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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(3): 964-967
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www.thepharmajournal.com
Received: 12-01-2021
Accepted: 18-03-2021

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Assessment of molecular diversity using RAPD markers in elite genotypes of cotton (*Gossypium hirsutum* L.)

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Abstract
The contemporary appraisal was to analyze eleven parental genotypes in cotton for their molecular diversity using RAPD markers so as to sort out the contrasting parents for developing mapping populations and study of different yield related traits for the development of varieties and heterosis breeding. Altogether 25 RAPD primers were utilized to contrast the parental genotypes out of which 15 showed transparent scorable distinct bands. Out of 15, 5 markers showed polymorphism and 10 showed monomorphism. Average number of bands per primer is 4.4. The genetic similarity coefficient ranged from 0.357 to 0.840 and the maximum dissimilarity of 0. 643. Clustering depicted that the 11 genotypes fell into two major clusters one formed by 8-1-2 genotype and the other include remaining 10 genotypes. Crosses between the genotypes based on their genetic distances which represent that genetic elements are in contrasting nature assist in creation of genetic variability and assist in hybrid breeding programmes.

Keywords: Cotton, RAPD markers, molecular diversity, UPGMA

Introduction
Cotton is a significant fiber yielding cash crop grown all around the world. Assessment of genetic diversity through conventional approaches may not provide necessary information for carrying out the crossing patterns in order to produce novel hybrids or varieties. certain traits are to be studied only after maturity which is a cumbersome and time-consuming procedure. There may be other trait which are neglected during diversity assessment as a result of which the outcome may be biased. Hence substitute technique which provide efficient and more reliable one is in need for the assessment of diversity. With the advent of the molecular marker technology it become easy to study the various genetic aspects at molecular level. Among the different marker systems, RAPD (Williams *et al*., 1990) [16], Zabeau and Vos, 1993 [17] given AFLP markers (Amplified fragment length polymorphism), RFLP (Restriction fragment length polymorphism) markers designed by Liu and Turner, 1993 and other marker systems like SSRs (Simple sequence repeats) (Akkaya *et al*., 1992) [1], SNPs(Single nucleotide polymorphism) (Bojinov and Lacape, 2003) [3] were used in various agricultural research platforms for crop improvement. Many eminent scientists utilized the RAPD marker system for various research purposes. For speedy recovery of recurrent parent genome Murtaza *et al*., 2005 utilized RAPD marker system. Welsh *et al*., (1991) [15], Lu and Myres (2004), Rana and Bhat, (2005) and Sheidail *et al.* (2007) [13] used the RAPD molecular marker technology for the identification of the cultivars, purity analysis and to make inter and intra-crosses based on their molecular diversity in cotton. Other significant food crops like rice (Haiyuan *et al*., 1998) [5], maize (Iva *et al*., 2005) [7] and wheat (Awan *et al*., 2008) [2] also RAPD markers were utilized for the assessment of molecular diversity. Current study was made to analyze the molecular diversity among parental genotypes in cotton in order to carry out further crosses among them.

Materials and Methods
Experiment was conducted at agriculture research station Hebballi, university of agricultural sciences Dharwad. Eleven parental genotypes of cotton namely Sahana, LH-2076, CPD-813, L-761, R-132, 1-2-1, 8-1-2, IC-6, DC-12-111, RAH-221 and ARBH-813 were analyzed for their molecular diversity. Parents of the hybrids which gave rise to the F3 lines being evaluated were sown in three blocks in Randomized Complete Block Design. Each entry was sown in two rows of 5 m length at a spacing of 90 x 20 centimeters.

Extraction of DNA and its quantification

DNA extraction

The DNA was extracted from the recombinant lines by following CTAB extraction method (Saghai-Maroof *et al*., 1984) with required modifications. CTAB buffer, (1% polyvinyl pyrrolidone, 2% cetyl trimethylammonium bromide, 20mM EDTA, 100mM Tris-HCl, 1.4M Nacl), TE Buffer (10mM Tris, 1mM EDTA at pH 8) isopropanol, RNase A solution, 70% ethanol, 2ml centrifuge tubes, centrifuge (14,000 x g) and Speed Vac. are the materials used. One or two fresh young leaves (not fully expanded and less than a week old) from shoot apex were harvested and the surface was cleaned with wet paper towel. The sample was ground to fine powder using liquid nitrogen with prechilled pestle and mortar. 500µl of CTAB extraction buffer was added to the homogenized tissue, thoroughly mixed and vortexed. The homogenate was subjected to hot water bath for 30 minutes at 60º C. after completion of incubation, the homogenate was centrifuged at 14000 rpm (revolutions per minute) for 5 minutes. Supernatant was collected and transferred to a new tube to which 5 µl of RNase A solution was added and incubated at 37º C for 30 minutes. Added chloroform and isoamyl alcohol in the ratio of 24:1 in an equal amount. Vortexed and centrifuged it for 2-3 minutes at 14000 rpm for 5 minutes. As a result of which the liquid in it got separated into different phases. Top phase which is aqueous in nature is collected in a separate tube. This step was repeated till the aqueous phase is with brevity. Solution was collected and incubated for 30 min at -20º C after addition of cold isopropanol of volume 0.75. samples are gently removed and subjected to centrifugation for 20 min at 14,000 rpm. 70% alcohol was used for the washing of the pellet and Speed Vac for drying the remaining alcohol. 20 µL TE buffer was added for the long-time preservation of the DNA.

