1546 Molecular Evaluation of elite cotton cultivars using DNA markers

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ABSTRACT

RAPD and ISSR marker systems were used for identification and genetic diversity analysis of elite *G.hirsutum, G. arboreum* and introgressed lines. PCR products were subjected to agarose gel electrophoresis and the banding patterns were compared among 12 elite cotton varieties of diploid , tetraploid and introgressed cotton. Out of 20 random primers tested, 15 primers produced reproducible results yielding 123 markers with 92 (74%) being polymorphic. The random primer OPA 17 generated the maximum number of polymorphic markers with a specific band of 300 base pairs for variety PA-255. Out of 55 ISSR primers tested, 15 were scorable, producing 101 marker bands with 83 being polymorphic. The primer IS-08 generated the greatest number of polymorphic markers. The ISSR markers were found to be more reproducible and polymorphic than RAPD markers. Both ISSR and RAPD techniques were thus found to be efficient methods for detecting DNA polymorphism useful for cultivar identification and molecular evaluation in cotton.

Cotton 'the white gold' is the world's leading natural fiber crop and it is the corner stone of textile industries world wide. The cultivated cottons include *Gossypium arboreum* (L) and *Gossypium herbacium* (L) (Old World species), both diploid species with an AA genome native to southern Asia, Africa and two allotetraploid species *Gossypium barbadense*(L) and *Gossypium hirsutum* (L) (New World species) with AD genome from Central, North and South America. Genetic diversity resulting from interspecific introgression can be evaluated with morphological characteristics, seed proteins, isozymes and DNA markers. To have reliable estimates of genetic relationship, a large number of polymorphic markers are required. This limits the use of morphological characteristics and isozymes, which are few, or lack adequate levels of polymorphism in *Gossypium* spp. Therefore there is a need to study polymorphism at the DNA level which can be indicative of genetic diversity in cotton. DNA markers have proven to be valuable in crop breeding especially in studies of genetic diversity and in cultivar identification. Polymerase chain reaction (PCR) based molecular markers, e.g. ISSR, RAPD, SSR, STS, AFLP etc. are useful for various applications in the plant breeding. Among these, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) are arbitrary markers in which only one primer is used. RAPD markers involve the amplification of random DNA segments using arbitrary sequences of 10-15 base pairs without any prior knowledge of DNA sequence. The ISSR technique involves amplification of a DNA segment present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite strands. This technique uses microsatellites, usually 16 to 25 bp long, as primers in the single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter SSR sequences of different size (Reddy *et al.*, 2002). The primers used can be repeats of di, tri, tetra or penta nucleotides anchored with one or two base sequences at 3'or 5' end (Zietkiewch *et al.*, 1994). Unanchored primers also can be used (Gupta *et al.*, 1994). ISSR are reproducible markers with 92-95 per cent efficiency (Reddy *et al.*, 2002). The present molecular diversity analysis was carried out for identification of elite cotton cultivars, to analyze genetic relationship and genetic diversity of the cultivars.

MATERIALS AND METHODS

The list of elite cotton cultivars used in the present study is as below.

Elite *G. hirsutum* cultivars.

1. PH-93 2. PH-325 3. PH-348 4. NH-452 5. NH-545

Elite *G. arboreum* cultivars.

1. PA-402 2. PA-255 3. PA-405

Elite introgressed cultivars.

1. PAIG- 8/1 2. PAIG-27

Parents of introgressed cultivars.

1. PA-140 (*G. arboreum*) 2. Poornima (*G. hirsutum*)

The seeds of the above 12 cotton cultivars were obtained from the Cotton Research Station, Nanded; Cotton Research Station, Mahboob Baugh Farm; and the Cotton Research Scheme, Marathwada Agricultural University, Parbhani. Total genomic DNA was extracted from 4gm of bulked leaf sample sample by a modified procedure of Edwards (1991) in which 2% PVP (polyvinyl pyrrolidone) was added to the DNA extraction buffer to avoid co-isolation of phenolics and polysaccharidesin the DNA. A total of 20 primers from the OPA series from Operon Technologies, USA were used for this study (OPA-1, OPA-2, OPA-3, OPA-4, OPA-5, OPA-6, OPA-7, OPA-8, OPA-9, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14, OPA-15, OPA-16, OPA-17, OPA-18, OPA-19, OPA-20). A total of 55 ISSR primers from Banglore Genie, India were used. Among them only 15 primers (IS-1, IS-2, IS-3, IS-4, IS-5, IS-6, IS-7, IS-8, IS-9, IS-10, IS-11, IS-12, IS-13, IS-14, IS-16) were scorable on 1.6 per cent agarose gel.

