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**Short Communication**

# Identification of Cotton Hybrid through the Combination of PCR Based RAPD, ISSR and Microsatellite Markers

**Ashok Dongre* and Vilas Parkhi**
Biotechnology Section, Central Institute for Cotton Research, Post Box No.2, Shankar Nagar P.O., Nagpur 440 010, India

**Hybrid cotton H '6' and its parents G.Cot.10 (male) and G.Cot.100 (female) were studied for identification with three PCR based molecular markers, RAPD, ISSR and microsatellite. Twenty RAPD primers, nineteen ISSR primers and twenty-five JESPR cotton microsatellite loci were used. RAPD primer OPA 11 was found to be useful in differentiating parents and hybrid. Two ISSR primers, IS4 and IS7 showed polymorphism in the parents. IS4 identified a female-specific amplicon of about 500bp and IS7 identified two female-specific amplicons of about 500 and 1200bp in the hybrid H '6'. Microsatellite loci JESPR-2 and JESPR-17 were found to be heteroallelic for parents. JESPR-2 identified one male-specific repeat of about 850bp, while JESPR-17 detected two male-specific repeats of about 800bp and 700bp in the hybrid H '6'. Results indicated that using all three markers - RAPD, ISSR and SSR - in combination is faster and more reliable than using the three in isolation, for the identification of cotton hybrid.**

***Key words***: *Gossypium,* ISSR, RAPD, SSR, hybrid.

The green revolution was based mainly on the use of high yielding varieties and hybrids of crop plants. In cotton (*Gossypium hirsutum* L.) a similar revolution was ushered in following the introduction of inter and intraspecific hybrids. Today, hybrid cotton covers about 45% of the total area under cotton and accounts for about 55% of the India's total production, i.e. 16 million bales in 2002-03. The success of hybrid cotton technology depends on the timely production and adequate supply of genetically pure hybrid seeds to the farmers. The genetic purity of a variety/ hybrid refers to absence of seeds of other genotype than the specific one. In order to determine the genetic purity, field test 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. Therefore, an alternative technique that offers efficient, quick and reliable assessment of genetic purity is urgently needed.

Isozymes, seed storage proteins and RFLP have been used for varietal purity determination (1, 2). PCR based DNA markers such as RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), SSR (Simple Sequence Repeat) have been proved to be advantageous over the RFLP as they require less time, low cost, small quantity of DNA for the analysis, and their dominant and co-dominant nature (3). The hybrid H'6' having good yield potential, short duration and high ginning percentage with better spinning count is grown in Gujarat, Maharashtra and Andhra Pradesh. The objective of present study was to identify molecular markers by using RAPD, ISSR and SSR in combination for F1 hybrid H'6' that could be used in the future to test the purity of commercial samples.

Authenticated breeder seeds of cotton F1 hybrid H '6' and the parents G.Cot.10 (male) and G.Cot.100 (female) were obtained from the plant breeder of Crop Improvement Division, CICR, Nagpur. The seeds were germinated in the pothouse and the authenticity of plants was confirmed phenotypically in flowering stage. Random samples from the seed lots were germinated in blotters and 3-day-old fifteen sprouts of each genotype were used for DNA extraction. A modified procedure of Edward *et al* (4) was used for DNA isolation from imbibed seeds, in which 2% polyvinyl pyrrolidone (PVP) was added in DNA extraction buffer to avoid co-isolation of phenolics and polysaccharides with DNA. 5-10 µg of RNase was added to it. The quality and quantity were estimated by measuring O.D. at 260/280 nm and 260 nm, respectively in UV spectrophotometer. Intactness of genomic DNA was checked on 0.8 % agarose gel.

Twenty random primers of OPA series from Operon Technogies (Almeda CA, USA) were used. Reaction

*Corresponding author. E-mail: abdcicr@rediffmail.com

mixture (25 µl final volume) contained 50 ng genomic DNA, 1x PCR buffer, 200 µmol of each dNTPs (Q-Biogene), 20 ng RAPD primers and 1U *Taq* polymerase (Q-Biogene, USA). An initial denaturing step of 6 min at 94 °C was followed by 36 PCR cycles (denaturing at 94 °C for 45 sec, primer annealing at 36 °C for 1 min and primer extension at 72 °C for 1 min). A final step of 10 min at 72 °C was carried out for polishing the ends of PCR products. Nineteen ISSR scorable primers (selected from 55 ISSR primers that were designed and screened for PCR amplification) were used for this analysis. The PCR reaction mixture (25 µl final volume) contained 50 ng of genomic DNA, 1x PCR buffer, 200 µmol of each dNTPs (Q-Biogene), 15 ng ISSR primers (Bangalore Genei Pvt. Ltd, India) and 1U *Taq* polymerase (Q-Biogene, USA). An initial denaturing step of 5 min at 94 °C was followed by 45 PCR cycles (denaturing at 94 °C for 1 min, primer annealing at 49 °C for 45 sec, and primer extension at 72 °C for 2 min). A final step of 5 min at 72 °C was carried out for polishing the ends of PCR products.

