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Full Research Article

DNA Fingerprinting of Upland Cotton (*Gossypium hirsutum*) Hybrids and their Parents Using RAPD and SSR Markers

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Abstract

Cotton, the white gold is also known as the king of fiber crops and is the main raw material for textile industry. Use of morphological differences, between true hybrids and off types in grow out test (GOT) for genetic purity analysis, are not always apparent and cannot be recognized easily. Further, morphological traits are costly, tedious to score and environment sensitive. Alternatively, it is suggested that recent breakthrough in molecular markers can be employed in genetic purity analysis. Molecular markers such as RAPD and SSR markers were used for the parental identification of different hybrids. The experiment was carried out at the Biotechnology Laboratory, Main Cotton Research Station, Surat, Gujarat during 2013-14. In present study, seven RAPD markers (RP10, RP20, RP21, RP22, RP25, SRT-6 and SRT-13) and six SSR markers (MGHES-62, TMB-1181, TMB-0409, TMB-1791, JESPR-153 and CM-45) were identified to be polymorphic between parents of the hybrids studied. Among these primers JESPR-153 is able to identify the true hybrid (Heteroallelic). These reported markers showed specific size of amplicons which helped in parental confirmation. Results indicated that, these two markers-RAPD and microsatellites individually or in combination are more reliable for identification and testing of genetic purity of cotton hybrids. These highly informative markers not only differentiated the parent genotypes but also confirmed the parentage of their true F_1 hybrids. Our findings revealed that RAPD and SSR procedures were excellent genomic tools for parentage confirmation and hybridity determination, and would also enhance efficiency of our breeding programmes through marker assisted selection.

1. Introduction

Cotton is the leading fiber crop the world. In India, cotton covers cultivated area of about 11.6 m ha^{-1}. It occupies second position in production with 375 lakh bales amongst all cotton producing countries in the world i.e. next to China (Anonymous, 2013). Average productivity of India is 552 k ha^{-1} which is low as compared to world average of 754 k ha^{-1} (Anonymous, 2013). India has unique feature of in the cotton cultivation in the sense that all the four species of *Gossypium, G. hirsutum, G. barbadense* (both tetraploid), *G. herbaceum* and *G. arboreum* (both diploid) and their hybrids are cultivated on commercial scale. Tetraploid cotton (*Gossypium hirsutum*) with the genome constitution 2 (AD) (2n=52) along with *G*. barbadense dominate the world cotton production. The genomes of *G. hirsutum* individually are referred to as Ah and Dh and their chromosomes as H_1-H_{13} and H_{14}-H_{26}, respectively (Menzel and Brown, 1978). Germplasm diversity is of concern to breeders as they rely on genetic variation between parents to create unique gene combinations necessary for new superior cultivars (Van Esbroeck and Bowman, 1998). For the improvement of agronomically and economically important traits, plant breeding generally recombines traits present in different parental lines of cultivated and wild species. Conventional breeding programmes reach this goal by generating an F_1 hybrid and F_2 segregating population and then screening the phenotypes of pooled or individual plants for presence of desirable traits, which is followed by a process of repeated backcrossing, selfing and testing. The success of hybrid cotton technology depends on the timely production and adequate supply of genetically pure hybrid seeds to the farmers. In this procedure, the hybrid nature of the plants is assessed by growing them in the field which is very laborious and prolonged method. Therefore, an alternative technique

