Indian J. Plant Physiol., Vol. 14, No. 2, (N.S.) pp. 105-110 (April-June, 2009)

GENETIC DIVERSITY ANALYSIS OF ELITE PARENTAL LINES OF COTTON USING RAPD, ISSR AND ISOZYME MARKERS

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Received on 21 March, 2009, Revised on 11 June, 2009

SUMMARY

The genetic diversity and similarity were investigated in nine elite parental lines of cotton using 25 RAPD, 3 ISSR and 2 isozyme markers. Total 189 amplicons were generated using all 30 markers, of which 117 bands were found to be polymorphic. Highest polymorphism was seen with ISSR primers (66.66%) while, it was least (42.85%) with the isozymes. Based on Jaccard's similarity coefficient, the similarity index was in the range of 0.11 to 1.00 among nine parental lines. The lowest similarity (11%) was found among LRA-5166, GCot.10 and 76 IH-20. The dendrogram of cotton lines showed two major clusters. The Parental line LRA-5166 was found in one cluster and the rest eight parental lines were found in different sub-sub cluster of another cluster. The results suggested that the genetic constituents of LRA-5166 is quite different than other tested parental lines.

Key words: **Cotton, genetic diversity, isozyme, ISSR, polymorphism, RAPD**

INTRODUCTION

Tetraploid upland cotton (*Gossypium hirsutum*, AD1; 2n=4x=52), is the most predominant cultivated cotton with high yield and wide adaptation, accounting for more than 90% of the world cotton production. Cotton breeding has made significant contributions in the past century, like increased cotton yield, improved fiber quality and enhanced biotic tolerance. Current and obsolete cultivars and strains of Upland cotton have been and still are the main sources in cotton breeding programs worldwide. However, the desirable and amenable genetic variations for breeders are limited, lacking or difficult to dissect. Due to the narrow genetic base of cotton germplasm that cotton breeders have been utilizing and low efficiency of traditional selection methods, cultivar improvement in cotton has slowed down in the past 10–15 years (May *et al*. 1995, Meredith 2000.)

Analysis of genetic diversity and relatedness between species and among genotypes is useful in plant breeding programs because it provides a tool for accurate organization of germplasm and efficient parental selection. Morphological and physiological features of plants have been used to understand the genetic variation. There are about 145 morphological markers identified in cultivated cotton but their utility in breeding programmes has remained limited because of their deleterious effect and the difficulties in accumulating multiple markers in a single genotype (Preetha and Raveendren 2008)

Therefore, more reliable markers such as protein, or more specifically, allelic variants of several enzymes so called isozymes are being used to understand the genetic variation. But the number of isozymes available is limited and their expression is often restricted to a specific developmental stage of the tissues. With the development of the polymerase chain reaction (PCR) technology, the

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number of useful DNA based marker types has been expanded considerably. Different PCR-based markers are available nowadays including Randomly Amplified Polymorphic DNA (RAPDs), ISSR (Inter Simple Sequence Repeat), Microsatellites (SSRs), Expressed Sequence Tags (ESTs) and Amplified Fragment Length Polymorphisms (AFLPs). Analysis of genetic diversity and relatedness between species and among genotypes is useful in plant breeding programs because it provides a tool for accurate organization of germplasm and efficient parental selection (Rana *et al*. 2005, Hussein *et al*. 2006). The present investigation was conducted to estimate the genetic polymorphism among nine elite parental lines of cotton using two types of PCR- based DNA markers,i.e., RAPDs, ISSRs, and isozymes as biochemical marker was to assess the genetic relationships among these parental lines.

MATERIALS AND METHODS

Seed material of nine elite cotton parental lines, *viz.* 1:GCOT-67, 2:American Nectariless, 3:GCOT-100, 4:GCOT-10, 5:Surat Dwarf, 6:LRA-5166, 7:BC68-2, 8:76IH-20 and 9:GCOT-16 was obtained from the Main Cotton Research Centre, Navsari Agricultural University, Navsari. Leaf samples of 500 mg were homogenized in 2.5 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4) with 1 mM EDTA and 1% (w/v) polyvinylpyrolidone (PVP) in a pre-chilled pestle and mortar under ice cold conditions. The homogenates were centrifuged at 10,000 rpm for 20 min and supernatant was used for the detection of SOD and esterases isozymes banding pattern. Isozymes were separated on ATTO vertical electrophoresis unit using 1 mm gel. Electrophoresis was performed at 30 mA until tracking dye moved at bottom. Enzyme extracts (100 µg protein) were loaded for each isozyme and mixed with 2 µl tracking dye. Protein concentration was determined by the method of Lowry *et al*. (1951).

