This strengthened the ability and accuracy of the ISSR analysis applied to chrysanthemum genotypes in the present study.

In conclusion, the ISSR markers with a relatively low number of primers could effectively distinguished the cultivars with the similarity coefficient ranged from 0.275 to 0.775 showing moderate to high diversity among the genotypes. Though the genotypes Punjab Gold and CO-3 were originated from widely divergent locations, they showed close association with 58% similarity. The overall dendrogram based on ISSR clearly indicated that there were considerable diversity and relationships among the 37 genotypes of chrysanthemum by clustering the closely related ones together and demonstrated significant levels of variation.

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Table 1: Details of ISSR primers and amplified bands of all the DNA samples as obtained from thirty seven genotypes of chrysanthemum

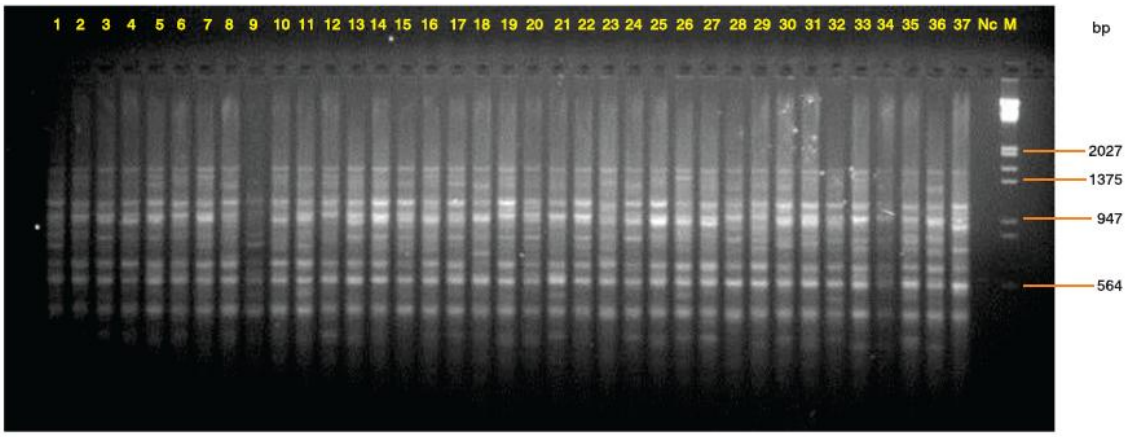
Primer	Annealing temperature (^{0}C)	DNA repeats	No. of total bands	Polymorphic bands(%)	Size range of amplified product(bp)
808	52^{0}C	$(AG)_8C$	15	15(100%)	250-1550
810	50^{0}C	$(GA)_8T$	8	8(100%)	220-1800
812	50^{0}C	$(GA)_8A$	9	9(100%)	400-1400
825	50^{0}C	$(AC)_8T$	7	5(71.43%)	400-1400
836	53^{0}C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

Table 2: Principal Component Analysis showing the contribution of ten ISSR primers among 37 chrysanthemum genotypes

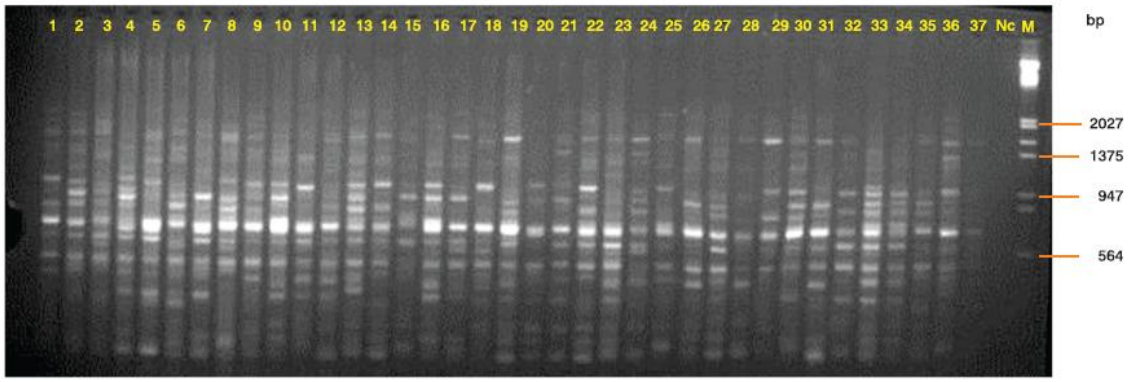
S.No.	Genotypes	Eigen value	Percentage variation explained	Cumulative variation (%)
1.	Aparajitha	18.09	48.89	48.89
2.	Punjab Gold	1.26	3.42	52.31
3.	CO-3	1.08	2.93	55.24
4.	Raichur	1.02	2.75	57.98
5.	Silper	0.91	2.46	60.44
6.	Yellow Gold	0.91	2.45	62.89
7.	Punjab Anuradha	0.82	2.21	65.10
8.	Rekha	0.74	1.99	67.09
9.	Chandrika	0.73	1.97	69.06
10.	Snow Cem	0.71	1.91	70.97
11.	Meera	0.67	1.80	72.77
12.	Shaffoli	0.66	1.78	74.55
13.	Terry	0.63	1.71	76.26
14.	Shintome	0.61	1.65	77.91
15.	Arka Ravi	0.55	1.49	79.40
16.	Ratlam Selection	0.53	1.44	80.83
17.	Neelima	0.51	1.39	82.22
18.	Flirtation	0.50	1.36	83.59
19.	Mother Teresa	0.48	1.31	84.89
20.	Autumn Joy	0.47	1.28	86.17
21.	Anjali	0.46	1.24	87.41
22.	Akitha	0.42	1.13	88.55
23.	PAU-B-107	0.40	1.09	89.64
24.	Farr	0.39	1.05	90.69
25.	Jaya	0.36	1.03	91.72
26.	Harvest House	0.34	0.93	92.65
27.	Lilith	0.32	0.87	93.52
28.	Asha	0.32	0.86	94.38
29.	Pusa Centenary	0.28	0.75	95.13
30.	Salora	0.27	0.74	95.87
31.	IIHR-13	0.26	0.71	96.59
32.	Basanthi	0.25	0.67	97.25
33.	Red Stone	0.23	0.63	97.89
34.	Geetanjali	0.22	0.60	98.49
35.	Red Gold	0.21	0.58	99.07
36.	Kalyani Mauve	0.19	0.51	99.58
37.	Local Button	0.16	0.42	100