DNA quantity and quality estimation

The concentration of DNA was assessed spectrophotometrically and also by gel electrophoresis using 0.8 per cent agarose with known concentration of uncut DNA. In spectrophotometric analysis, 5µl of DNA sample diluted with TE buffer and volume made up to 3000µl was subjected to spectrophotometer readings at absorbance of 260nm. Concentration of DNA (µl/ml) = O.D at 260 x 50. To test the quality of DNA, samples were run on 0.80 per cent agarose gel 1x TAE (Tris Acetic Acid EDTA) buffer and stained with ethidium bromide and checked for contamination by RNA (which usually runs ahead) and the DNA was evaluated by comparing it with a standard undigested DNA sample.

Amplification of DNA in PCR: 30 ng of DNA, 3mM of MgCl2, 0.2µl of Taq polymerase were taken for the production of consistent bands. Remaining reagents concentrations were kept constant so that each replicate may be produced with maximum reproducibility and consistency in development of bands. Amplification reaction mixture was prepared in 1.2ml thin walled PCR tubes containing following components in a total volume of 25µl. For one reaction 10x assay buffer with 15 mm MgCl2 of 2.0µl, MgCl2 of 1.0µL, dNTPs mix (2.5 mm each) of 1.0µl, primer (5 pm/µl) of 1.0µl, template (15 ng/µl) of 2.0µL, sterile distilled water 17.66µl, and Taq. DNA polymerase (3U/µl) of 0.34(µl) was taken. The PCR amplification for RAPD analysis was performed according to Williams *et al.*, (1990) [16] with certain modifications. The amplification conditions were as follows, thermal cycler was organized at 45 cycles at 94ºC for 1 min, annealing for 1 min at 36ºC, extension for 2min at 72ºC, accompanied by 95ºC for 5min and final extension at 72ºC for 10 min. this was done before and after 45 cycles severally. After the completion of the PCR, the products were stored at 4 ºC until the gel electrophoresis was done.

Separation of amplified products by Agarose gel electrophoresis: The amplified products from each tube along with 2µl of loading dye (bromophenol blue) were separated on 1.2 per cent agarose gel at 70 volts (<5 volts per centimeters of gel) using 1x TAE buffer of pH 8.0 containing ethidium bromide. Lambda DNA double digest was used as DNA molecular weight marker. The gel was photographed by using documentation system (Hero lab, EASY. 440k). The amplified fragments were scored as '1' for presence of single band, '2' for presence of two bands and '0' for the absence of a band generating the 0,1 and 2 matrix and per cent polymorphism was calculated by using the following formula.

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \text{ X } 100$$

Pair wise genetic similarities (Sij) between genotypes were estimated by DICE similarity coefficient. Clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on un weighted pair group arithmetic mean (UPGMA) using NTSYS-PC. The similarity measurements were converted to genetic distance measurements as (1-SM) X 100 (Spooner *et al*., 1996) [14].

Results and discussion

Table 1: Analysis of RAPD banding pattern in cotton parental genotypes at ARS Dharwad during kharif

Sl. No	Name	Sequence (5'-3')	Bands	Polymorphic bands	% Polymorphism
1	OPI-1	ACCTGGACAC	4	3	75
2	OPI-2	GGAGGAGAGG	4	3	75
3	OPI-4	AAGGCGGCAG	4	4	100
4	OPI-6	TTTGCCCGGT	5	4	80
5	OPI-8	ACAACGCGAG	5	5	100

