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Hybrid authentication in upland cotton through RAPD analysis

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Abstract

The investigation pertaining to the hybrid identification in *Gossypium hirsutum* L. through Random amplified polymorphic DNA (RAPD) analysis was conducted on 3 genotypes (CIM-511, SLS1 and Paymaster) and their hybrids (SLS1 × CIM-511, Paymaster × CIM-511, Paymaster × SLS1) at University of Agriculture, Faisalabad, Pakistan during 2007. Sixteen RAPD markers developed by Genelink, Company were utilized for this purpose. DNA was extracted from young leaves of the plants and quantified by spectrophotometer and subjected to RAPD analysis. These 16 primers amplified a total of 518 fragments in the parents and hybrids and out which 76 loci were polymorphic. On an average of 7.13 bands per primer were observed with maximum of eight bands and minimum of six. The primer GLG-17 was found to produce 87.5 % polymorphic fragments. The lowest polymorphism (42.85%) was seen in primer GLH-2. Comparison of the RAPD banding pattern of the parents with the respective hybrids clearly identified genuine hybrids. The cluster dendrogram based on similarity matrix obtained by unweighted pair group method using arithmetic average (UPGMA), also revealed the same results. This study suggested that RAPD analysis can be utilized for both reliable and less time consuming identification of hybrids.

***Key words*:** *Gossypium hirsutum*; Hybrid authentication; RAPD analysis

Introduction

Cotton is the leading fibre and food crop of the world. The green revolution was mainly attributed to the development and adoption of high yielding varieties in grain crops. However, a similar revolution in cotton was ushered by the introduction of inter and intraspecific hybrids. The success of hybrid cotton technology depends on the timely production and adequate supply of genetically pure hybrid seeds to the farmers. In order to determine the genetic purity, field Grow out Test (GOT) is conducted. The GOT is an expensive and time consuming procedure delaying planting and leading to the loss of seed viability. 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. RAPD marker system has been proved to be positive over the RFLP as it is simple and requires less time, low cost, small quantity of DNA for the analysis and the ability to generate polymorphisms (Williams *et al.*, 1990). 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). RAPD system of molecular markers has been utilized by many scientists. Nybom and Hall, (1991), Welsh *et al.*, (1991), Iqbal *et al.* (1997), Khan *et al.* (2000), Dighe *et al.* (2001), Rahman *et al.* (2002), Lu and Myres (2004), Mehetre *et al.* (2004a), Mehetre *et al.* (2004b), Dongre and Parkhi, (2005), Rana and Bhat, (2005) Hussain *et al.* (2007), and Sheidail *et al.* (2007) used RAPD marker technology for DNA fingerprinting in cotton and suggested that this technique is reliable for the detection of various cultivars and inter and intraspecific crosses on the base of polymorphic sequences present in their genetic makeup. Ming *et al.* (2004) identified trait variance in the progenies of Gossypium hirsutum transferred from G. barbadense by SSR markers. RAPD analysis has also been utilized for hybrid identification and assessment of genetic diversity in wheat (Awan *et al.*, 2008), rice (Haiyuan *et al.*, 1998), maize (Iva *et al.*, 2005), Leucadendron (Lui *et al.*, 2007), muskmelon (Park and Crosby, 2004) Cyrtandra (Gesneriaceae) (Smith *et al.* 1996) and Theobroma (Wilde *et al.*, 1992).

By the use of molecular techniques, it is now possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related species. The objective of this study was the identification of hybrids of cotton (*Gossypium irsutum* L.) cultivars through RAPD marker system.

Materials and Methods

Plat material and glasshouse experiment

The studies pertaining to the DNA fingerprinting for identification of cotton hybrids using RAPD marker system were carried out at the Department of Plant Breeding and Genetics, and the Centre of Agricultural Biochemistry & Biotechnology (CABB), University of Agriculture, Faisalabad during the years 2004-06. The plant material for this study comprised of three genotypes (CIM-511, SLS1 and Paymaster) and their hybrids (SLS1 × CIM-511, Paymaster × CIM-511, Paymaster × SLS1).