Twenty-five polymorphic microsatellite primers named JESPR [isolated and characterized by Reddy *et al* (5)] selected from Cotton Database (http://algodon.tamu.edu/cottondb.html) were used for PCR amplification. The PCR reaction mixture (25µl final volume) contained 50 ng of genomic DNA, 1x PCR buffer, 200 µmol of each dNTPs (Q-Biogene), 15 ng each reverse and forward primers (custom synthesized from Bangalore Genei Pvt Ltd, India) and 1U *Taq* polymerase (Q-Biogene, USA). An initial denaturing step of 5 min at 94 °C was followed by 40 PCR cycles consisting each of a denaturing step of 15 sec at 94 °C, a primer annealing step of 30 sec at 55 °C and a primer extension step of 2 min at 72 °C. A final step of 30 min at 72 °C was given for polishing the ends of PCR products.

The products of the above reactions were electrophoresed on 1.4% (RAPD), 1.6 % (ISSR) and 2% (SSR) agarose gels containing 0.1 µg $ml^{-1}$ of ethidium bromide for about 5 h at 60 Volts. Gel was photographed under UV light with Tracktel GDS-2 gel documentation.

Genetic purity of cotton F1 hybrid H '6' (*G. hirsutum)* and its parental lines, G.Cot.10 (male) and G.Cot.100 (female) were carried out by using a combination of RAPD, ISSR and SSR markers. Out of 20 RAPD primers assessed only 5 primers, OPA 8, 9, 11, 17 and 19 showed polymorphic amplification pattern between the parents. Further, only one primer, OPA 11, [CAATCGCCGT], showed reproducible polymorphic DNA banding pattern. Only two markers were generated by OPA 11, of which one amplicon of about 700bp was found only in the male parent (Fig. 1 A). All the seeds in bulk of hybrid H'6' from single cross were tested for presence of the male-parent-specific band and a band, of about 700 bp specific to the male parent G.Cot.10 was amplified in the hybrid seeds (Fig.1 A).

Two primers IS4 [5´$(AGC)_5$GC3´] and IS7 [5´$(CA)_7$GT3´] out of 19 primers showed polymorphism in parents. IS4 generated a polymorphic marker of about 500bp in the female parent, G.Cot.100 and Hybrid H '6'. In addition, a 600bp amplicon was also obtained in both the parents and the hybrid. Primer IS7 generated three markers - polymorphic amplicons of about 1200bp and 500bp were amplified in the female parent, G.Cot.100 and hybrid 'H6', while a marker of about 800bp was monomorphic (Fig.1 B).

Out of 25 JESPR microsatellite primers, 18 JESPR pairs were scorable on agarose gel. Only two loci were found heteroallelic, JESPR-2 [F 5´GAGGCAATGTCGGATGTGGGC3´R-5´GCAAGTAGGTGGTGGCCGGAG3´] and JESPR-17 [F-5CCACCCAAATTTTTCATGGAGAG3´ R-5´ CCTTCCTCATGTATGACATTGATGG3´] and were used to identify the hybrid H '6' and its parents. JESPR-2 amplified two repeats of different length, out of which a repeat of about 850bp was G.Cot.10 specific and was also amplified in H '6'. The other repeat of about 350bp was homoallelic for both the parents. JESPR-17 generated three repeats. Two repeats of about 800bp and 700 bp were heteroalleic and detected in male parent G.Cot.10 and in hybrid H '6'. The other repeat of about 350bp was homoallelic for both the parents (Fig.1 C).

Pendse *et al* (6) have used RAPD for identification of cotton hybrids and parental lines. However, the RAPD markers have come under criticism because they lack reproducibility. To overcome this problem, reproducible random primer OPA 11 showed polymorphism among the parents and the hybrid. Instead of relying on RAPD only, present study was augmented with the additional reliable, fast, and cost effective ISSR and microsatellite markers. ISSR marker was found to be potentially useful for studying genetic diversity, introgression analysis and identification of cotton germplasm (7). In the present study, 17 bp ISSR primer IS4 of AGC repeat anchored with GC and 16 bp primer IS7 of CA repeat anchored with GT expressed dominant markers of female parent in hybrid 'H6'. This led to confirmation of hybridity and hence can be used as a