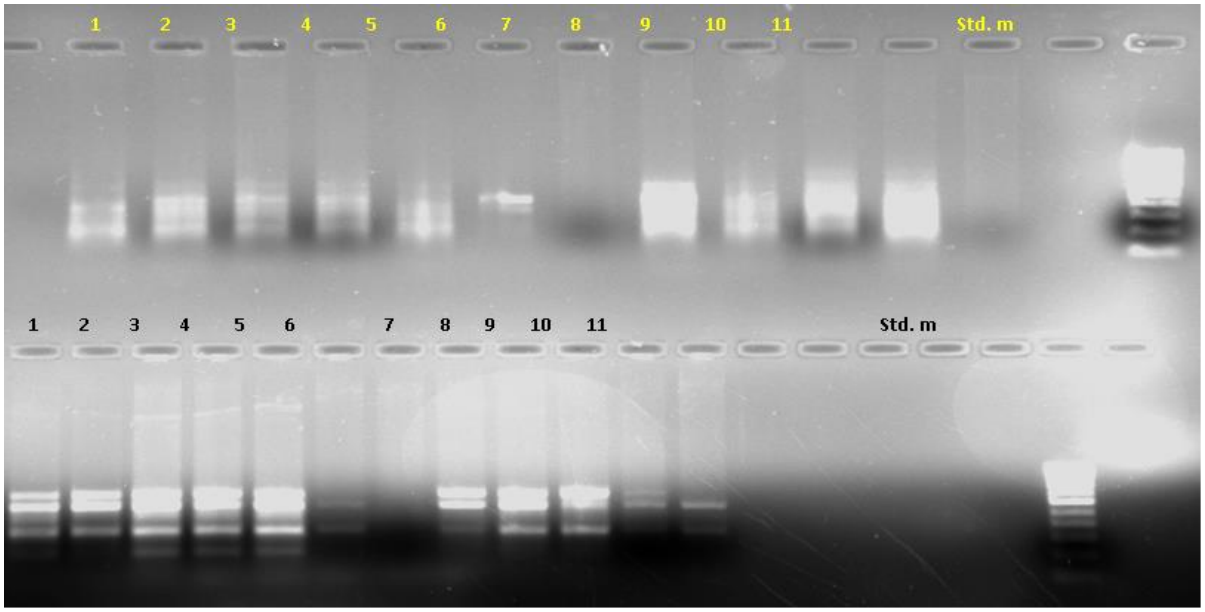
Gel picture depicting amplification for primers OPI-1 and OPI-2 was shown in *figure* 1. Out of five primers OPI-6 and OPI-8 had shown the highest polymorphism of 100% and lowest shown by OPI-1 and OPI-2, 75%. In total five primers produced 22 bands out of which 19 bands were of polymorphic developing a polymorphism of 86.36 per cent, with an average polymorphic band 3.8 and bands per primer 4.4. Khan *et al*. (2000) [8] while working with 31 different species of *Gossypium* recorded a polymorphism of 99.8% with RAPD marker system. Hussein *et al*. (2006) used 28 RAPD primers for studying 21 cotton accessions and recorded a polymorphism of 59.1%, Polymorphism on behalf of RAPD markers is mainly due to addition deletion, substitution of bases at the primer attaching site.

Similarity matrix and dendrogram

Based on the binary data generated by the polymorphic bands of different markers used for study, pairwise comparison was made for the common bands to the total bands in that pair which has given the information of extent of similarity between them. Similarity coefficient ranged from 0.357 to 0.840 with maximum dissimilarity between RAH 221 and 8-1-2 genotypes and minimum dissimilarity between L-761 and Sahana. Genetic similarity had been assessed by RAPD along with AFLP markers in cotton by Multani and Lyon, 1995 and for wild relatives of cotton by Khan *et al*., 2000 [8]. Similarity matrix studies were used for the assessment if genetic relatedness among hybrids and parents by Dongre and Parkhi, 2005 [4]. From the similarity matrix generated by the RAPD diversity depicts that the parental genotypes were diverse from one another and have different genetic background developed due to the variation in different genetic elements (*Table* 3). Unweighted pair group method using arithmetic mean (UPGMA) was used for the generation of dendrogram which depicted the clustering of the parental genotypes into two major clusters group A and group B further group B was divided into different C and D. group D was divided into E and F. Group E was further divided into two groups G with two genotypes and H with one. Group F was further divided into two clusters I and J. Group I was with two groups K and L where L with two genotypes and K with one. Thus, there was a remarkable molecular diversity among the parental lines studied. (*Fig 2*) The banding pattern generated by the utilization of RAPD markers had given considerable amount of information about the genetic diversity present among the parental genotypes of cotton. Crossed to be made based on the dendrogram and similarity matrix studies in order to carry out further research the experiment also proven the effectiveness of RAPD marker system for the assessment of genetic diversity. Considerable amount of genetic variation can be released by crossing the parental genotypes based on their molecular divergence.