The parents were sown in 12×12 cm earthen pots in the glasshouse in November 2004. Optimum temperature (35 ºC) and photoperiod (about 14 hours) were maintained by heaters and electric lights in the glasshouse. At the time of flowering, crosses were made following all the necessary precautionary measures to avoid any contamination of the genetic material. Maximum pollinations were made in order to produce sufficient quantity hybrid seed. Hybrid seed was planted in pots to get fresh young leaves for DNA extraction.

DNA extraction and quantification

DNA extraction was carried out according to Iqbal *et al.* (1997). Young leaves of the F_1 plants were ground into a very fine powder using liquid nitrogen. Material was transferred to an eppendorf tube and added an equal volume of hot 65 ºC 2XCTAB [2% CTAB (W/V), 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 NaCl and 1% PVP]. The mixture was kept for 30 minutes at 65°C in water bath. Equal volume of chloroform /isomyl alcohol (24:1) was added to the mixture. Emulsion was mixed gently and centrifuged in a micro centrifuge for 10 min. at 13000 rpm.

The supernatant was isolated in a new eppendorf tube and discarded the rest of organic portion. An equal volume of chilled isopropanol was added to precipitate the DNA. Precipitated DNA was centrifuged for 10 minutes at 13000 rpm to make the pellet. The supernatant was discarded bearing the pellet at the bottom of the eppendorf tube. It was washed by 70% ethanol. The solution was centrifuged for 2 minutes at 13000 rpm and ethanol was discarded. The pellet was dried in vacuum dryer. The pellet was rehydrated in double distilled deionized water. RNase was added to the sample (100 µl DNA: 1 µl RNase). The sample was incubated at 37° C for 1 hour. DNA was quantified by spectrophotometer and properly diluted for PCR amplifications.

Table 1. Some primers with their level of polymorphism.

Primers	No. of Polymorphic bands	Percent polymorphism
GLE1	4	57.14
GLE2	3	42.86
GLE3	4	50.00
GLF1	6	85.71
GLF2	5	71.43
GLF3	8	80.00
GLF4	6	75
GLF5	4	57.14
GLG1	6	85.71
GLG17	7	87.52
GLG18	10	83.00
GLH2	3	42.85
GLH3	4	57.14
GLH5	5	71.43
GLH19	3	42.86
GLH20	6	75.00
Total	76	

PCR amplification

Different concentrations of template DNA, Taq polymerase and $MgCl_2$ were used for the optimization of PCR to obtain bright and reproducible RAPD patterns. Different DNA concentrations 5, 7, 10, 15, 18 and 25ng/25µL were studied. The concentration of 25ng/25µL was found to produce the most consistent and reproducible banding patterns. Of the 12 concentrations of MgCl2 studied (0.5, 0.7, 0.9, 1.5, 1.8, 2.0, 2.3, 2.5, 2.8, 3.0, 3.2, 3.5mM), 3mM was found optimum for consistent results. Similarly, among the Taq DNA polymerase concentrations studied (0.2, 0.5, 0.7, 1.0 and 2 unit/25µL reaction), 0.2 unit/25µL concentration of Taq was found optimum for better amplification of genomic DNA. Other reaction conditions were kept constant to obtain consistent and reproducible amplified bands in each replicate.

PCR amplification was performed with 16 random decamer primers (Gene Link Co. USA) (Table 4). Amplification was performed in a 25 µl reaction volume containing 8.3 µl of d H_2O, 2.5 µl of 10x Taq polymerase buffer (Fermentas), 2.5 µl of gelatin, 3.0 µl of MgCl2 (Fermentas), 4.0 µl of d NTPs (dATP, dTTP, dCTP, dGTP) (Fermentas), 2.0 µl of oligonucleotide primer (Gene Link Co. USA), 0.2 µl of Taq polymerase (Fermentas) and 2.5 µl of 25ng of template DNA. Amplification conditions were maintained at 94 ºC for about 2 minutes and thermal cycler was programmed for 45 cycles of 1 min at 94 ºC (denaturation), 35 ºC for 1 min (annealing) and 72 ºC for 2 min (elongation) followed by 94 ºC for 4 min and 72 ºC for 10 min before and after 45 cycles respectively. Amplified products were electrophoresed in 1.5% agarose gel in 1X TBE buffer (100 mM Tris-HCl, pH 8.3; 83 mM boric acid; 1 mM EDTA) at 60 V. The gels were stained with 1 % ethidium bromide solution and visualized under UV light.

