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Cotton genetic diversity study by AFLP markers

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Abbreviations: AFLP: amplified fragment length polymorphism
PCR: polymerase chain reaction

Amplified fragmentlength polymorphism (AFLP) markers have been used to ascertain the intensity of inherent diversity and relatedness in cotton (*Gossypium* spp.) plants. The effectiveness of this method to distinguish inter and intra specific difference in cotton could be handy in cultivar recognition and in marker assisted parental selection tool for plant breeders. Twenty cotton cultivars belonging to *Gossypium hirsutum L.*, and *G. arborium L.* from the Pakistan and US origin were used for AFLP based genetic diversity estimates. The objective of this study was to assess the level of genetic variation among some cotton cultivars belonging to the old and new world species of cotton. Four *Eco*RI-*Mse*I primer-pair combinations were used forthe AFLP analysis. The AFLP data assigned the genotypes into groups that corresponded with their origin and lineage relationships and showed a narrow genetic base among these cultivars.

Cotton and cotton products occupy a pivotal position in the world economy. Pakistan is the fifth largest producer of cotton in the world, the third largest exporter of raw cotton, the fourth largest consumer of cotton, and the largest exporter of cotton yarn. It was important to study the genetic diversity of Pakistan's present cotton cultivars and hybrids, which will be used for the development of new cotton genotypes. Previous studies (Wendel, 1989; Brubacker and Wendel, 1994; Multani and Lyon, 1995; Tatineni et al. 1996) revealed that cultivated cotton displays a very low level of genetic diversity. While need for cultivar specific DNA markers in a cotton breeding program for cultivar registration, plant patents,and breeder's right protection and early detection of agronomic and economic traits as an aid to marker assisted selection is still needed (Brubaker and Wendel, 1994).

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Polymerase chain reaction (PCR) technology has promoted the development of a range of molecular assay systems which detect polymorphism at the DNA level and offer an alternative to the hybridization-based method of RFLP. In this study we used the most widely adopted PCR-based marker technologies, AFLPs for characterizing the natural variations of cotton cultivars. The AFLP technique is based on the amplification of short restriction endonuclease digested genomic DNA fragments onto which adaptors have been ligated at both ends. Primers complementary to the adaptors and possessing 30 selective nucleotides of one to four bases are used in a selective amplification reaction. The presence or absence of these selective nucleotides in the genomic fragments being amplified provides the basis for revealing polymorphism. AFLPs tend to generate dominant markers due to the differences in the DNA sequence in the selective 30 nucleotides immediately adjacent to the restriction enzyme site.

AFLPs have been used to estimate genetic relationships in many studies including cotton (Pillay and Myers, 1999) lentil (Sharma et al. 1996), soybean (Maughan et al. 1996), and barley (Becker et al. 1995). The objective of this study was to evaluate the genetic diversity in cotton cultivars and hybrids by the use of AFLP marker technology.

MATERIALS AND METHODS

Plants used in this study were consisted of 19 accessions of *G. hirsutum* and one accession from *G. arborium* (Table 1). Seed material was obtained from the cotton-breeding program at Bahauddin Zakaryia University, Multan and Cotton Research Institute, Faisalabad, Pakistan.

Table 1. Cotton cultivars.

Sr. no.	Cultivar/ Cross/ Parentage	Species	Origin
1	Rohi (D9 x Local Pakistani Exotic)	*Gossypium arborium*	Old world
2	AC134 (148F x 199F)	*Gossypium hirsutum*	New world
3	B557 (268F x L5)	*Gossypium hirsutum*	New world
4	FH87 (AC134 x Paymaster)	*Gossypium hirsutum*	New world
5	S14 (Selection from S12)	*Gossypium hirsutum*	New world
6	SA100	*Gossypium hirsutum*	New world
7	Laokra 5.5 x SA100	*Gossypium hirsutum*	New world
8	SA100 x Laokra 5.5	*Gossypium hirsutum*	New world
9	S14 x SA100	*Gossypium hirsutum*	New world
10	SA100 x S14	*Gossypium hirsutum*	New world
11	SA100 x Stoneville 857	*Gossypium hirsutum*	New world
12	Stoneville 857 x SA100	*Gossypium hirsutum*	New world
13	SA100 x DPL-7340-424	*Gossypium hirsutum*	New world
14	DPL-7340-424 x SA100	*Gossypium hirsutum*	New world
15	Laokra 5.5 x Stoneville 857	*Gossypium hirsutum*	New world
16	Stoneville 857 x Laokra 5.5	*Gossypium hirsutum*	New world
17	S14 x Stoneville 857	*Gossypium hirsutum*	New world
18	Stoneville857 x S14	*Gossypium hirsutum*	New world
19	B557 x AC134	*Gossypium hirsutum*	New world
20	AC134 x B557	*Gossypium hirsutum*	New world

