

IDENTIFICATION OF RAPD MARKERS FOR HYBRID SEED PURITY TEST IN COTTON

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Abstract

Cotton is an important crop that plays a key role in India's economic and social affairs. The variety characterization with morphological features has several undesirable properties, such as seasonal dependence, large space and time requirements, tedious and influenced by the environment. The main advantages of the RAPD technique include simple and rapid analysis: it does not require production of genomic or cDNA clones, restriction enzyme, Southern digestion or hybridization of DNA with radioactively labeled probes of agarose). Other advantages relate to the use of universal sets of commercially available primers, their minimal requirement for substrate DNA and a negligible number of primers, and their lower cost. Because of all these properties, RAPD is particularly well suited for detecting polymorphic situations in unknown genetic material where no pre-existing set of RFLP clones is available.

Keywords: RAPD, DNA, Tetraploid cotton

Introduction

Cotton is the "king" of fiber culture and the most important Commercial culture in the world. Cotton production in India is progressing throughout the year Three decades ago, it reached a production of over 140 lakh bales per Year that has made India not only self-sufficient, but also a surplus country. This is mainly due to the commercial use of heterosis, especially tetraploid cotton. Cotton plays an important role in the Indian economy. More than 30% of foreign exchange revenue comes from exports of cotton and cotton textiles. Cotton accounts for 80% of the raw materials needed by the Indian textile industry and is therefore widely distributed and traded on domestic and international markets.

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 random amplified polymorphic DNA (RAPD) amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) 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. Hybrid identification in a crop species through DNA fingerprinting is an effective tool for increasing the speed and quality of backcross conversion, thereby reducing the time required to produce plant varieties with desired characteristics (Farooq and Azam, 2002; Murtaza et al., 2005). The RAPD system of molecular markers has been used 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 the RAPD marker technology for DNA fingerprinting of cotton and pointed out that this technique is reliable in the detection of multiple inter- and intraspecific varieties and crosses based on polymorphic sequences in their genetic makeup. , Ming et al. (2004) identified feature variance in progeny of *Gossypium hirsutum*, which was transferred from *G. barbadense* by SSR markers. The RAPD analysis has also been used for the hybrid identification and assessment of genetic diversity of wheat (Awan et al., 2008), rice (Haiyuan et al., 1998) and corn (Iva et al., 2005). *Leucadendron* (Lui et al., 2007), Melon (Park and Crosby, 2004), *Cyrtandra* (Gesneriaceae) (Smith et al., 1996) and *Theobroma* (Wilde et al., 1992). Using molecular techniques, it is now possible to accelerate the transfer of desired genes between varieties and introduce new genes from related species. The aim of this study was to identify cotton species hybrids (*Gossypium irtsutum* L.) by the RAPD marker system.

Material and Methods

The studies pertaining to the DNA fingerprinting for identification of cotton hybrids using RAPD marker system were carried out at CICR, Regional Station, Coimbatore. 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).

DNA extraction and quantification

Extraction of the DNA was performed according to Iqbal et al. (1997). The young leaves of F1 plants were ground to a very fine powder with liquid nitrogen. The material was transferred to an Eppendorf tube and an equal volume of hot 65 °C 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 held at 65 °C for 30 minutes in a water bath. An equal volume of chloroform / iso-methyl alcohol (24: 1) was added to the mixture. The emulsion was mixed gently and

centrifuged in a microfuge for 10 minutes. at 13000 rpm. The cited DNA was centrifuged at 13,000 rpm for 10 minutes to prepare the pellet. The supernatant was discarded with the sediment in the lower part of the Eppendorf tube. It was washed with 70% ethanol. The solution was centrifuged for 2 minutes at 13,000 rpm and the ethanol was discarded. The sediments were dried in a vacuum dryer. The sediments were rehydrated in bidistilled deionized water. The sample was spiked with RNase (100 µl DNA: 1 µl RNase). The sample was incubated for 1 hour at 37 ° C. The DNA was quantified by spectrophotometer and properly diluted for PCR amplifications.

PCR amplification

Different concentrations of template DNA, Taq polymerase and MgCl₂ were used to optimize the CR to obtain bright and reproducible RAPD motifs. Different DNA concentrations of 5, 7, 10, 15, 18 and 25 ng / 25 µl were investigated. It was found that the concentration of 25 ng / 25 µm. l produces the most consistent and reproducible band patterns. Of the 12 investigated MgCl₂ concentrations (0.5, 0.7, 0.9, 1.5, 1.8, 2.0, 2.3, 2.5, 2.8, 3.0, 3, 2. 3.5 mM), 3 mM were found to be optimal for consistent results. Similarly, between the concentrations of Taq DNA polymerase tested (0.2, 0.5, 0.7, 1.0 and 2 units / 25 µl reaction), the concentration was found to be 0.2 units / kg 25 µl of Taq was optimal for better amplification of genomic DNA. The other reaction conditions were kept constant to obtain coherent and reproducible enhanced bands in each replica.