RAPD analysis and statistical procedure

The bands were counted from top of the lanes to their bottom. All visible and unambiguously scorable fragments amplified by the primers were scored under the heading of total scorable fragments. Amplification profiles of three lines of cotton were compared with each other and bands of DNA fragments were scored as present (+) or absent (-). The data were used to estimate genetic similarity on the basis of number of shared amplification products (Nei & Li, 1979). The coefficients were calculated by the following statistical equation (Mehetre *et al.*, 2004a; Wilde *et al.*, 1992) $F= 2Nxy/ (Nx + Ny)$ Where, F is the similarity coefficient in which Nx and Ny are the number of fragments in population x and y,

Table 2. Six types of RAPD markers observed in hybrids and their parents

Type Marker	Male (M)	Hybrid (H)	Female (F)	No. of polymorphic bands	Percent (%)
1	+	+	-	65	44.83
2	+	-	+	7	4.83
3	-	+	+	25	17.24
4	+	-	-	15	10.34
5	-	+	-	15	10.34
6	-	-	+	18	12.41
Total				145	

respectively, where Nxy is the number of fragments shared by the two populations. Cluster analysis was based on similarity matrix obtained with unweighted pair group method using arithmetic average (UPGMA), and the relationships between genotypes were displayed as dendrogram.

Results and Discussion

Extent of polymorphism

Gel pictures showing amplifications in the parents and hybrids are shown in Fig. 2. The parents and the F1 plants were carefully observed on the basis of morphology to see if they were true hybrids. In total, 31 primers amplified 518 fragments in the parents and hybrids. Out of which 76 loci were polymorphic (Table 1). On an average of 7.13 bands per primer were observed with maximum of eight bands and minimum of six. The primer GLG17 was found to produce 87.5 % polymorphic fragments and the lowest monomorphic bands i.e. 12.5%. The lowest polymorphism (42.85%) was seen in the primer GLH2. Similarly, the primers GLE2 and GLH2 produced highest monomorphic ones i.e. 57.14%. 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 (Mehetre *et al.*, 2004a). Hussein *et al.* (2002) utilized 49 RAPD primers to investigate the genetic diversity among 13 cotton genotypes and detected a level of polymorphism of 30.4%. Lu and Myers (2002) evaluated DNA variation among ten upland cotton varieties using RAPD data. Out of 86 random decamer primers screened, 63 generated a total of 312 DNA fragments. Forty two bands were polymorphic, which showed a low percentage (13.5%) of DNA variation. Hussein *et al.* (2006) examined 21 cotton accessions utilizing 28 RAPD primers. The total number of amplicons detected was 323, while, the number of polymorphic amplicons was 191. Thus, the level of polymorphism among the 21 accessions was 59.1%. On the other hand, Khan *et al.* (2000) working on 31 *Gossypium* species, recorded a level of polymorphism of 99.8%. Sources of polymorphism in RAPD assay may be due to deletion, addition or substitution of base within the priming site sequence (Williams *et al.*, 1990).

Hybrid Identification

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 (Fig. 2a-e). Two primers, GLF3 and GLF4 identified the F_1

Table 3. Nei's genetic similarity matrix in the parents and hybrids

Population	CIM-511 (1)	SLS1 (2)	Paymaster (P.M) (3)	SLS1 × CIM-511 (4)	Paymaster × CIM-511 (5)	Paymaster × SLS1 (6)
(2)	0.6140					
(3)	0.6140	0.5439				
(4)	0.7719	0.6842	0.5263			
(5)	0.8070	0.5965	0.6667	0.7193		
(6)	0.6667	0.8246	0.6491	0.6842	0.7018	

Table 4. Information regarding primers used for RAPD analysis of cotton genotypes