that offers efficient, quick and reliable assessment of genetic purity is urgently needed. Molecular marker analysis offers an efficient alternative to this approach as genetic relationships are estimated on the basis of genotype and not phenotype. Among these marker techniques, DNA based markers which include restriction fragment length polymorphism (RFLP) (Liu and Turner, 1993), random amplified polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993), microsatellite or simple sequence repeat (SSR) (Akkaya et al., 1992) and single nucleotide polymorphism (SNP) (Bojinov and Lacape, 2003) are of utmost significance for crop improvement. Among these, RAPDs being relatively simple, less expensive and reliable and are the most widely used molecular technique well suited for estimating similarity and differences among different cotton cultivars (Preetha and Raveendran, 2008; Sheidai et al., 2007; Rana and Bhat, 2005; Abdel Ghani and Zaki, 2003; Lu and Myers, 2002). Microsatellites or SSR are typically the repeat unit of 1-6 nucleotides and its analysis is performed by using pairs of specific primers flanking tandem arrays of microsatellite repeats. SSR markers are codominant and extremely polymorphic (Liu et al., 2002). Molecular markers not only allow the easy and reliable identification of breeding lines, hybrids and cultivars (Bastia et al., 2001; Asif et al., 2005, 2006; Tabbasam et al., 2006) but also facilitate the monitoring of introgression, mapping of QTLs (Jiang et al., 2000), marker assisted selection (MAS) (Ribaut and Hoisington, 1998; Zhang et al., 2003) and estimation of genetic diversity (Mukhtar et al., 2002; Rahman et al., 2002, 2007). Hybrid identification in a crop species through DNA fingerprinting is an effective tool to increase the speed and quality of backcrossing conversion, thus reducing the time taken to produce crop varieties with desirable characteristics (Farooq and Azam, 2002; Murtaza et al., 2005). The proposed research work was conducted to study the efficiency of RAPD and SSR assay for hybridity determination, which would be a valuable genomic tool for the cotton breeders.s

2. Materials and Methods

2.1. Plant materials

The studies pertaining to the DNA fingerprinting for identification of cotton hybrids using RAPD and SSR marker system were carried out at the Biotechnology Laboratory, Main Cotton Research Station, Surat, Gujarat during 2013-14. The plant material for this study comprised of four parent genotypes (G.Cot.16, H-1353/10, BS-30 and H-1452/10) and their twelve hybrids.

2.2. DNA extraction

Young and tender leaves from twenty one days old seedling of 16 lines included in the present study were used for DNA isolation using CTAB method as suggested by Saghai Maroof et al. (1984) with minor modification. High salt concentrations and polyvinyl-polypyrrolidone were added separately to remove polysaccharides and polyphenol compounds. Extraction was performed by an extended RNase treatment and a phenol-chloroform method. DNA was then finally dissolved in 100 µl of 1X TE and mixed thoroughly. Content was centrifuged at 12000 rpm for 2 min to collect the dissolved DNA at bottom. An aliquot of stock was stored in refrigerator at -20 °C till further use.

2.3. PCR amplification

The genomic DNA was subjected to polymerase chain reaction. Random decamers and SSR primers (including EST-SSRs) were surveyed for screening four cotton parents (G.Cot.16, H-1353/10, BS-30 and H-1452/10) and subsequently screening their F_1 with polymorphic RAPDs and SSRs. Fifty oligonucleotide primers obtained from Bangalore GeNei, India (GeNei™) were used for RAPD analysis. For RAPD assay PCR amplification was performed in 25 µl reaction volumes containing 20 ng μl^{-1} genomic DNA, 10X PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs (each of dATP, dGTP, dCTP and dTTP), 10 pmole μl^{-1} Random primer and 3U μl^{-1} *Taq* DNA Polymerase in 200 µl PCR tube. PCR profile was 94°C for 5 min., then 30 cycles of 94°C for 45 sec, 38°C for 1 min, 72°C for 1 min and finally 72°C for 10 min. A set of 29 SSR primer pairs belonging to JESPR, MGHES, TMB, CIR and BNL series synthesized from Eurofins Genomics India Pvt. Ltd. were used to study parental identification of hybrids. SSR amplification was performed in 25 µl reaction volumes containing similar reagents used for RAPD assay except primers. PCR profile for SSR amplification was similar to RAPD profile but cycles were 35 instead of 30 and annealing temperature ranged from 55 to 60 °C.

2.4. Gel electrophoresis

All the RAPD PCR products were run in 1.8% agarose gel, while SSR products were run in 2.0% agarose gel containing 4 µl of ethidium bromide (1 mg ml^{-1}). Running buffer containing Tris-buffer, boric acid and EDTA (pH 8.0) was used for electrophoresis. The standard DNA marker (100 bp- 3 kb) was also run along with the samples. The separated bands were documented under UV transilluminator and photographed by Gel documentation system (BIORAD) and analyzed.