Isozymes of SOD were separated on a 10% non-denaturing polyacrylamide gels. After electrophoresis, SOD isoforms were visualized by the method of Beauchamp and Fridovich (1971). Gels were stained in 50 mM sodium phosphate buffer, pH 7.8 containing 0.24 mM NBT and 28 µM riboflavin for 20 min in the dark followed by immersing in 50 mM sodium phosphate buffer, pH 7.8 containing 28 mM TEMED, which were then exposed to a light source at room temperature until white bands appeared in blue background.

Esterases isoforms were separated on 10% native polyacrylamide gels. The gels were visualized by incubating in a solution containing 1.4 g sodium dihydrogen phosphate, 550 mg disodium hydrogen phosphate, 0.1 g fast blue RR salt and 15 mg a-napthyl acetate (dissolved in chilled acetone) in 100 ml Milli Q water (Sadasivam and Manickam 1996). The gels were incubated with shaking until bands were visualized. After that, gels were immersed in stop solution containing Methanol: MilliQ water: Acetic acid: Ethanol (10:10:2:1) and scanned.

Total genomic DNA was isolated from young leaf material following the modified CTAB method described by Keim *et al*. (1988). To avoid co-isolation of phenolics and polysaccharides 2% polyvinyl pyrrolidone (PVP) was added in DNA extraction buffer. The quality and quantity were estimated by measuring O.D. at 260/280 nm and 260 nm, respectively, in Nano drop spectrophotometer. Intactness of genomic DNA was checked on 0.8 % agarose gel. The genomic DNA was amplified using random primers of OPE, OPF, OPG, OPH, OPI and OPJ (Operon Tech., California, USA) and 10 UBC ISSR primers. Twenty five decamers (Table 1) were selected after screening a set of 120 primers from OPE, OPF, OPG, OPH, OPI and OPJ to obtain RAPD profile. Three ISSR primers UBC-807, UBC-843 and UBC-873 were selected after screening of 15 UBC primers.

The PCR reactions for RAPD and ISSR were carried out in a 25 µl of reaction mixture. The PCR mixture consisted of 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.8 pmoles primer, 50 ηg genomic DNA and 1.5 U Taq polymerase (Biogene,USA). All the PCR reactions were carried out in a 200 µl thin walled PCR tubes. The amplification carried out in an Biometra gradient thermo-cycler with heated lid to reduce evaporation. The cycling programme for RAPD involved an initial denaturation at 95 °C for 5 minutes followed by 40 cycles at 95 °C for 1minute, 38 °C for 50 seconds, 72 °C for 1.5 minute and then a final extension at 72 °C for 10 minutes. Whereas, the

cycling programme for ISSR involved an initial denaturation at 95 °C for 5 minutes followed by 40 cycles at 95 °C for 1 minute, annealing temperature for ISSR primers were varied according to GC content (Ta for 45 seconds), extension at 72 °C for 2 minutes and then a final extension at 72 °C for 7 minutes. Amplification products were electrophoresed in 1.5% (RAPD) and 1.8% (ISSR) agarose gel containing 5 µg ethidium bromide/ 100ml. The electrophoresis was carried out at 60 mA (constant) in 0.5 TBE buffer to separate the amplified bands. The standard DNA marker (DNA logic ladder, Biogene USA) was also run along with the samples. The separated bands were visualized under UV light and photographed by Gene Genius Bio Imaging System.

Only well-separated and intense bands that were observed in all the three independent amplifications were selected for scoring. Band positions for each cotton genotype and primer combination were scored as either present (1) or absent (0). The scores were entered into a database programme (Microsoft Excel) and compiled in a binary matrix for phylogenetic analysis using NTSYS-pc (Numerical Taxonomy and Multivariate analysis) system version 2.2 by Exeter Software (Rohlf, 2004). The SIMQUALK programme was used to calculate Jaccard's similarity coefficient and a graphical phenogram (dendrogram) of the genetic relatedness among the 9 genotypes was produced by means of the unweighted pair group method with arithmetic average (UPGMA) analysis (Sneath and Sokal 1973).

