Genetic Diversity Analysis of Antirrhinum (*Antirrhinum majus* L.) Inbreds Using RAPD Markers

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Genetic diversity analysis of 15 *Antirrhinum majus* L. inbreds was carried out using RAPD markers to assess genetic diversity present amongst them. A total of 80 DNA amplicons were scored using nine primers. Inbreds shared 63% genetic similarity among themselves. Inbreds were distinguishable from each other based on their banding pattern which is evident by the fact that none of the inbreds shared 100% similarity between each other. UPGMA cluster analysis grouped the inbreds into four clusters at 85% similarity. The co-phenetic correlation coefficient (r) was 0.9322, exhibiting best fit between dendrogram and similarity matrix based on RAPD data. The polymorphism information content (PIC) was in the range of 0.00 to 0.962, while average expected gene diversity ranged from 0.00 to 0.425. Jaccard's pair wise similarity coefficient ranged from 55 to 98.8%.

Key Words: Antirrhinum; Cluster; Genetic diversity; Polymorphism information content; RAPD

Introduction

Snapdragon (*Antirrhinum majus*) native of the Mediterranean region, from Morocco and Portugal north to Southern France and east to Turkey and Syria is amongst most beautiful ornamental flowering plants. The genus *Antirrhinum* ($2n = 16$) has >20 species, is mostly found around the Mediterranean Sea and in North America (Stubbe, 1966). Genetic variability considered to be the basis of plant breeding (Simmond 1983) is the essential pre-requisite for initiation of breeding work as improvement of any crop is possible only when wide genetic variability exists in the population of that species. Molecular marker analysis is a powerful tool for distinguishing one individual from another on the basis of DNA banding pattern using molecular markers and RAPD molecular markers discovered by Welsh and McClelland (1990) and Williams *et al*. (1990) can be used for assessment of genetic variability in populations as it is simple, fast, requires small quantity of template DNA and involves no radioactivity or southern blotting and hybridization for distinguishing individuals. Thus, present investigation was taken up to distinguish *Antirrhinum majus* inbreds from each other and to assess genetic variation among them using RAPD molecular markers.

Materials and Methods

Plant Material

A total of 15 *Antirrhinum majus* L. inbred lines were sampled from Model Floriculture Centre (MFC), GB Pant University of Agriculture and Technology (GBPUAT), Pantnagar, India (Table 1). Twelve inbreds were from flower seed companies located in India at New Delhi, Lucknow, Guwahati and Dimapur in North-East and three inbreds *viz.* SA-1, Sant-11 and Anti-331 were introduced from Thompson and Morgan (Ipswich, United Kingdom). These inbreds were selfed for six generation to maintain their genetic purity.

DNA Isolation

DNA was extracted using the procedure reported by Torres *et al.* (1993) with some modifications. Leaf material (500 mg) was ground to a fine powder in liquid nitrogen, mixed with 8 ml of extraction buffer [CTAB (Cetyl trimethyl Ammonium bromide) (2% w/v), Tris-HCl (100 mmol/l pH 8.0), EDTA (20 mmol/l pH 8.0), NaCl (1.4 mol/l), sodium bisulfite (0.1% w/v) and 2-mercaptoethanol (0.4% v/v)] and incubated at 60°C for 45 min. The samples were mixed with an equal volume of chloroform-iso amyl alcohol (24:1) and centrifuged at 9,000 xg for 5 minutes under

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Table 1. Details of inbreds of *Antirrhinum majus* used for DNA fingerprinting