Cultivars and their special characters

Rohi old world cotton belonging to *G. arborium.* AC134 (obsolete local cultivar), B557 (obsolete local cultivar), FH87 (high oil content), S14 (high ginning outturn), SA100 (red leaves), these are belongs to the new world *G. hirsutum L.*

Crosses

Laokra 5.5 x SA100, SA100 x Laokra 5.5, S14 x SA100, SA100 x S14, SA100 x Stoneville 857, Stoneville 857 x SA100, SA100 x DPL-7340-424, DPL-7340-424 x SA100, Laokra 5.5 x Stoneville 857, Stoneville 857 x Laokra 5.5, S14 x Stoneville 857, Stoneville x S14, B557 x AC134, AC134 x B557.

Seeds were germinated in 20 cm plastic pots in the greenhouse and Genomic DNA was extracted from young expanding leaves using the Nucleon Plant DNA isolation and purification kit.

The AFLP procedure (Vos et al. 1995) and as modified by Pillay and Myers (1999) was used to access the genetic diversity. DNA of 250 ng was extracted from all the 20 cultivars. The DNA was digested with *Eco*RIand *Mse*RI at 37ºC for 2 hrs. A small aliquot of the digested DNA was run on a 1.5% (w/v) agarose gel to check if the DNA

digestion was complete. The digested samples were incubated at 70ºC for 15 min to inactivate the restriction endonucleases. *Eco*RI and *Mse*RI adapters were ligated to the digested DNA samples to generate template DNA for amplification. Preamplification was carried out with +1-primers each carrying one selective nucleotide (*Eco*RI + A, *Mse*RI + C) in a thermocycler for 20 cycles set at 94ºC denaturation (30 sec), 56ºC annealing (60 sec), and 72ºC extension (60 sec). The initial denaturation was done at 94ºC for 30 sec and the final extension at 72ºC for 8 min. The amplification products were diluted 20-fold in TE buffer and stored at -20ºC. Selective AFLP amplification was carried out with *Eco*RI + 3 primers and *Mse*RI +3 primers and 5 µL of the diluted PCR products from the preamplification.Four primer pair combinations of *Eco*RI + 3 (E-plus three nucleotides) and *Mse*RI + 3 (M-plus 3 nucleotides) were tested and include: (i) E-AAG/M-CTC, (ii) E-AAG/M-CTG, (iii) E-ACC/M-CAC, and (iv)E-AAG/M-CAA. The PCR amplifications were carried out as follows: one cycle at 94ºC for 30 sec, 65ºC for 30 sec, and 72ºC for 60 sec; followed by 12 cycles of touchdown PCR in which the annealing temperature was decreased by 0.7ºC every cycle until a touchdown annealing temperature of 56ºC was reached. Once reached, another 20 cycles were conducted as described above for preamplification. The reaction product (2 µL) was mixed with an equal volume of formamide loading buffer (98% [v/v] formamide, 10 m*M* EDTA,0.005% [v/v] of each of xylene cyanol and bromophenol blue) denatured by incubating at 90ºC for 5 min and quickly cooled on ice. The products were analyzed on 5% (w/v) denaturing polyacrylamide gels. The gel was run at constant power (50-55 W) until the xylene cyanol was about two-thirds down the length of the gel. The gel was silver stained.

Bands that showed clear polymorphisms were scored visually as present (1) or absent (0). Cluster analysis was performed to provide a statistical basis to establish the number of cluster represented by the 20 genotypes. A clustering procedure (hierarchical cluster analysis) was performed using the unweighted pair group mean with arithmetical averages (UPGMA) method (Sneath and Sokal, 1973) by computer programme of SPSS. The output of this analysis was used to derive a dendrogram, which showed the phylogenetic relationships between all the cultivars.

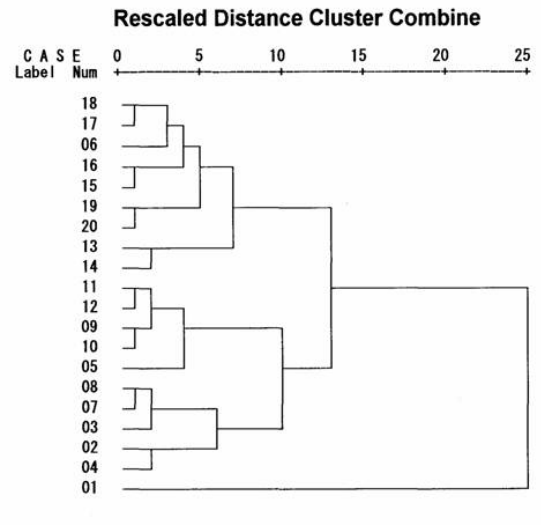

Figure 1. Dendrogram using hierarchical cluster analysis.