Oligo Name	Sequence(5'-3')	Size	MW	TM	EC	%GC	nmol/A260	ug/A260
GL DecamerE-01	CCCAAGGTCC	10	2972.97	33.6	99.5	70	10.1	29.9
GL DecamerE-02	GGTGCGGGAA	10	3133.05	33.6	115.9	70	8.6	27
GL DecamerE-03	CCAGATGCAC	10	2996.98	29.5	107.5	60	9.3	27.9
GL DecamerF-01	ACGGATCCTG	10	3028	29.5	104.9	60	9.5	28.9
GL DecamerF-02	GAGGATCCCT	10	3028	29.5	104.9	60	9.5	28.9
GL DecamerF-03	CCTGATCACC	10	2947.96	29.5	96.7	60	10.3	30.5
GL DecamerF-04	GGTGATCAGG	10	3108.04	29.5	113.1	60	8.8	27.5
GL DecamerF-05	CCGAATTCCC	10	2947.96	29.5	96.7	60	10.3	30.5
GL Decamer G-01	CTACGGAGGA	10	3077.02	29.5	115.7	60	8.6	26.6
GL Decamer G-17	ACGACCGACA	10	3005.98	29.5	114.2	60	8.8	26.3
GL Decamer G-18	GGCTCATGTG	10	3059.02	29.5	102.3	60	9.8	29.9
GL Decamer H-02	TCGGACGTGA	10	3068.02	29.5	109	60	9.2	28.1
GL Decamer H-03	AGACGTCCAC	10	2996.98	29.5	107.5	60	9.3	27.9
GL Decamer H-05	AGTCGTCCCC	10	2963.97	33.6	92.8	70	10.8	31.9
GL Decamer H-19	CTGACCAGCC	10	2972.97	33.6	99.5	70	10.1	29.9
GL Decamer H-20	GGGAGACATC	10	3077.02	29.5	115.7	60	8.6	26.6

Product Manual. GL RAPD Decamer Sets. Catalog No. 40-0001-XX. Gene Link. Co. USA. www.genelink.com

hybrids. GLF3 generated a polymorphic marker of approximately 650bp in the male parent CIM-511 and in hybrid but not in the female parent, SLS 1. In addition, a 750bp amplicon was also produced by GLF4, which helped to identify the hybrid. It was found that Paymaster × SLS1 offspring was 82.46 % similar to the male parent (SLS1) and about 65 % similar to the female parent, Paymaster (Table 3). The present study shows that the higher degree of similarity between male parent and offspring compared to female parent and offspring along with male-specific bands of Type 1 markers is an indication that the offspring is a successful cross and true hybrid of Paymaster × SLS1 (Table 2). In contrast, Mehetre *et al.* (2004a) documented highest coefficient of relationship between the interspecific

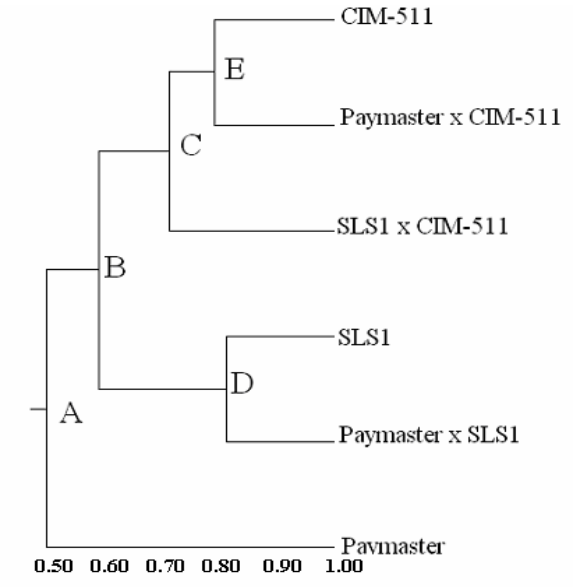

Fig 1. Dendrogram of genotypes along with their hybrids developed from RAPD data using unweighted pair group of arithmetic means (UPGMA). The scale is based on Nei and Li's coefficients of similarity.

hybrid and its parents G. *hirsutum* and *G. raimondii* but the hybrid was more similar to it female parent. Variation in marker from the parents to hybrids may have originated due recombination, deletion, mutation or random segregation of the chromosomes at meiosis during the process of hybrid formation (Williams *et al.* 1990; Huchett and Botha, 1995; Smith *et al.*, 1996; Mehetre *et al.*, 2004a; Mehetre *et al.*, 2004b). However a variety of markers in combination could be used to assess more consistent consequences. Dongre and Parkhi (2005) conducted a research on the identification of cotton hybrid through the combination of PCR based RAPD, ISSR and Microsatellite markers and suggested that using all three markers in combination was faster and more In the conventional breeding it is difficult to identify hybrids during early stages. The success of identification of a true hybrid can be established using morphological basis at maturity (Sheidail *et al.*, 2007).