Sl. No. (Sample code)	Name of Genotype	Source of collection
1.	65 G	New Delhi, India
2.	135 G	Lucknow, Uttar Pradesh, India
3.	109 G	New Delhi, India
4.	98 G	Guwahati, Assam, India
5.	Sant 11	Ipswich, United Kingdom
6.	97 G	Lucknow, Uttar Pradesh, India
7.	128 G	New Delhi, India
8.	92 G	Lucknow, Uttar Pradesh
9.	Anti-331	Ipswich, United Kingdom
10.	328 G	Dimapur, Nagaland, India
11.	113 G	Guwahati, Assam, India
12.	99 G	Lucknow, Uttar Pradesh
13.	SA-1	Ipswich, United Kingdom
14.	90 G	New Delhi, India
15.	61 G	Dimapur, Nagaland, India

refrigeration. The DNA was precipitated by adding one volume of chilled isopropanol for 1 h. The DNA pellet was washed in ethanol (70%). Finally, the DNA pellet was air-dried and suspended in an appropriate volume of 1 X TE buffer and quantified using UV spectrophotometer.

PCR Amplifications and Electrophoresis

PCR amplifications were performed in a volume of 25 µl reaction containing 1µl template DNA (100 ng/µl), dNTPs (2.5 µM each) 2 µl, *Taq* polymerase (3 U/µl) 0.25 µl, Reaction buffer (10 X) 2.5 µl, Primer 1 µl. Amplifications were performed in a PCR thermal cycler (Eppendorf, Hamburg, Germany). The thermal cycles performed were programmed as follows: 3 cycles of 94°C (2 min)/36°C (1 min 30 s)/72°C (2 min) and 41 cycles of 94°C (20 s) /36°C (40 s)/72°C (2 min) and final extension at 72^0C (6 min). RAPD primers were same as those used by Gomez *et al.,* 2002 and were custom synthesized from Ocimum Biosolutions Pvt. Ltd., Hyderabad, India (Table 2). DNA Fragments generated by amplification were separated on 1.5% agarose gel run in 1 X Tris-borate EDTA buffer, stained with ethidium bromide (0.5 µg/ml), and visualized with ultraviolet light and photographs. "Low range DNA ruler" (Bangalore Genei) was included in the gels as a molecular size reference.

Molecular Markers Data Analysis

DNA banding pattern were scored for the presence (1) or absence (0) of bands of various molecular sizes in the form of binary matrix. Data were analyzed to obtain Jaccard's similarity coefficients among the inbreds by using NTSYS-PC (version 2.02e; Exeter Software, Setauket, NY, USA; Rohlf, 1990). Dendrogram was constructed using the Unweighted Pair Grouping Method with Arithmetic Averages (UPGMA) and Sequential Agglomerative Hierarchical and Nested (SAHN) clustering methods. The reliability of the dendrogram was tested by co-phenetic correlation coefficient between similarity and co-phenetic value matrix based on RAPD data using Mxcomp of NTSYS-PC (version 2.02e; Rohlf, 1990).

Average expected Gene Diversity (H_j)

Bands were scored in the gel and converted to numbers. For this, each of the band (in the same row) was scored and transformed to 1, if it was present or to 0, if it was absent. The presence of band corresponds to the dominant/ heterozygous genotype (AA/Aa) and the absence to the recessive genotype (aa) for dominant marker and were scored 1 and 0, respectively. On the basis of this scoring pattern, allelic frequencies were calculated.

Polymorphism Information Content

The genetic diversity of each RAPD locus was estimated using Polymorphism Information Content (PIC) that refers to the value of a marker for detecting polymorphism within a population of inbreds, depending on the number of detectable alleles and the distribution of their frequency (Anderson *et al.,* 1993) as follows

$$PIC = 1 - \sum_{j=1}^{n} P^2_{ij}$$

Where, P_{ij} is the frequency of j^{th} allele in the i^{th} primer and summed up over *n* alleles.