RAPD Amplification.

RAPD amplification reaction was carried out with 25µl reaction volume containing 40ng genomic DNA, 2.5 µl of reaction buffer (10X *Taq* polymerase buffer with 15mM $MgCl_2$), 200 µM of each dNTPs (Q Biogene), and 20 ng of 10 Mer RAPD primers (from Operon Technologies). Amplification was performed in a thermocycler (Biometra UNO – Thermoblock). The reaction profile was preceded by a single 94 ^{o}C soak for 6 minutes, 36 cycles consisting each of a denaturing step of 45 seconds at 94 ^{o}C, a primer annealing step of 1 minute at 36 ^{o}C and a primer extension step of 1 minute at 72 ^{o}C. At the end of 36 cycles, a single 72 ^{o}C extension was applied for 10 minutes for polishing the ends of PCR products. All completed reaction were held at 4 ^{o}C.

ISSR Amplification.

ISSR amplification was performed in 25 µl reaction volume containing 40 ng genomic DNA, 2.5 µl of reaction buffer (10X *Taq* polymerase buffer with 15 mM $MgCl_2$), 200 µM of each dNTPs (Q -Biogene), 15 ng of ISSR primers (synthesized by Bangalore Genie, India), 1.5 U *Taq* polymerase (3U/µl, Bangalore Genie, India). The reaction profile was preceded by a single 94^{o}C soak for 5 minute, 45 cycles consisting each of a denaturing step of 1 minute at

94°C, a primer annealing step of 45 seconds at 49°C and a primer extension step of 2 minutes at 72°C. At the end of 45 cycles, a single 72°C extension was applied for 5 minutes for polishing the ends of PCR products. All completed reactions were held at 4°C.

Amplified products were separated on a 1.6 % agarose gel containing 0.1 µg/µl of ethidium bromide for about 5 hours at 60 Volts. Gels were photographed under UV light with a Tracktel GDS-2 gel documentation system.

Data analysis.

DNA fragment size was estimated by comparing the DNA bands against a *Lambda/ Hind III*, *pUC* 18 /*Sau* 3A-*pUC* 18/ *Taq I* base pair ladder (Bangalore Genie, India). The amplified DNA bands were scored on gel under a UV transilluminator as 1 for the presence and 0 for the absence of bands and assembled in the data matrix table. The pair wise comparisons were calculated using Nei and Li's coefficient (1979). The similarity values found were utilized to group individuals via the unweighted pair group method with arithmetic average (UPGMA). NTSYS-PC (Rolf, 1993) was used to perform all the analysis.

RESULTS

RAPD analysis.

Out of 20 random OPA series primers, of, 15 produced reproducible results (Table 1). These fifteen reproducible primers generated a total 123 markers of which 92 were polymorphic i.e. 74 per cent amplified loci were polymorphic. The average number of polymorphic markers generated per primer was 6.13. The size of RAPD amplicons was between 150 bp in OPA 06 and OPA 12 and 3000 bp in OPA 12, OPA 15, OPA 16 and OPA 17.

Highly polymorphic profiles were obtained with 10 of the total primers used such as OPA 06, OPA 17 and OPA 19. These 10 primers detected 79 polymorphic markers. However, none of the primers individually was so informative as to differentiate all the genotypes. The random primer OPA 17 generated the maximum number of markers i.e. 16 and OPA 03 generated the least number of markers (Table 2). The primer OPA 17 showed a specific band of 3000 bp for the cultivar PA-255 (Plate 1a). The primer OPA 16 generated 7 polymorphic markers and 3 monomorphic markers. OPA 16 also generated a specific band of 700 bp for the cultivar PA-255 (Plate 1b).