RESULTS AND DISCUSSION

RAPD analysis using 25 decamers (Table 1) gave 154 loci, out of which 97 bands (62.99%) were found to be polymorphic. Among the twenty five primers, OPF-10 gave the maximum number of bands (10), whereas, maximum polymorphism was generated by OPI-4 (100 %). The number of amplicons /primer ranged from 2 (OPE-3) to 10 (OPF-10), whereas the number of polymorphic bands per primer ranged from 1 (OPE-16, OPG-5 and OP-P03) to 10 (OPF-10). Hussein *et al*. (2007) characterized 11 cotton genotypes using 15 selected RAPD primers and observed a total of 112 polymorphic bands out of 177 reproducible products. This corresponds to a level of 63.2% polymorphism. On the other hand a high level of polymorphism (97.2%) among cotton genotypes was observed by Rana and Bhatt (2005). They utilized all four cultivated species of cotton. Three ISSR primers (Table 2) UBC-807, UBC-843 and UBC-873 were selected after screening a total of 15 different ISSR primers to generate PCR profiles. A total of 21 bands were obtained, showing 66.66%

Table 1 . List of random primers and their per cent polymorphism revealed by RAPD analysis of elite cotton parental lines.

Primer	Total number of bands	Total number of poly-morphic bands	Total number of mono-morphic bands	% polymorphism
OPE-1	9	6	3	66.67
OPE-2	6	5	1	83.33
OPE-3	2	0	2	0.00
OPE-9	4	2	2	50.00
OPE-12	7	2	5	28.57
OPE-14	3	0	3	0.00
OPE-16	4	1	3	25.00
OPF-1	9	7	2	77.78
OPF-2	8	6	2	75.00
OPF-4	8	5	3	62.50
OPF-5	5	3	2	60.00
OPF-6	9	5	4	55.56
OPF-10	10	9	1	90.00
OPF-12	6	3	3	50.00
OPG-4	8	7	1	87.50
OPG-5	4	1	3	25.00
OPG-6	7	5	2	71.43
OPH -20	7	3	4	42.86
OPI-2	3	2	1	66.67
OPI-3	9	3	6	33.33
OPI-4	9	9	0	100.00
OPI-7	5	4	1	80.00
OPI-11	2	1	1	50.00
OPI-13	7	6	1	85.71
OPJ-11	3	2	1	66.67
Total	**154**	**97**	**57**	**62.99**

Table 2. List of ISSR primers and their per cent polymorphism revealed by ISSR analysis of elite cotton parental lines.

Primer	Total number of bands	Total number of poly-morphic bands	Total number of mono-morphic bands	% polymorphism
UBC-807	8	3	5	37.50
UBC-843	6	5	1	83.33
UBC-873	7	6	1	85.71
Total	**21**	**14**	**7**	**66.66**

Table 3. Isozymes and their per cent polymoephism in elite cotton parental lines.

Primer	Total number of bands	Total number of poly-morphic bands	Total number of mono-morphic bands	% polymorphism
Esterase	8	5	3	62.50
SOD	6	1	5	16.66
Total	**14**	**6**	**8**	**42.85**

polymorphism. Two isozymes viz., SOD and esterase generated 14 bands with 42.85% polymorphism. The Jaccard's similarity coefficient analysis of Joint RAPD, ISSR and isozyme showed 100% similarity between G. Cot-67 and American Nectariless (Table 4). Whereas, LRA 5166 had similarity value less than 25 % with all the parental lines, it was altogether different from GCot-100 and Surat dwarf (0% similarity); moreover, it had mere 11 % similarity with GCot-10, 76 IH-20 and GCot-16.

Two major clusters were formed on the basis of UPGMA clustering analysis (Fig 1). The first cluster was having only single parental line *viz* LRA 5166 whereas, second cluster contained remaining eight genotypes. G-Cot-10, BC-68-2 and 76 IH-20 having 44% similarities with GCot-67 and American Nectariless.

Similarly, low level of DNA variation among ten varieties of *G. hirsutum* was observed by Lu and Myers (2002), they observed only 13.5% polymorphism. Although, we found 63% polymorphism but five parental lines showed 100% similarity. Most of the primers produced polymorphic amplification products but degree of variation among different parental line is very less except LRA 5166. Our results are closely consistent with results observed by Patil *et al*. (2007), they observed two major clusters among tested genotypes. The two genotypes, LRA-5166 and Abadhita were found in one cluster and the genotype Jayadhar and Rahs-14 were found in other cluster. Moreover, they categorized LRA-5166 and Abadhita as drought susceptible genotypes