Results

A total of 10 primers were used for genetic diversity analysis of *Antirrhinum majus* inbreds. Out of 10 primers, nine produced reproducible polymorphic bands. Each of the primers varied greatly in their ability to resolve variability among the inbreds. The individual primer produced bands in a range of 2 (Primer- OP-020) to 15 (Primer- UBC-543) bands. A total of 80 DNA amplicons were scored using these nine primers with an average of 8.90 bands per primer (Table 2). The amplified fragments ranged from 100 bp to 3500 bp. The polymorphism percentage was in the range of 0.00% to 100% for these primers (Nadarajan *et al.,* 1999). All primers based on their % polymorphism and unique band amplification were considered as highly informative primers. A moderate

to high proportion of polymorphic amplified products were detected. PIC values ranged from 0.00 for primer 6 to as high as 0.962 for primer 10, with an average of 0.338 for nine primers (Table 2). A gel scoring was done using the 0-1 matrix for band profile of 80 DNA amplicons produced by 9 polymorphic RAPD primers, which clearly distinguished *Antirrhinum majus* inbreds from each other at molecular level. The results clearly showed that 15 *Antirrhinum majus* inbreds were genetically diverse from each other at molecular level in spite of having similarities at morphological level, which is evident by similarity coefficient matrix between inbreds as none of the inbreds shared 100% similarity with each other (Table 3) and by the dendrogram generated using similarity matrix as well (Fig. 2).

Average Expected Gene Diversity

The average expected gene diversity ranged from 0.00 for primer UBC-540 followed by 0.156 for primer OP-020 to a maximum of 0.425 for primer UBC-543 followed by 0.415 for primer UBC-557 with an average of 0.216 for all the random primers among all the inbreds at all the locus studied (Table 2).

Jaccard's Similarity Coefficient

Pair-wise Jaccard's similarity coefficient for the genetic similarities among 15 inbreds is presented in Table 3. The value of the coefficients were estimated on the basis of nine primers which ranged from 55% between inbreds 98G and 328G followed by 56.3% between inbreds 98G and 113G and by 57.5% between inbreds 109G and Anti-331

Table 2. Number of RAPD loci, primer sequence, amplified fragment size, PIC and average expected gene diversity generated by 9 random primers

Primer Code	Primer Name	Nucleotide sequence (5′-3′)	Tm (^{0}C)	Fragment size range	PIC	Average expected gene diversity
109761	UBC-507	AGA CGT ACT C	30.0	250-3250	0.045	0.062
109762	UBC-511	GAA TGG TGA G	30.0	350-3050	0.509	0.425
109763	UBC-514	CGG TTA GAC G	32.0	275-3050	0.102	0.041
109764	UBC-523	ACA GGC AGA C	32.0	300-3050	0.442	0.400
109766	UBC-540	CGG ACC GCG T	34.0	350-1400	0.000	0.000
109767	UBC-557	GTG TAG AGC C	32.0	450-3050	0.589	0.415
109768	UBC-543	CGC TTC CGG T	34.0	150-2750	0.272	0.218
109769	OP-001	GGC ACG TAA G	32.0	500-2500	0.124	0.232
109770	OP-020	ACA CAC GCT G	32.0	1500-2000	0.962	0.156

Table 3. Jaccard's similarity coefficient between fifteen *Antirrhinum majus* inbreds

	65G	135G	109G	98G	Sant11	97G	128G	92G	Anti-331	328	113G	99G	SA-1	90G	61G
65G	1.000-														
135G	0.863	1.000													
109G	0.700	0.788	1.000												
98G	0.675	0.763	0.975	1.000											
Sant11	0.788	0.750	0.688	0.663	1.000										
97G	0.800	0.738	0.650	0.625	0.938	1.000									
128G	0.763	0.700	0.613	0.588	0.925	0.963	1.000								
92G	0.813	0.750	0.638	0.613	0.925	0.988	0.950	1.000							
Anti-331	0.813	0.725	0.613	0.588	0.875	0.888	0.875	0.900	1.000						
328	0.825	0.738	0.575	0.550	0.763	0.775	0.763	0.788	0.888	1.000					
113G	0.863	0.750	0.588	0.563	0.800	0.863	0.850	0.875	0.875	0.913	1.000				
99G	0.875	0.763	0.600	0.575	0.763	0.825	0.813	0.838	0.838	0.900	0.963	1.000			
SA-1	0.863	0.750	0.638	0.613	0.875	0.888	0.850	0.900	0.925	0.838	0.850	0.888	1.000		
90G	0.850	0.738	0.625	0.600	0.888	0.875	0.863	0.888	0.913	0.825	0.838	0.875	0.988	1.000	
61G	0.850	0.763	0.625	0.600	0.863	0.850	0.838	0.863	0.888	0.775	0.813	0.825	0.938	0.950	1.000