Similarly, in crossing program under greenhouse conditions space is limited to grow plants. It is necessary to select true hybrids for establishing breeding program but it is difficult before flowering and bolls formation. By the use of RAPD technique, it is easier to identify true hybrids at early stages. This technique can be adopted for large scale screening of hybrids in cotton (Mehetre *et al.*, 2004b; Dongre and Parkhi, 2005). In addition, RAPD and amplified fragment length polymorphic (AFLP) markers have been used successfully to estimate genetic similarity and for cultivar analysis of various Australian cotton cultivars (Multani and Lyon, 1995), local Pakistani cotton cultivars (Iqbal *et al.*, 1997), and their wild relatives (Khan *et al.*, 2000).

Hybrid authentication by genetic similarity coefficients and Cluster analysis

The RAPD markers in the parents and F_1 were classified into six types according to the presence or absence of bands (Table 2). RAPD fingerprinting was utilized to determine the relatedness parent and their hybrids (Mehetre *et al.*, 2004a; Mehetre *et al.*, 2004b; Dongre and Parkhi, 2005). The varieties (CIM-511, SLS1 and Paymaster) and their hybrids (SLS1 × CIM-511, Paymaster × CIM-511 and Paymaster × SLS1) were selected to study the variation at the DNA level. The data were used to estimate genetic similarity on the basis of number of shared amplification products which were denoted by the Nei and Li (1979) coefficients of similarity (Table 3). Nei's coefficients of similarity showed that the genotype Paymaster and the hybrid SLS1 × CIM-511 had the lowest value (0.5263). It means that these two populations were diverse from each other and had very little relationship due to different parentage and different evolutionary areas. This divergence was seemed to be mostly contributed by the second factor because the parents of the hybrid were locally developed cultivars whereas Paymaster was evolved in American region. This suggested that the breeders of different breeding centre provided with divergent gene pool are able to evolve genotypes with significant variability (Rahman *et al.*, 2002).