The amplification was carried out in a reaction volume of 25 µm. l, which was 8.3 .mu.m. l d H 2 O, 2.5? l Taq 10 x polymerase buffer (Fermentas), 2.5 µM. l gelatin, 3 µL l contained MgCl₂ (Fermentas), 4.0 µl dNTP (dATP, dTTP dCTP, dGTP) (Fermentase), 2.0 µl oligonucleotide primer (Gene Link Co. USA), 0.2 µl Taq polymerase (Fermentas)) and 2.5? Template DNA Amplification conditions were maintained at 94 ° C for about 2 minutes and the cycler was programmed for 1 minute at 94 ° C (denaturation) and 35 ° C for 1 minute for 45 minutes. Minute (glow) and 72 cycles. For 2 minutes (elongation), followed by 94 ° C for 4 minutes and 72 ° C for 10 minutes before and after 45 cycles. The amplified products were electrophoresed in a 1.5% agarose gel in 1 x TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 60V. The gels were stained with 1% ethidium bromide solution and visualized under ultraviolet 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. The coefficients were calculated by the following statistical equation (Mehetre et al., 2004a; Wilde et al., 1992) $F = \frac{2N_{xy}}{N_x + N_y}$ Where, F is the similarity coefficient in which N_x and N_y are

the number of fragments in population x and y, respectively, where N_{xy} 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.

Result and Discussion

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 were polymorphic (Table 1). On an average of 7.13 bands per primer were observed with maximum of eight bands and minimum of six. It was found that the primer GLG17 produced 87.5% polymorphic fragments and the lowest monomorphic bands at 12.5%. The lowest polymorphism (42.85%) was observed in the GLH2 primer. Similarly, the 57.14% GLE2 and GLH2 primers produced the highest monomorphs. The monomorphic bands are those that are present in both parents and their hybrids. The polymorphs are present in one or more individuals, but not all, and the only ones are present in at least one individual, in no other (Mehetre et al., 2004a). Hussein et al. (2002) used 49 RAPD primers to study the genetic diversity of 13 cotton genotypes and found a polymorphism rate of 30.4%. Lu and Myers (2002) evaluated the DNA variations of ten highland cotton varieties using RAPD data. From 86 Selected random decameric primers 63 generated a total of 312 DNA fragments. 42 bands were polymorphic and showed a small percentage (13.5%) of the DNA variation. Hussein et al. (2006) investigated 21 cotton accessions with 28 RAPD primers. The total number of amplicons detected was 323 while the number of polymeric amplicons was 191. Therefore, the total polymorphism of the 21 patients was 59.1%. On the other hand, Khan et al. (2000) who worked on 31 *Gossypium* species had a polymorphism rate of 99.8%. The polymorphism sources in the RAPD may be due to the elimination, addition or substitution of the base in the sequence of the priming site.

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. Two primers, GLF3 and GLF4 identified the F1 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 \times SLS1 offspring was 82.46% similar to the male parent (SLS1) and about 65% similar to the female parent, Paymaster 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 \times SLS1.

Table 1. 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	12.1

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.

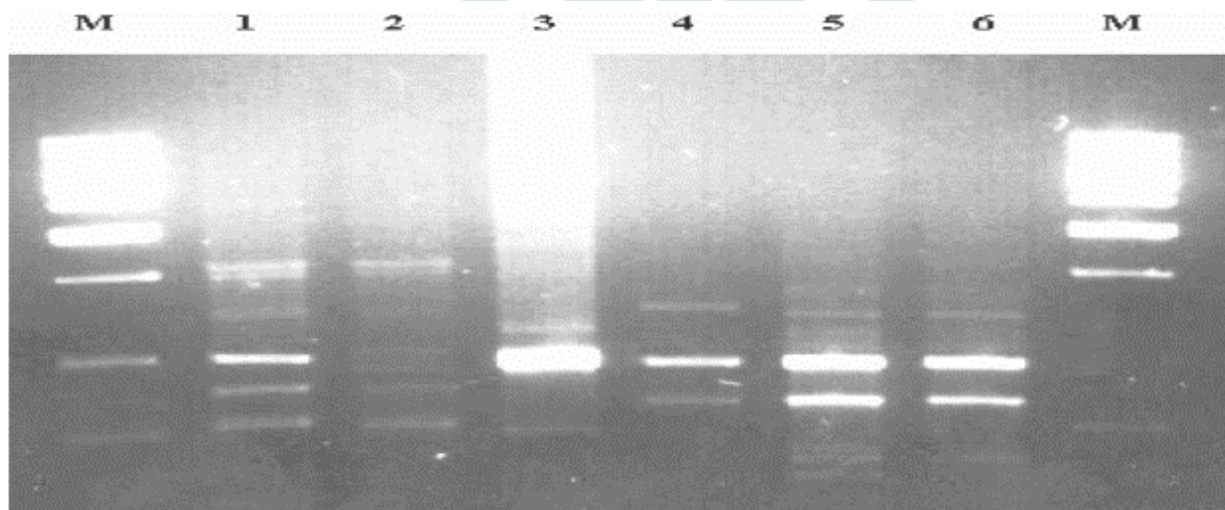


Figure 1. RAPD amplifications of the genotypes

Hybrid authentication by genetic similarity coefficients and Cluster analysis

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. 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).

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

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.

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