ISSR- 855:



Amplicon size rangedfrom 250bp-1600bp

ISSR - 857 :



Amplicon size rangedfrom 220bp-1550bp

M-marker, EcoR 1- Hind III double digest of λ DNA; NC-negative control (no DNA), 1-37 represent the genotypes

Name of the genotypes:

1. Aparajitha
2. Punjab Gold
3. CO-3
4. Raichur
5. Silper
6. Yellow Gold
7. Punjab Anuradha
8. Rekha
9. Chandrika
10. Snow Cem
11. Meera
12. Shaffoli
13. Terry
14. Shintome
15. Arka Ravi
16. Ratlam Selection
17. Neelima
18. Flirtation
19. Mother Teresa
20. Autumn Joy
21. Anjali
22. Akitha
23. PAU-B-107
24. Farr
25. Jaya
26. Harvest House
27. Lilith
28. Asha
29. Pusa Centenary
30. Salora
31. IIHR-13
32. Basanthi
33. Red Stone
34. Geetanjali
35. Red Gold
36. Kalyani Mauve
37. Local Button

Fig 1 ISSR profile of Chrysanthemum cultivars

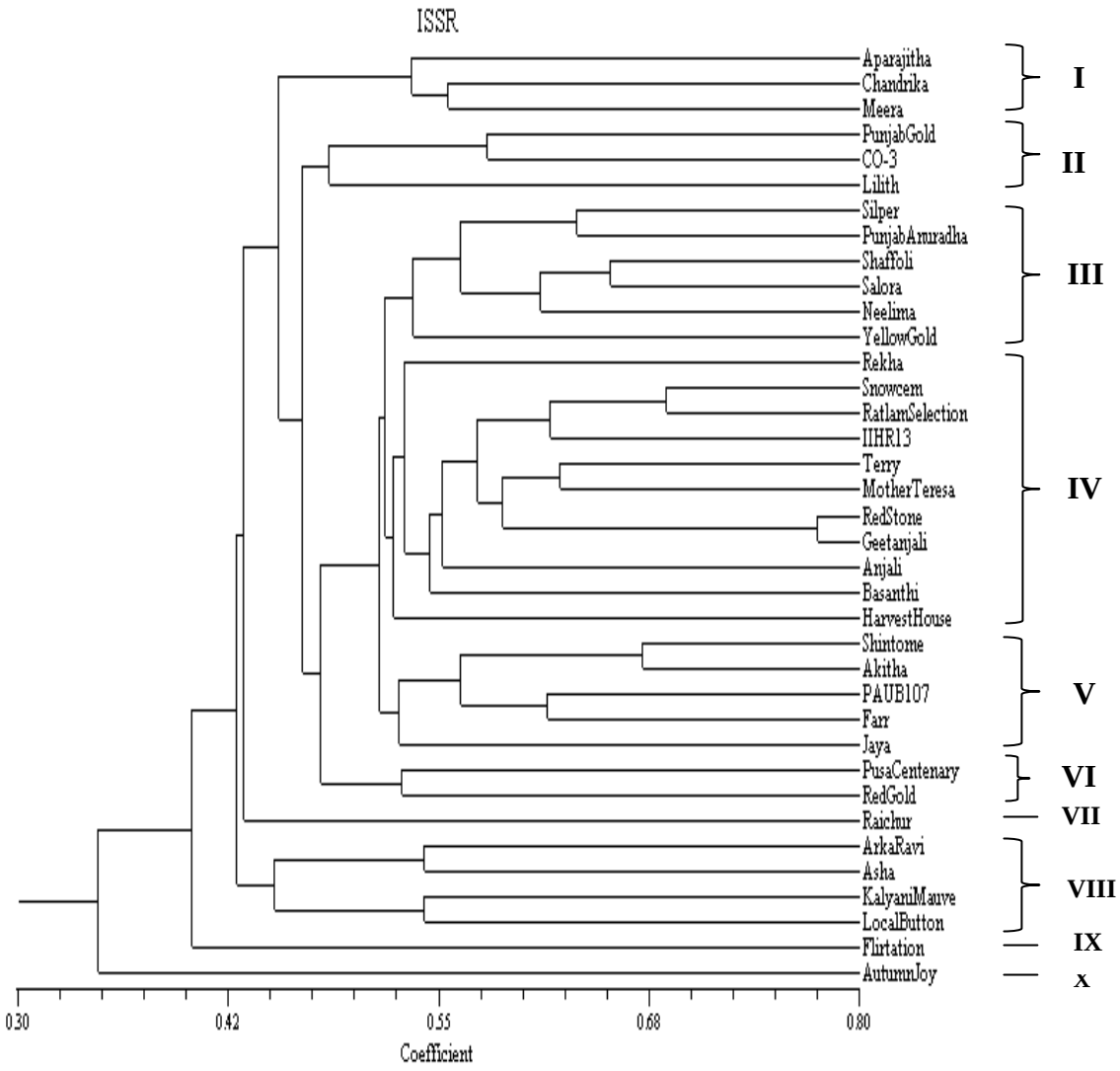
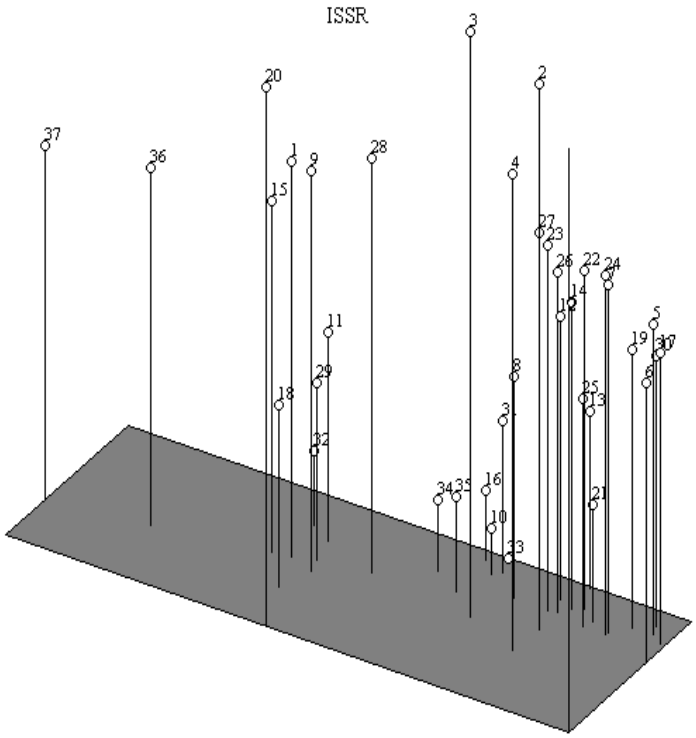


Fig 2. Dendrogram generated using UPGMA analysis showing the genetic relationship among Chrysanthemum genotypes using ISSR data



1. Aparajitha	9. Chandrika	18.Flirtation	27. Lilith
2. Punjab Gold	10. Snow Cem	19.Mother Teresa	28. Asha
3. CO-3	11. Meera	20.Autumn Joy	29. Pusa Centenary
4. Raichur	12. Shaffoli	21.Anjali	30. Salora
5. Silper	13. Terry	22.Akitha	31. IIHR-13
6. Yellow Gold	14. Shintome	23.PAU-B-107	32. Basanthi
7. Punjab Anuradha	15. Arka Ravi	24.Farr	33. Red Stone
8. Rekha	16. Ratlam Selection	25.Jaya	34. Geetanjali
9. Chandrika	17. Neelima	26. Harvest House	35. Red Gold
36. Kalyani Mauve	37. Local Button		

Fig 3. Principal component analysis of 37 chrysanthemum genotypes based on ISSR markers (Three dimensional)