Table 4. Jaccard's similarity index for elite parental lines of cotton

	G-67	AMERICAN NECTARILESS	GCOT-100	GCOT-10	SURAT DWARF	LRA-5166	BC68-2	76IH-20	GCot-16
G-67	1.00								
AMERICAN NECTARLESS	1.00	1.00							
GCOT-100	0.33	0.33	1.00						
GCOT-10	0.44	0.44	0.44	1.00					
SURAT DWARF	0.20	0.20	0.00	0.22	1.00				
LRA-5166	0.25	0.25	0.00	0.11	0.00	1.00			
BC68-2	0.43	0.43	0.43	0.67	0.33	0.00	1.00		
76IH-20	0.44	0.44	0.44	1.00	0.22	0.11	0.67	1.00	
GCot-16	0.44	0.44	0.44	1.00	0.22	0.11	0.67	1.00	1.00

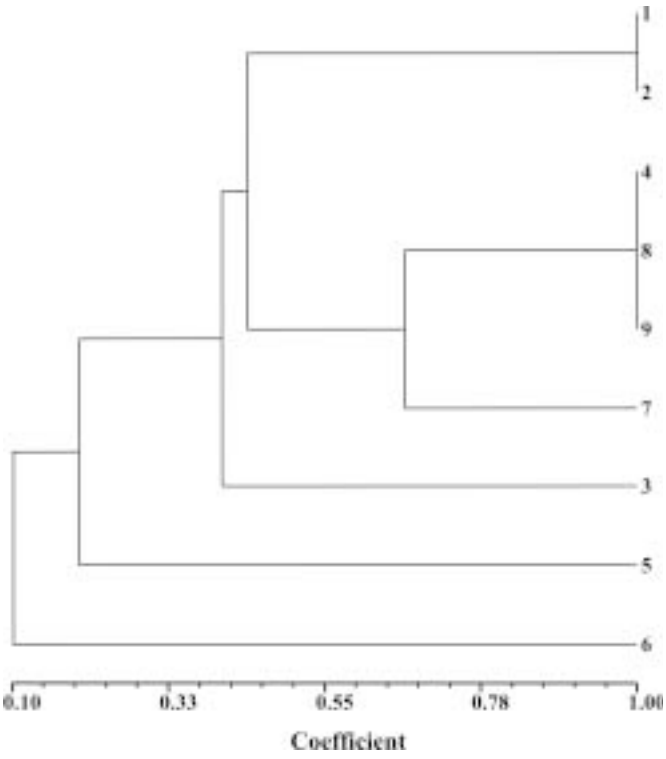

Fig. 1. Dendrogram depicting the genetic relationship among the elite parental lines of cotton based on pooled RAPD, ISSR and isozymes data
Number for cotton parental lines: (1) G cot-67, (2) American Nectariless, (3) GCOT-100, (4) GCOT-10, (5) SURAT DWARF, (6) LRA-5166, (7) BC68-2, (8) 76IH-20 and (9) GCot-16

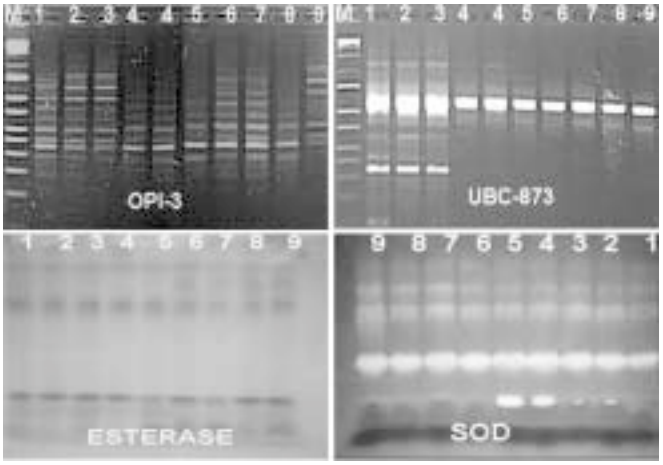

Fig. 2. Banding pattern of nine elite parental lines of cotton generated by RAPD(OPI-3), ISSR (UBC-873) and isozymes (esterase and SOD)
Lanes are (1) GCOT-67, (2) American Nectariless, (3) GCOT-100, (4) GCOT-10, (5) SURAT DWARF, (6) LRA-5166, (7) BC68-2, (8) 76IH-20 and (9) GCOT-16

based on yield and physio-biochemical observations *viz.* LAD, SLW, RWC, CSI, proline content, chlorophyll fluorescence and leaf water potential. This grouping pattern was further confirmed by molecular analysis. Interestingly, we also found LRA 5166 in separate cluster with higher genetic diversity among tested parental lines this might be due to drought susceptible character of this line.

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