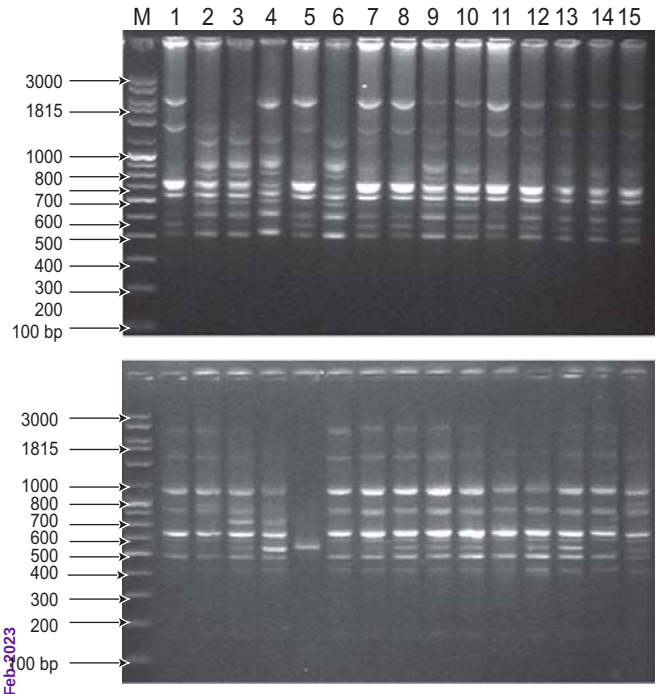

Fig. 1. DNA profiles of 15 *Antirrhinum majus* inbreds obtained with primers (a)UBC-523 and (b)UBC-511. Marker Lane corresponds to low range DNA rular as fragment size marker

to a maximum of 98.8% between inbreds SA-1 and 90G followed by 97.5% between inbreds 109G and 98G and by 96.3% between inbreds 97G and 128G and between 113G and 99G. This maximum similarity between these inbreds indicates the relatedness of these inbreds by ancestry. These similarity coefficients, thus, also indicate that sufficient amount of variability is present between the fifteen inbreds and that the inbreds were different from one another.

Cluster Analysis

The UPGMA cluster analysis method was followed for the construction of the phylogenetic tree (Fig. 2). The result showed that at 0.85 similarity coefficient four major clusters were formed. Out of these clusters, two clusters were having two inbreds. These genotype clusters were Cluster I (inbreds 65G and 135G) and IV (inbreds 109G and 98G) clearly indicating distinctness of these inbreds from the others. Cluster II was having seven inbreds *viz*. inbreds (Sant-11, 97G, 92G, 128G, Anti-331, SA-1, 90G and 61G) while Cluster III was having three inbreds (328G, 113G and 99G). This dendrogram analysis has grouped inbreds into different clusters based on their similarity coefficient and thereby on the basis of genetic variability. Inbreds included in single cluster are genetically closer