2.5. Data scoring and analysis

The weak and spurious bands were excluded from the analysis. Bands were score as 1 (present) or 0 (absent). Similarity coefficient was calculated using the Jaccard index and a cluster analysis was performed by Unweighted Pair Group Method using Arithmetic Average (UPGMA), using the NTSYS-pc

analytical software (Rohlf, 1998). A dendrogram was generated based on the cluster analysis.

3. Results and Discussion

3.1. Molecular Marker analysis

It is important to select true hybrids for establishing breeding program but it is difficult to identify true hybrids based on morphological parameters, due to availability few number of traits as well as influence of environment on the these traits. By the use of molecular marker technique, it is easier to identify true hybrids at early stages which are independent to environmental influence. This technique can be adopted for large scale screening of hybrids cotton (Mehetre et al., 2004b; Dongre and Parkhi, 2005). Molecular markers have been used successfully to estimate genetic similarity and for cultivars analysis of various cotton cultivars (Multani and lyon, 1995; Iqbal et al., 2000).

3.1.1. RAPD analysis

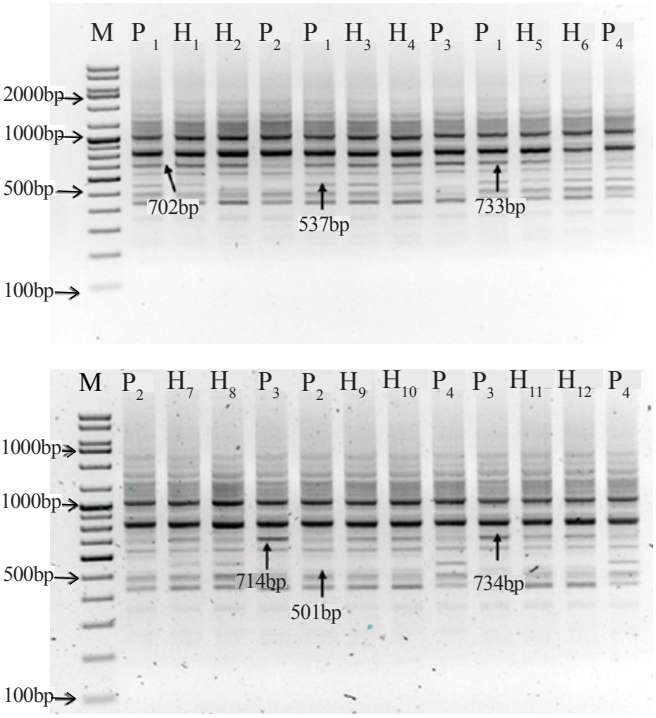
Twelve cotton hybrids (F_1) of parents G.Cot.16, H-1353/10, BS-30 and H-1452/10 were subjected to RAPD analysis using 50 decamers. All the primers responded for amplification. Amongst them RP10, RP20, RP21, RP22, RP25, SRT-06 and SRT-13 have shown polymorphism.

Gel pictures showing amplifications in the parents and hybrids are shown in Figure 1-6. The parents and the F_1 plants were carefully observed on the basis of morphology to see if they were true hybrids. Among the 50 decamer primers, seven primers amplified 77 fragments in the parents and hybrids. Out of which 24 loci were polymorphic (Table 1). The primer RP 10 was found to produce 80% polymorphic fragments and the lowest monomorphic bands. The lowest polymorphism (9.1%) was seen in the primer RP22. Monomorphic bands are those which are present in both parent and their hybrids, polymorphic are present in one or more but not all individuals and unique ones are present in at least one individual not in any other. The polymorphisms observed between the parents are used as markers for hybrid identification. Comparing the RAPD banding pattern of parents with respective hybrids, genuine hybrids were confirmed. Two primers, RP21 and RP25 identified the F_1 hybrids (Figure 1-2). RP 21 generated polymorphic bands in the range of approximately 501-734bp. Similarly, primer RP 25 also produced polymorphic bands in the range of approximately 252-652bp, which helped to identify the hybrid. Therefore, these RAPD markers not only produced unique banding pattern and also discriminated cotton parents but also their true hybrids (Asif et al., 2009; Macha et al., 2010; Dongre et al., 2011; Dongre et al., 2012; Farzaneh et al., 2010). Variation in markers from parents to hybrid may have originated due to recombination, deletion, mutation or random segregation of the chromosomes at meiosis during the process of hybrid formation (Williams et al., 1990).