ISSR analysis.

Initially 55 ISSR primers were screened, of these 15 primers (IS-1, IS-2, IS-3, IS-4, IS-5, IS-6, IS-7, IS-8, IS-9, IS-10, IS-11, IS-12, IS-13, IS-14, IS-16) were scorable on 1.6 per cent agarose gel were scorable. A total of 101 ISSR markers were amplified, out of which 83 were polymorphic (82.1%). Average number of polymorphic loci amplified per primer was 5.5. The results obtained from ISSR-PCR analysis are presented in Table 3. The size of ISSR amplicons was between 100 bp in IS 09 to 3000 bp in IS 07. The number of polymorphic markers generated by each ISSR primer are given in Table 4. The ISSR primer IS 16 generated the least number of markers (3). The maximum number of polymorphic markers (12)was generated by IS 8 (Plate 2a). Highly polymorphic profiles were obtained with 9 of the primers used viz., IS 4, IS 5, IS 6, IS 7, IS 8, IS 9, IS 11, IS 12 and IS 14. None of the primers individually was so informative as to differentiate all the cultivars. Primers IS 7 and

IS 8 were polymorphic in all the cultivars. IS 7 generated a specific band of 800 bp for the cultivar PH 93 and another of 585 bp for cultivar PH 348 (Plate 2b). IS 9 produced a specific band of 1 kb for cultivar PA 140 (Plate 2c).

Introgression Study

These two introgression cultivars were developed from interspecific crosses between *G. arboreum* (PA-140) and *G. hirsutum* (Poornima). Polyploidy was induced in the diploid cotton species *G. arboreum*(2n=26) by colchicine treatment prior to their being crossed with *G. hirsutum*(2n=52). To recover the maximum genes from *G. arboreum*, the resultant F_1 was then back crossed with 4n *G. arboreum* in the C_4 generation. PAIG 8/1 and PAIG-27 are the selection lines from back crossed F_1 populations (Deshpande and Baig, 2002). Since the maximum number of genes of *G .arboreum* have been recovered in the introgressed cultivars, these showed the highest level of genetic similarity with *G. arboreum;* although a few genes like those for fibre quality, boll size and ginning outturn have been introgressed from *G.hirsutum*.

RAPD analysis.

Two introgressed cultivars PAIG-8/1 and PAIG –27 and their parents Poornima (*G.hirsutum*) and PA-140 (*G.arboreum*) were analyzed for detecting introgression using 20 RAPD primers (Table 5). The number of parent specific bands present in both introgressed cultivars shows that they have the greatest homology with parent PA 140. Primer OPA 12 showed a specific band of 750 bp which is present in both introgressed cultivars and the parent Poornima (Plate 3a). The primer OPA 6 revealed a 1500 bp band in both introgressed cultivars which is specific to the parent Poornima (Plate 3b). The primer OPA 17 revealed a 800bp band in both cultivars which is specific to parent Poornima but absent in parent PA 140 (Plate 3c).

ISSR analysis.

The ISSR analysis revealed that both introgressed cultivars had more specific band homology with parent PA-140 (9), only one specific band for Poornima was generated by the primer IS-14 (Table 6). The primer IS-14 generated a 800bp band in both introgressed cultivars which is specific to parent Poornima (Plate 3d).

These two analyses make it clear that the introgressed cultivars PAIG 8/1 and PAIG-27 are more similar to PA-140 than the *G. hirsutum* parent Poornima.

Cluster Analysis.

RAPD.