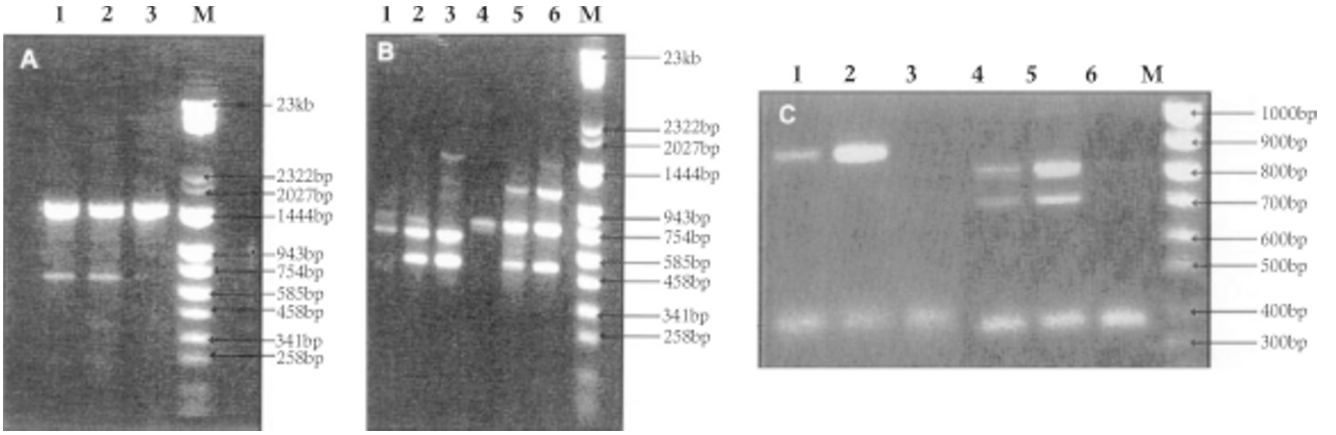


**Fig.1.** Identification of cotton F1 hybrid H '6' (*G. hirsutum)* and its parental lines, G.Cot.10 (male) and G.Cot.100 (female). (**A**) RAPD profile of OPA 11. Lane 1, male; lane 2, hybrid; lane 3, female; lane M, Lambda/ *Hin*d III, pUC 18/ *Sau*3A I - pUC 18/ *Taq* I Digest, (**B**) ISSR profile of IS4 (lane 1, male; lane 2, hybrid; lane 3, female) and IS7 (lane 4, male; lane 5, hybrid; lane 6, female). Lane M, Lambda/ *Hin*d III, pUC 18/ *Sau*3A I - pUC 18/ *Taq* I Digest, and (**C**) Microsatellite profile of JESPR-2 (lane 1, male; lane 2, hybrid; lane 3, female) and JESPR-17 (lane 4, male; lane 5, hybrid; lane 6, female). Lane M, 100 bp DNA Ladder.

discriminating marker for testing of genetic purity of hybrids. It may be noted that ISSR-PCR markers have previously been used as a legal evidence for settling proprietary claim of disputed chilli sample (8). SSR is more reliable for identification and genetic purity testing of citrus (9), and rice (10). In the present study, SSR primer JESPR-2 had shown homoallelic and heteroallelic repeats. Since heteroallelic repeat of character in both loci expresses only in male parent, G.Cot.100 and hybrid, H '6', it strongly supports the hybridity and overrule the possibility of inbred as it was not amplified in the female parent. Thus, SSR can also be used in testing the genetic purity of hybrid and its parents.

In conclusion, present study indicates that RAPD, ISSR and SSR markers individually have their own merits in the identification of parents and their hybrid. However, a combination of all the three PCR based markers can be used for testing the genetic purity of cotton seed which will be more reliable substitute for GOT and a tool for seed certification.

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## References

1 **Tanksley SD & Jones RA,** *Hort Sci*, **16** (1981) 179.

2 **Livneh O, Vardi E, Stram Y, Edelbaum O & Sela H,** *Euphytica*, **62** (1992) 97.

3 **Powel W, Morgante M, Andre C, Hanafey M, Vogel J, Rafalski T & Rafalski A,** *Mol Breeding,* **2** (1996) 225.

4 **Edward K, Johnstone C & Thomson C,** *Nucleic Acids Res*, **19** (1991) 1349.

5 **Reddy O U K., Pepper A E, Abdurakhmonov I, Saha S, Jenkins J N, Brooks T, Bolek Y & El-Zik K M,** *J Cotton Sci,* **5** (2001) 103.

6 **Pendse R, Malhotra S, Pawar SE & Krishna T G,** *Seed Sci Technol,* **29** (2001) 503.

7 **Dongre A, Parkhi V & Gahukar G,** *Indian J Biotech* (2003) (In press).

8 **Kumar L D, Kathirvel M, Rao G V & Nagaraju J,** *Forensic Sci Int,* **116** (2001) 63.

9 **Oliveira A C & Garcia A G,** *Euphytica,* **128** (2002) 397.

10 **Yashitola J & Sonti R V,** *Proc 8th National Rice Biotechnology Network Meeting*, 21-25 Oct. 2001 held at Aurangabad, India (2001) p175.