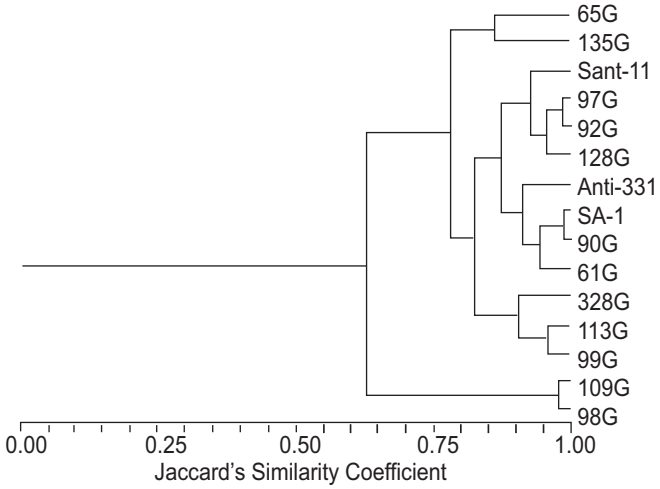

Fig. 2. UPGMA dendrogram of 15 inbreds of *Antirrhinum majus* based on Jaccard's similarity coefficient

than the inbreds of other clusters. The co-phenetic correlation coefficient (r) was 0.9322, exhibiting best fit between similarity and co-phenetic value matrix based on RAPD data.

Discussion

Out of the 10 primers used for diversity analysis of *Antirrhinum majus* inbreds nine produced reproducible amplifications. These primers revealed variability among the *Antirrhinum majus* inbreds at DNA level. The polymorphism percentage exhibited by the RAPD primers was in the range of 0.00% to 100% for these primers (Nadarajan *et al.,* 1999 reported 77.4% polymorphism between seven *Japonica*, two *Indica* and one tropical *Japonica* rice varieties by RAPD markers). All primers based on their % polymorphism and unique band amplification were considered as highly informative primers. A moderate (*A. majus*, 58.04%; this study) to high (*A. microphyllum*, 90%; Torres, 1999) proportion of polymorphic amplified products were detected. As expected, the PIC distributions revealed that, in terms of genetic distance, dominant markers had lower levels of polymorphism as compared to codominant markers, which agrees with the results published by Becker *et al.* (1995); Russell *et al.* (1997) and Pejic *et al.* (1998). The average expected gene diversity ranged from 0.00 to a maximum of 0.425 with an average of 0.216 for all the random primers among all the inbreds at the entire locus studied (Lu *et al.,* 2005).

Pair-wise Jaccard's similarity coefficient for the genetic similarities among 15 inbreds presented in Table 3 ranged from 55% between inbreds 98G and 328G to a maximum of 98.8% between inbreds SA-1 and 90G.

Similar values of similarity coefficients were obtained among rapeseed inbreds (Cartea *et al*., 2005). This maximum similarity between these inbreds indicates the relatedness of these inbreds by ancestry. These similarity coefficients, thus, also indicate that sufficient amount of variability is present between the fifteen inbreds. The genetic relationship among the inbreds using UPGMA cluster analysis based on the RAPD-derived data was studied. Our results indicated that it is easy to distinguish all the snapdragon inbreds from others by using molecular markers. Similar studies were also done by many other workers e.g. in *Antirrhinum* by Torres *et al.* (2003) and in wheat inbreds by Wei *et al.* (2005). The cluster analysis based on Jaccard's similarity coefficient clearly indicated distinctness of inbreds grouped in single cluster from the others. This dendrogram analysis has grouped inbreds into four different clusters based on their similarity coefficient and thereby on the basis of genetic variability (Jimenez *et al.,* 2002). Inbreds included in single cluster are genetically closer than the inbreds of other clusters. The co-phenetic correlation coefficient (r) was 0.9322, exhibiting best fit between similarity and cophenetic value matrix based on RAPD data (Uma *et al.,* 2008).

In a wider context, the speed, efficiency and reliability of the RAPD methodology are important considerations for the development of strategies for effective management of germplasm collection in terms of identification of duplicates, the estimation of diversity, monitoring genetic erosion and enhancing the use of these collections. RAPD markers have been used for the DNA fingerprinting for identification of cultivars and for assessment of genetic relationships among cultivars (Li and Nelson, 2002). The results suggest that RAPD molecular markers can be used efficiently for analyzing genetic diversity and phylogenetic analysis (Ni *et al.,* 2003; Nagaraju *et al.,* 2002).

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