RESULTS AND DISCUSSION

Four primer-pair combinations were used to assay 20 cotton plants from each of the 20 accessions for AFLP analysis. An average of 40 to 80 scorable bands was detected after selective PCR amplification with each primer combination. The bands ranged in size from 50 to 500 bp. The results of agglomeration schedule (Table 2) and the dendrogram of genetic relationshipsfrom UPGMA cluster analysis (Figure 1) resulted in five major groups. The *G. hirsutum* genotypes were clustered into four major groups depending upon their origins, while the *G. arboreum* formed one separate group. The AFLP dendrogram assigned the genotypes into groups corresponding with their origin and pedigree relationships.

This study showed that AFLP is a very sensitive technique for detecting markers for genetic studies in cotton. Banding patterns obtained with AFLP were highly reproducible when the same sample DNA is used in independent experiments (Pillay and Myers, 1999). We used silver staining, the silver stained AFLP gels are considered to produce a larger number of better-defined bands than ^{32}P-labeled gels (Cho et al. 1996).

Figure 1 shows that the cross Stoneville 857 x S14 (18) and cross S14 x Stoneville 857 (17) are reciprocal crosses of the parents Laokra 5.5 and SA100 and combined at the first level. Cultivar SA100 (6) joined this group at level three to form one group. The second group consisted of three sisters groups i.e. cross Stoneville 857 x Laokra 5.5 (16) and cross Laokra 5.5 x Stoneville 857 (15) were reciprocal crosses, while cross B557 x AC134 (19) and cross AC134 x B557 (20) were also reciprocal crosses of parents Laokra 5.5 and Stoneville 857, while third sister group consisted of crosses SA100 x DPL-7340-424 (13) and DPL-7340-424 x SA100 (14), which were reciprocal crosses of parents SA100 and DPL-7340-424. These two groups comprised of all the genotypes of US origin; this could explain the formation of this cluster.

The third cluster consisted of both US and Pakistan originated cultivars and crosses. The first subgroup consisted of crosses SA100 x Stoneville 857 (11) and Stoneville 857 x SA100 (12), while the second subgroup consisted of crosses S14 x SA100 (9) and SA100 x S14 (10), while cultivar (5) S14 combined with this cluster at stage 4. S14 cultivar was a selection from cultivar S12 which was a hybrid of Pakistan and US origin varieties (MNH93 x 7203-14-4 Arizona).

Table 2. Agglomeration schedule.

Stages	Cluster Combined		Coefficients	Stage Cluster First Appeared		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	2	18	2.000	0	0	11
2	1	9	2.000	0	0	14
3	8	15	3.000	0	0	12
4	4	17	4.000	0	0	10
5	5	12	4.000	0	0	10
6	6	7	4.000	0	0	7
7	6	20	7.000	6	0	15
8	14	19	7.000	6	0	15
9	3	10	7.000	0	0	16
10	4	5	8.000	4	5	13
11	2	16	9.000	1	0	12
12	2	8	12.167	11	3	14
13	4	11	13.500	10	0	17
14	1	2	16.600	2	12	16
15	6	14	20.833	7	8	17
16	1	3	22.357	14	9	18
17	4	6	33.280	13	15	18
18	1	4	45.400	16	17	19
19	1	13	86.000	18	0	0

The fourth cluster consisted of the crosses SA100 x Laokra 5.5 (8) and Laokra 5.5 x SA100 (7) formed the first subgroup and these were reciprocal crosses of cultivars SA100 and Laokra 5.5, this subgroup was combined with cultivar (3) B557, whose parent was (268F x L5).

The cultivars AC134 (2) and FH87 (4) formed one group and these were the Pakistan local cultivars. The parentage of cultivar FH87 was (AC134 x Paymaster) while that of cultivar AC134 was (148F x 199F).

The fifth group consisted of the only one cultivar Rohi (1) which originated from a cross (D9 x Local exotic) belongs to diploid spp. *G. arborium* and stands alone from the other cultivars of *G. hirsutum* spp.

In the early 1970, high yielding tetraploid cotton varieties of US origin were introduced into Pakistan, and of these, the varieties that were better adapted were released directly for general cultivation. Those that were less adapted were crossed with local breeding lines. The same gene pool was used repeatedly and resulted in a narrow genetic base (Iqbal et al. 1997).

A unique feature of the AFLP data is its ability to discriminate all the taxa used in this study making it a very promising marker system in cotton especially since modern

cotton cultivars are closely related and have a high level of genetic uniformity (Van Esbroeck et al. 1998). AFLP data clearly differentiated the diploid and tetraploid species from each other (Figure 1).

In conclusion this study showed that with the help of AFLP technique one can easily discriminate closely related taxa in cotton and provides sufficient evidence in the shape of numbers of polymorphic markers for variety identification.

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