Table 1: Results of RAPD analysis

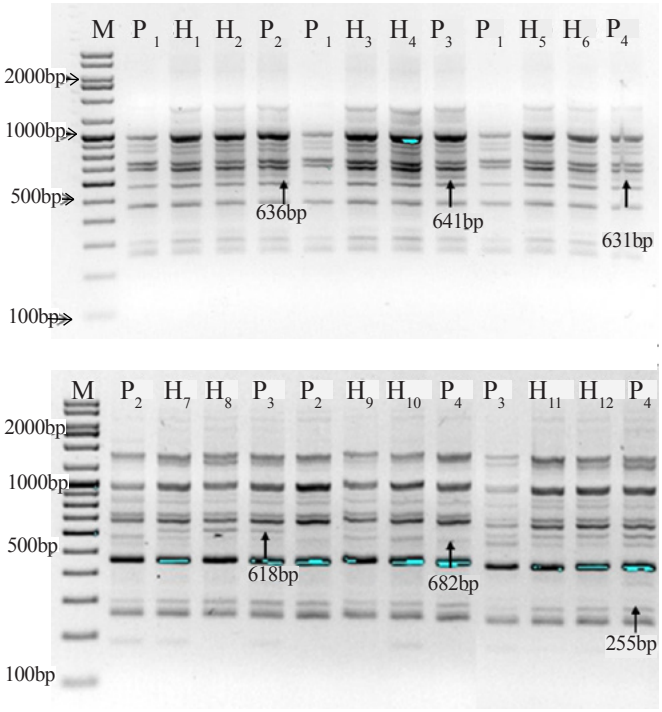
Sr. no.	Primers	Product range (bp)	TNB	NPB	NMB	% polymorphism	PIC value
1.	RP 10	580-2060	10	8	2	80	0.88
2.	RP 20	539-1738	12	4	8	33.3	0.92
3.	RP 21	501-734	9	2	7	22.2	0.89
4.	RP 22	548-561	11	1	10	9.1	0.91
5.	RP 25	255-652	10	2	8	20	0.90
6.	SRT 6	739-1839	11	2	9	18.2	0.91
7.	SRT 13	880-1617	14	5	9	35.7	0.92
	Total		77	24	53		

TNB: Total number of bands; NPB: Number of polymorphic bands; NMB: Number of monomorphic bands



P_1-G.Cot. 16, P_2-H-1353/10, P_3-BS-30, P_4-H-1452/10, H_1-G.Cot.16 (P_1)×H-1352/10 (P_2), H_2-H-1353/10 (P_2)×G. Cot.16 (P_1), H_3-G.Cot.16 (P_1)xBS-30 (P_3), H_4-BS-30 (P_3)×G. Cot.16 (P_1), H_5-G.Cot.16 (P_1)×H-1452/10 (P_4), H6- H-1452/10 (P_4)×G.Cot.16 (P_1), H_7-H-1353/10 (P_2)×BS-30 (P_3), H_8-BS-30 (P_3)×H-1353/10 (P_2), H_9-H-1353/10 (P_2)×H-1452/10 (P_4), H_{10}-H-1452/10 (P_4)×H-1353/10 (P_2), H_{11}- BS-30 (P_3)×H-1452/10 (P_4), H_{12}-H-1452/10 (P_4)×BS-30 (P_3).

Figure 1: Amplification patterns of different cotton hybrids and their parents produced by primer RP-21



P_1-G.Cot. 16, P_2-H-1353/10, P_3-BS-30, P_4-H-1452/10, H_1-G. Cot.16 (P_1)×H-1352/10 (P_2), H_2-H-1353/10 (P_2)×G.Cot.16 (P_1), H_3-G.Cot.16 (P_1)×BS-30 (P_3), H_4-BS-30 (P_3)×G.Cot.16 (P_1), H_5-G.Cot.16 (P_1)×H-1452/10 (P_4), H_6- H-1452/10 (P_4)×G.Cot.16 (P_1), H_7-H-1353/10 (P_2)xBS-30 (P_3), H_8-BS-30 (P_3)×H-1353/10 (P_2), H_9-H-1353/10 (P_2)×H-1452/10 (P_4), H_{10}-H-1452/10 (P_4)×H-1353/10 (P_2), H_{11}-BS-30 (P_3)×H-1452/10 (P_4), H_{12}-H-1452/10 (P_4)×BS-30 (P_3).