Nei and Li's (1979) similarity coefficient between 12 cotton cultivers using RAPD markers ranged from 0.5 to 0.92. *G. arboreum* cultivar PA-255 and the introgressed cultivar PAIG-8/1were highly similar. Similarly, a high degree of similarity was evident between cultivar PA-405 (*G. arboreum*) and PA-402 (*G. arboreum*). Maximum RAPD diversity was evident between the introgressed cultivar PAIG 27 and *G. arboreum* varieties such as PA-405 and PA-402. A dendrogram resulting from cluster analysis based on similarity values of 12 cotton cultivars generated from RAPD data (Fig 1) revealed that the12 cotton cultivars could be separated into 2 major groups (I and II). The first major group consisted of 6 *G. hirsutum* cultivars and the second major group consisted of the 4 *G*. *arboreum* cultivars and

the 2 introgressed lines. Each of these major groups could be further subdivided. In group I, 2 *G. hirsutum* cultivars, NH-545 and PH-93, clustered separately from rest of the *G. hirsutum* cultivars viz., PH-325, PH-348, Poornima and NH 452. In group II, the 4 *G. arboreum* cultivars and the introgressed cultivar PAIG-8/1 clustered separately from the cultivar PAIG-27.

ISSR.

Nei and Li's (1979) similarity coefficient between 12 cotton cultivars using ISSR markers ranged from 0.49 to 0.93. *G.hirsutum* cultivars PH 325, NH 452 and Poornima were highly similar. Moreover, high similarity was found between the two introgressed cultivars PAIG – 8/1 and PAIG-27. Maximum ISSR diversity was evident between PA-402 (*G. arboreum*) and the *G. hirsutum* varieties PH 325, NH 425 and Poornima. A dendogram based upon cluster analysis of similarity coefficients from the ISSR analysis revealed the same two major groups (I and II) as that of RAPD analysis (Figure 2). The only difference was that in group I, 5 *G. hirsutum* cultivars: NH 545, PH 93, PH 325, NH 425 and Poornima clustered separately from the *G. hirsutum* cultivar PH 348. In group II, the 2 introgressed cultivars PAIG 8 /1, PAIG 27 and 3 *G. arboreum* cultivars: PA 405, PA 140 and PA 402 clustered separately from the *G. arboreum* variety PA 255. DISCUSSION

In the present study, 20 RAPD and 55 ISSR markers were used for identification and genetic diversity analysis of 12 elite cotton cultivars. In order to apply molecular techniques for cultivar identification one needs to know the level of polymorphism revealed by the different techniques in the species under study. Due to lack of abundant intra-specific polymorphism in cotton, only seven cultivars were analysed in this study. No unique marker was observed for the rest of the cultivars. This is similar to the results of Chowdhary *et al.* (2002) who were able to identify only 6 chickpea cultivar specific markers out of 19 cultivars studied due to low level of intra-specific polymorphism in chickpea. However, the marker profiles of the 12 cultivars produced by 15 RAPD and 15 ISSR primers were different from each other. This indicated that cotton cultivars that are indistinguishable by cultivar specific markers could still be differentiated by a profile generated from several markers..

On average, RAPD primers revealed more bands than ISSR primers. However, the level of polymorphism was higher using ISSR primers than RAPD primers (Tables 2 and 4). A higher level of polymorphism for ISSR compared to RAPD primers was reported in chickpea (Chowdhari *et al*., 2002) and in citrus (Fung and Roose, 1997). Reproducibility of the ISSR markers was found to be greater than that of RAPD markers. We feel, therefore, that ISSR markers are superior to RAPD markers in cotton in term of cost and speed. Yang *et al.* (1996) in their comparison of DNA marker systems report a lower relative cost and time for ISSR's than RAPDs.

Both RAPD and ISSR markers clustered the *G. hirusutum* and *G. arboreum* cultivars into separate groups. Iqbal *et al.* (1997) also found distinct cluster formation of accessions belonging to *G. hirsutum* and *G .arboreum* groups except for one variety based on RAPD analysis. Although two separate groups are formed but there are differences in the intra-hirsutum and intra-arboreum clusters (Figures 1 and 2). The cultivar PH-348, a *G. hirsutum* genotype is most diverse from the other *G. hirsutum* cultivars based on ISSR markers. But based on RAPD markers PH-348 grouped closely to PH-325 (*G. hirsutum*). Two introgressed cultivars PAIG-8/1 and PAIG-27 also clustered in the *G. arboreum* group.