Table 2: Similarity matrix based on RAPD profile analysis of parental genotypes at ARS Dharwad

Genotypes	Sahana	LH-2076	CPD-813	L-761	R-132	1-2-1	8-1-2	IC-6	DC-12-111	RAH-221	ARBH-813
Sahana	1										
L-761	0.840	1									
CPD-813	0.714	0.750	1								
LH-2076	0.668	0.838	0.727	1							
R-132	0.665	0.709	0.606	0.812	1						
1-2-1	0.500	0.500	0.600	0.551	0.551	1					
8-1-2	0.545	0.538	0.500	0.518	0.592	0.416	1				
IC-6	0.600	0.705	0.668	0.800	0.742	0.560	0.664	1			
DC-12-111	0.662	0.645	0.787	0.562	0.687	0.482	0.668	0.628	1		
RAH-221	0.428	0.625	0.705	0.662	0.606	0.733	0.357	0.611	0.600	1	
ARBH-813	0.592	0.645	0.727	0.625	0.625	0.551	0.518	0.742	0.625	0.606	1



1) Sahana 2) LH-2076 3) CPD813 4) L-761 5) R-132
6) 1-2-1 7) 8-1-2 8) IC-6 9) DC-12-111 10) RAH-221
11) ARBH-813

Fig 1: RAPD pattern of parental lines for primers OPI-1, OPI-2

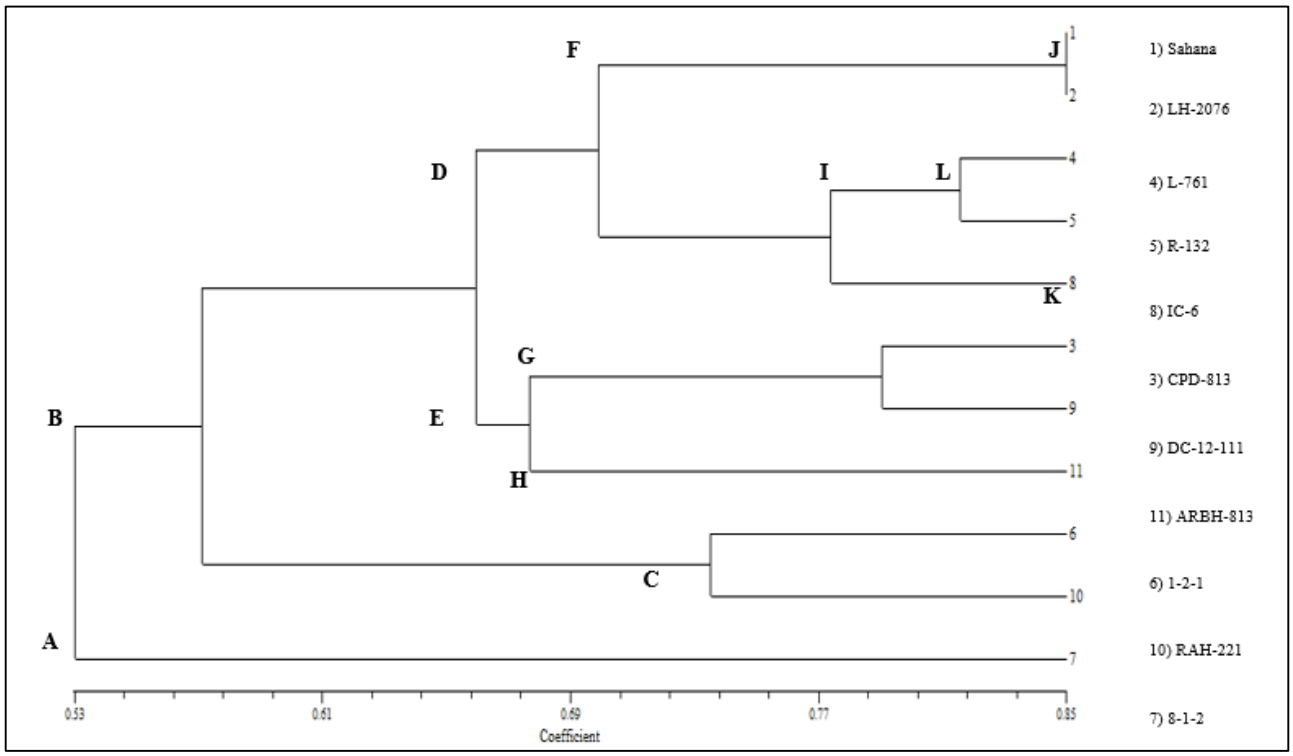


Fig 2: Dendrogram generated from the pooled data of RAPD profile using UPGMA analysis

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