Figure 2: Amplification patterns of different cotton hybrids and their parents

3.1.2. Simple sequence repeats (SSR)

Microsatellites are typically the repeat unit of 1-6 nucleotides and SSR analysis is performed by using pairs of specific primers flanking tandem arrays of microsatellite repeats. SSR markers are co-dominant and extremely polymorphic (Liu et al., 2002). These are more informative and highly polymorphic and their polymorphism is based on differences in number of repeats in amplified regions.

The polymorphisms observed between the parents were used as markers for hybrid identification. For the present study, EST based microsatellite (SSR) markers were used to analyze the parentage identification among 12 hybrids (F_1) and their four parents of cotton. A total of 29 SSR were used for hybrid identification. All the primers responded for amplification. Amongst them MGHES-62, TMB-0283, TMB-1181, TMB-1791, TMB-0409, JESPR-153 and CM-45 have shown polymorphism (Table 2). The primer JESPR 153 was found to produce 100% polymorphic fragments with the PIC value of 0.67 and lowest polymorphism in MGHES-62 with PIC value of 0.49. These highly informative primes not only differentiated parents but also confirmed the parentage of their F_1 hybrid. The SSR marker, TMB 1791 produced two distinguishable alleles of different base pair sizes, in which allele one was common in both parents. Size of allele two was different in different hybrids such as 202 bp, 195 bp, 187 bp, 195 bp, 188 bp and 178 bp which are specific to different parents and their respective hybrids. Similarly, JESPR 153 produced polymorphic allele in which one common allele present in both the hybrids and parents. Allele two was specific to respective parent in different sizes such as 132 bp, 125 bp, 124 bp, 121 bp, 135 bp and 137 bp, which is specific to their respective parents (Figure 4). Therefore, codominant nature of SSR marker with highly reproducible polymorphism makes it as a marker choice for the genetic studies in crops (Asif et al., 2009; Selvakumar et al., 2010; Wu et al., 2010). Microsatellite analysis have been successfully employed for parentage verification, hybrid identification, cultivars characterization and purity testing in other crop plants (Asif et al., 2006; Bertini et al., 2006; Tabbasam et al., 2006). Commercial cotton cultivars characterized and evaluated with microsatellites found some specific alleles for discriminating cotton germplasm (Zhang et al., 2005).