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Table 1. Result obtained from RAPD analysis of 12 *Gossypium* varieties

RAPD Analysis	Observation
Total number of Primers used	20
Number of reproducible primers	15
Total number of bands amplified from reproducible primers	123
Total number of polymorphic bands identified	92
Average number of polymorphic bands per reproducible primer	6.13
Percentage of polymorphic bands	74
Size range of amplified products	150-3000bp

Table 2. List of RAPD primers used, their sequence and level of polymorphism detected among 12 cotton genotypes studied

Primer	Primer sequences 5′-3′	Markers generated	Polymorphic marker
OPA 01	5′-CAGGCCCTTC-3′	4	3
OPA 02	5′-TGCCGACGTG-3′	3	2
OPA 03	5′-AGTCAGCCAC-3′	1	0
OPA 04	5′-AATCGGGCTG-3′	N R	
OPA 05	5′-AGGGGTCTTG-3′	N R	
OPA 06	5′-GGTCCCTGAC-3′	9	9
OPA 07	5′-GAAACGGGTG-3′	9	8
OPA 08	5′-GTGACGTAGG-3′	6	4
OPA 09	5′-GGGTAACGCC-3′	N S	
OPA 10	5′-GTGATCGCGC-3′	13	9
OPA 11	5′-CAATCGCCGT-3′	11	10
OPA 12	5′-TCGGCGATAG-3′	8	5
OPA 13	5′-CAGCACCCAC-3′	5	5
OPA 14	5′-TCTGTGGCGG-3′	N S	
OPA 15	5′-TTCCGAACCC-3′	10	5
OPA 16	5′-AGCCAGCGAA-3′	10	7
OPA 17	5′-GACCGCTTGT-3′	16	12
OPA 18	5′-AGGTGACCGT-3′	N R	
OPA 19	5′-CAAACGTCGG-3′	12	9
OPA 20	5′-GTTGCGATCC-3′	6	4
Total		123(100%)	92(74%)

NR – Non reproducible, NS – Non scorable

Table 3. Result obtained from ISSR analysis of 12 *Gossypium* varieties

ISSR Analysis	Observation
Total number of Primers used	55
Number of scorable primers	15
Total number of bands amplified from reproducible primers	101
Total number of polymorphic bands identified	83
Average number of polymorphic bands per reproducible primer	5.5
Percentage of polymorphic bands	82.1
Size range of amplified products	100-3000bp

Table 4. List of ISSR primers used, level of polymorphism detected among 12 cotton genotypes studied

Primer Code	Markers generated	Polymorphic marker
IS-1	4	3
IS-2	6	4
IS-3	4	3
IS-4	7	6
IS-5	10	9
IS-6	7	6
IS-7	8	8
IS-8	12	12
IS-9	9	7
IS-11	7	5
IS-12	6	4
IS-13	8	6
IS-14	6	5
IS-15	4	3
IS-16	3	2
	Total=101(100%)	**Total=83(82.1%)**

Table 5. Parent specific bands generated by introgressed lines by RAPD analysis

Primer	Introgressed variety PAIG-8/1	Parent-1 (Poornima)Specific band	Parent-2 (PAI40) specific band	Introgressed variety PAIG-27	Parent-1 (Poornima) Specific band	Parent-2 (PA-140) Specifier band
OPA17	8	2	3	8	2	3
OPA10	5	0	2	4	0	1
OPA06	3	1	1	3	1	1
OPA08	1	0	1	1	0	1
OPA12	7	2	3	7	2	2
OPA15	7	0	1	7	0	1
OPA16	5	0	1	5	0	1
OPA11	6	0	1	4	0	2
TOTAL	42	5	13	39	5	12

Table 6. Parent specific bands generated by introgressed lines by ISSR analysis

Primer	Introgressed variety PAIG-8/1	Parent-1 (Poornima)Specific band	Parent-2 (PAI40) specific band	Introgressed variety PAIG-27	Parent-1 (Poornima) Specific band	Parent-2 (PA-140) Specific band
IS-01	2	0	1	2	0	1
IS-14	3	1	0	3	1	0
IS-07	2	0	2	2	0	2
IS-09	7	0	5	6	0	4
IS-12	3	0	0	2	0	0
IS-15	1	0	0	1	0	0
IS-03	1	0	0	1	0	0
IS-13	2	0	1	3	0	2
Total	21	1	9	20	1	9