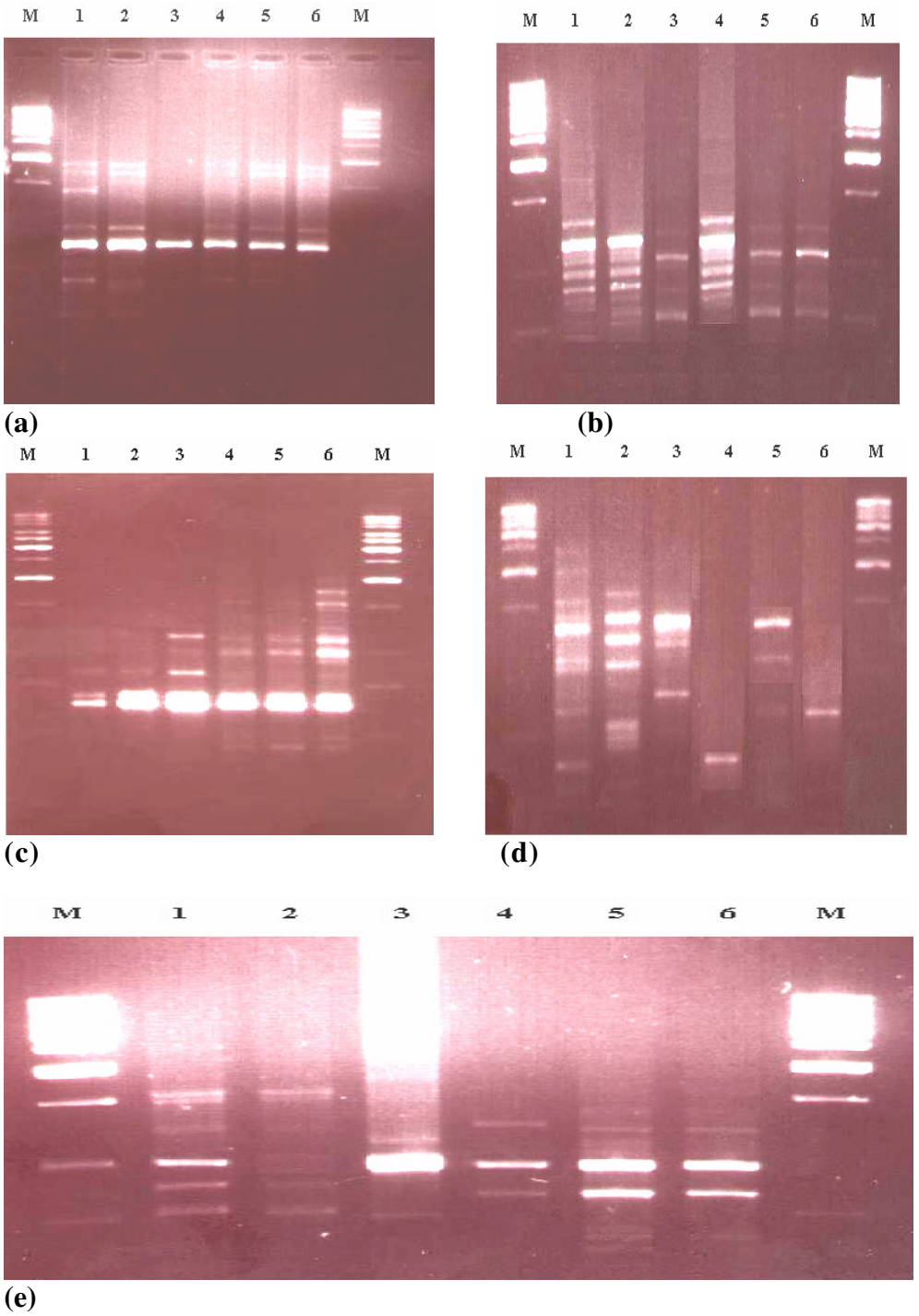

Fig 2. RAPD amplifications of the genotypes, CIM-511 (1), SLS1 (2), Paymaster (3) and their hybrids, SLS1 × CIM-511 (4), Paymaster × CIM-511 (5) and Paymaster × SLS1 (6) by the primers GLG17 (2a), GLF3 (2b), GLH20 (2c), GLG18 (2d) GLF4 (2e).

The genotype SLS1 was 82.46% similar to its hybrid Paymaster × SLS1 which exhibited the highest value of Nei's coefficient.

Cluster dendrogram based on similarity matrix obtained with unweighted pair group method using arithmetic average (UPGMA), and the relationships between genotypes displayed two main clusters namely A and B (Fig. 1). The cluster B was further divided into C and D, whereas C was further divided into E. The cluster A was genetically more diverse to all the other clusters. The cluster B was genetically similar to the clusters C and D. The cultivar CIM-511 and its hybrid, Paymaster × CIM-511 in the cluster E, however, SLS1and its hybrid, Paymaster × SLS1 in the cluster D showed very much similarity to each other. The cluster C the hybrid SLS1 × CIM-511 was found to be deviating from the variety CIM-511 and its hybrid, Paymaster × CIM-511. It indicated that breeding material for the development of these cultivars could be shared between different breeding regions and cotton improvement programs (Rana and Bhat, 2005).

The cluster tree based on similarity coefficients from UPGMA revealed that the variety paymaster belonging to different geographical region was different from all other cultivars and their hybrid contributing some diversity to the genetic material. It The dendrogram suggested that Paymaster × CIM-511 offspring was very much analogous to its parent CIM-511 and also Paymaster × SLS1 hybrid was very much similar to its parent, SLS1. It may be proposed that pointed cross combinations should be made in breeding programs to increase the genetic diversity was low in the population (Fouilloux and Bannerot, 1988). Conical crosses would broaden the genetic window and should aid breeding for high yield and disease resistance by creating better segregating populations (Rahman *et al.*, 2002).

Conclusion

Although the other DNA marker techniques like AFLP and SSR are of good importance for assessment of genetic variability and hybrid identification among cotton cultivars, this research concluded that RAPD banding patterns of the parents compared with their respective hybrids clearly recognized true hybrids. The results also inveterate the effectiveness of the RAPD markers for the detection of polymorphism among cotton genotypes based on estimation of similarity coefficients for the identification of genotypes and hybrids by distinctive fingerprints.

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