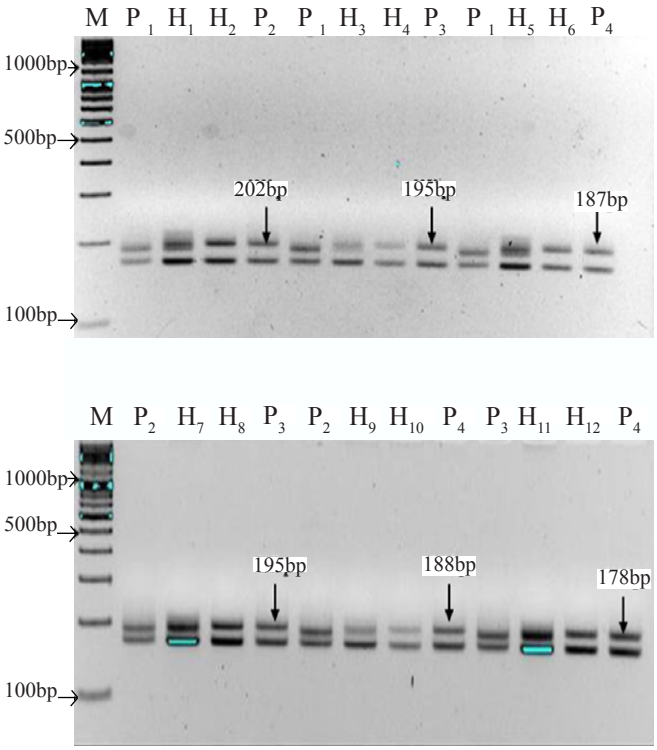
Table 2: Result of SSR analysis

Sr. no.	Primers	Product range (bp)	Tm (°C)	TNB	NPB	% polymor-phism	PIC value
1.	MGHES-62	142-165	55	2	1	50	0.49
2.	TMB-1181	223-240	55	3	2	66.7	0.59
3.	TMB-1791	178-202	55	2	1	50	0.50
4.	TMB-0409	133-241	55	2	2	100	0.47
5.	JESPR-153	121-137	56	3	3	100	0.67
6.	CM-45	126-140	55	3	3	100	0.58
	Total			15	12		

TNB: Total number of bands; NPB: Number of polymorphic bands

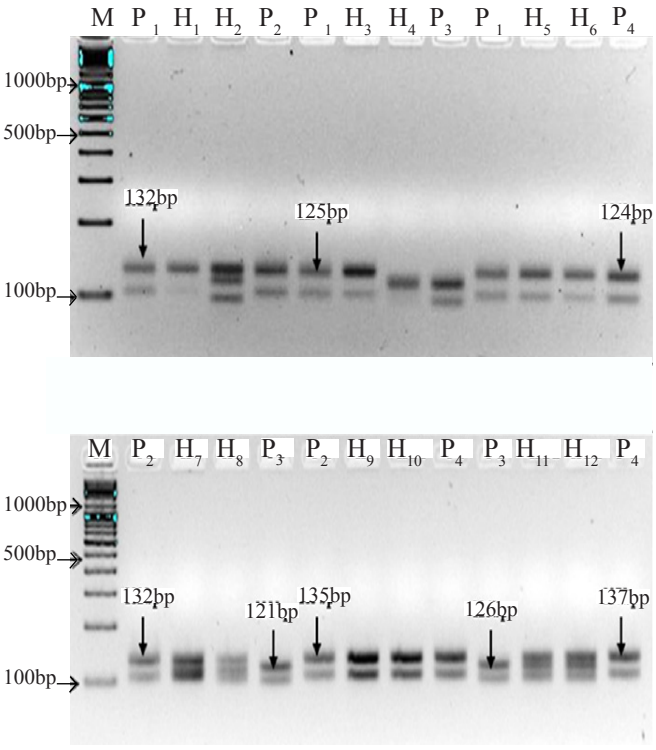
3.1.3. Similarity coefficient and clustering pattern

The NTSYSpc programme was used to calculate Jaccard's similarity coefficient. Similarity coefficient of different cotton F_1 hybrids and their parents is presented in Table 3. Genetic similarity matrix revealed, similarity values ranging from 0.78 to 0.99. Maximum genetic similarity (0.99) was found between P_3 (BS-30) and H_3 (G.Cot.16xBS-30) and least genetic



P_1- G.Cot.16, P_2-H-1353/10, P_3-BS-30, P_4-H-1452/10, H_1-G.Cot.16 (P_1)×H-1352/10 (P_2), H2-H-1353/10 (P_2)×G.Cot.16 (P_1), H_3-G.Cot.16 (P_1)×BS-30 (P_3), H_4-BS-30 (P_3)×G.Cot.16 (P_1), H_5-G.Cot.16 (P_1)×H-1452/10 (P_4), H_6-H-1452/10 (P_4)×G.Cot.16 (P_1), H_7-H-1353/10 (P_2)×BS-30 (P_3), H_8-BS-30 (P_3) ×H-1353/10 (P_2), H_9-H-1353/10 (P_2)×H-1452/10 (P_4), H_{10}-H-1452/10 (P_4)×H-1353/10 (P_2), H_{11}-BS-30 (P_3)×H-1452/10 (P_4), H_{12}-H-1452/10 (P_4)×BS-30 (P_3).

Figure 3: Amplification patterns of different cotton hybrids and their parents produced by TMB-1791



P_1- G.Cot.16, P_2-H-1353/10, P_3-BS-30, P_4-H-1452/10, H_1-G.Cot.16 (P_1)×H-1352/10 (P_2), H_2-H-1353/10 (P_2)×G.Cot.16 (P_1), H_3-G.Cot.16 (P_1)×BS-30 (P_3), H_4- BS-30 (P_3)×G.Cot.16 (P_1), H_5- G.Cot.16 (P_1)×H-1452/10 (P_4), H6-H-1452/10 (P4)×G.Cot.16 (P_1), H_7-H-1353/10 (P_2)×BS-30 (P_3), H_8-BS-30 (P_3)×H-1353/10 (P_2), H_9-H-1353/10 (P_2)×H-1452/10 (P_4), H_{10}-H-1452/10 (P_4)×H-1353/10 (P_2), H_{11}- BS-30 (P_3)×H-1452/10 (P_4), H_{12}-H-1452/10 (P_4)×BS-30 (P_3).

Figure 4: Amplification patterns of different cotton hybrids and their parents produced by JESPR- 153

similarity (0.74) was observed between P_2 (H-1353/10) and P_4 (H-1452/10).

The clustering pattern of dendrogram constructed by Jaccard's coefficient of similarity indicated differences among the different cotton F_1 hybrids and their respective parents. The dendrogram showed four major clusters such as A, B, C and D were formed on the basis of their similarity coefficient (Figure 5). The cluster A consists of only parent G.Cot.16. Cluster B consist of P_2 (H-1353/10), H_1 (G.Cot.16×H-1353/10), H_3 (G.Cot.16×BS-30) and H_5 (G.Cot.16×H-1452/10). Cluster C consist of P_3 (BS-30), H_4 (BS-30×G.Cot.16), H_2 (H-1353/10×G.Cot.16), P_4 (H-1452/10) and H_6 (H-1452/10×G.Cot.16). Cluster D consist of H_7 (H-1353/10×BS-30), H_8 (BS-30×H-1353/10), H_9 (H-1353/10×H-1452/10), H_{10} (H-1452/10×H-1353/10), H_{11} (BS-30×H-1452/10) and H_{12} (H-1452/10×BS-30). The dendrogram denoted that, cluster A shows its clear genetical divergence from other cluster. Cluster B near to cluster A

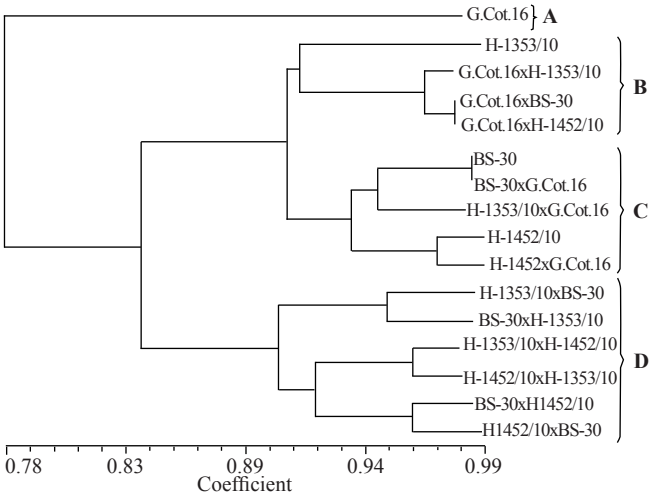


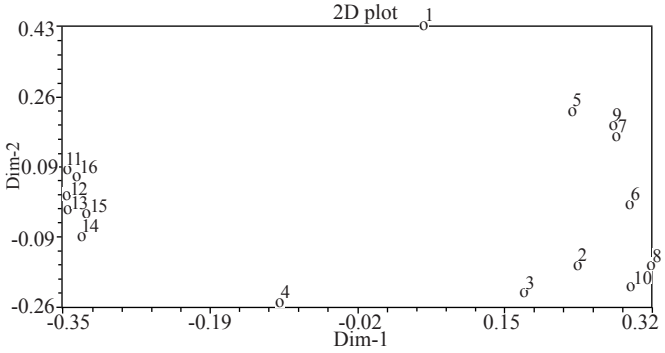
Figure 5: Dendrogram depicting the genetic relationship among the different cotton hybrids and their parents based on RAPD and SSR data

Table 3. Jaccard's similarity coefficient among different cotton F1 hybrids and their parents based on RAPD and SSR data analysis.

Genotype	G. cot.16	H-1353 /10	BS-30	H-1452 /10	G.cot.16× H-1352/10	H-1353 /10× G.cot.16	G.cot.16 ×BS-30	BS-30× G.cot.16	G.cot.16× H-1452/10	H-1452 /10× G.cot.16	H-1353 /10 × BS-30	BS-30× H-1353 /10	H-1353 /10× H-1452/10	H-1452 /10× H-1353/10	BS-30× H-1452/10	H-1452 /10× BS-30
G.cot.16	1.00															
H-1353/10	0.74	1.00														
BS-30	0.77	0.91	1.00													
H-1452/10	0.74	0.89	0.93	1.00												
G.cot.16x H-1352/10	0.81	0.91	0.89	0.88	1.00											
H-1353/10 xG.cot.16	0.78	0.89	0.94	0.92	0.95	1.00										
G.cot.16x BS-30	0.80	0.92	0.91	0.89	0.97	0.94	1.00									
BS-30x G.cot.16	0.78	0.89	0.99	0.94	0.91	0.94	0.92	1.00								
G.cot.16x H-1452/10	0.81	0.91	0.89	0.88	0.96	0.93	0.99	0.91	1.00							
H-1452/10 xG.cot.16	0.75	0.88	0.94	0.97	0.89	0.93	0.91	0.95	0.89	1.00						
H-1353/10 xBS-30	0.81	0.80	0.83	0.82	0.85	0.84	0.86	0.84	0.85	0.83	1.00					
BS-30x H-1353/10	0.80	0.85	0.84	0.83	0.86	0.85	0.87	0.85	0.86	0.86	0.95	1.00				
H-1353/10x H-1452/10	0.80	0.83	0.84	0.79	0.84	0.83	0.83	0.83	0.82	0.80	0.91	0.92	1.00			
H-1452/10x H-1353/10	0.78	0.79	0.84	0.81	0.84	0.87	0.81	0.83	0.80	0.82	0.88	0.89	0.96	1.00		
BS-30x H-1452/10	0.78	0.89	0.86	0.85	0.84	0.85	0.85	0.85	0.84	0.82	0.88	0.92	0.94	0.89	1.00	
H-1452/10x BS-30	0.78	0.85	0.84	0.83	0.86	0.87	0.85	0.83	0.84	0.82	0.88	0.92	0.92	0.92	0.92	1.00

consist of hybrids having parent G.Cot.16. Whereas, cluster C encompasses the hybrids of parent BS-30 and H-1452/10. Cluster D consist of genetically diverge hybrids encompassing hybrids of parents H-1353/10, BS-30 and H-1452/10.

The genetic relationships among the 16 cotton hybrids and their respective parents were also revealed by PCA (Figure 6). The results of PCA analysis were in accordance with the clustering pattern of the dendrogram. The first three most informative principal components explained 57.85% of the total variation. The cophenetic correlation values for the dendrogram based on RAPD and SSR data was high (r=0.98).



G. Cot. 16, 2:H-1353/10, 3:BS-30, 4: H-1452/10, 5: G.Cot.16×H-1352/10, 6: H-1353/10×G.Cot.16, 7: G.Cot.16×BS-30, 8: BS-30×G.Cot.16, 9: G.Cot.16×H-1452/10, 10: H-1452/10×G. Cot.16, 11: H-1353/10×BS-30, 12: BS-30×H-1353/10, 13: H-1353/10×H-1452/10, 14: H-1452/10×H-1353/10, 15: BS-30×H-1452/10, 16: H-1452/10×BS-30

Figure 6: Two-dimensional plot of 16 cotton hybrids and their respective parents obtained using principal component analysis of RAPD and SSR

4. Conclusion

Molecular markers such as RAPD and SSR markers were used for the parental identification of different hybrids. Out of 79 markers, seven RAPD markers (RP10, RP20, RP21, RP22, RP25, SRT-6 and SRT-13) and six SSR markers (MGHES-62, TMB-1181, TMB-0409, TMB-1791, JESPR-153 and CM-45) were found to be polymorphic between parents of the hybrids studied. Amongst these primers JESPR-153 is able to identify the true hybrid (Heteroallelic). These reported markers showed specific size of amplicons which helps in the parental conformation.

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