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MOLECULAR SIMILARITY AMONG THE *GOSSYPIUM* RESTORER LINES USING RANDOMLY AMPLIFIED POLYMORPHIC DNA

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Random amplified polymorphic DNA (RAPD) analysis was used to evaluate the genetic diversity among the identified restorer lines in cotton. Twelve genotypes belonging to *Gossypium hirsutum* L. and one to *G. barbadense* L. were analysed with 23 random decamer primers using the polymerase chain reaction (PCR). All the 23 primers used in the analysis showed polymorphism between the 13 lines. Among the 188 RAPD markers amplified, 5 (2.66%) were unique to *G. barbadense* cultivar Pima. The amplified products varied in number and intensity among the selscted genotypes. Cluster analysis by the unweighted pair group method of arithmetic means (UPGMA) showed that 13 genotypes can be placed in two groups with a similarity ranging from 43.2% to 95.4%. The genotypes ALBAR-51, DPL-15, PK-863 and LL-60 which had the potentiality to be used as the restorer based on the pollen fertility, pollen abundancy and selfed seed set per cent, also showed their similarity at molecular level, by grouping themselves in a cluster having atleast one stable restorer. The results reveal the genetic relationship of the identified partial restorers with the already available stable restorers so that selection of restorers for testing the expression of heterosis when crossed with the CMS line can be undertaken.

Keywords : Cotton; Molecular similarity; Restorer lines; RAPD.

Introduction

The analysis of genetic diversity and relation between genotypes is a pre-requisite for the exploitation of hybrid vigour in any crop improvement programme. Standard breeding procedures utilize the genetic variability present within the available gene pool of crop species to synthesize new varieties or hybrids. Morphological features are indicative of the genotype but are represented by only a few loci because there are not a large enough number of characters available. Moreover, they can also be affected by environmental factors and growth practices. To have an accurate and reliable estimate of genetic relationships and genetic diversity, a large number of polymorphic markers are essentially required. Biochemical markers such as isozymes have been used to distinguish between homozygous and heterrozygous individuals and to estimte the level of genetic variability in plant populations[1]. The genetic distances of a large number of accessions of upland cotton from different locations by isozyme analysis was studied[2]. However, isozyme analysis has certain limitations due to the availability of limited number of marker loci, a general lack of polymorphism for these loci in elite breeding materials, and the chance of variability in banding patterns being due to plant development[3]. The random amplified polymorphic DNA (RAPD) technique[4] provides an unlimited number of markers, which can be used for assessing the extent of genetic diversity of genetic similarities and the cultivar analysis of various plant species including rice[5,6], banana[7,8], *Brassica*[9,10], *Triticum*[11,12] and coffee[13]. The objective of present work was to evaluate the level of genetic similarities between already available stable restorers and newly identified restorers in cotton.

Materials and Methods

Seeds of the cotton genotypes used for analysis are shown in Table 1. These genotypes include ten *Gossypium hirsutum* lines (BAR7/8-1, H-105, 0737-3, ALBAR-51, A 618-GF, DPL-15, PK863, LL 60, B-50 and AC-133) which were identified as partial restorers based on the extent of pollen fertility, pollen abundance and selfed seed set percentage when crossed with the CMS line, and the three already existing stable

restorer lines Mex-685-9, Demeter and Pima.

The genomic DNA was isolated from the selfed seeds of all the above-mentioned thirteen genotypes as per the protocol[14]. The DNA isolation procedure involved crushing about 100 mg seed tissue (imbibed for 16 hrs) in a 1.5 ml eppendorf tube with a spatula or a metal rod in 200 µl of extraction buffer (o.1M Tris-HCI, pH 8.0; 0.05M EDTA, pH 8.0; 0.5M NaCl). To this, 20 µl of 20 per cent sodium dodecyl sulfate (SDS) was added. The tube was maintained at 65°C for 10 min. The tubes were later cooled and 70 µl of 5M potassium acetate was added and vortexed. The tubes were maintained at 4°C for 20 min at 14,000 rpm. About 100 µl of the clear supernatant was taken in a fresh tube. To this tube was added 25 µl of 10 M ammonium acetate and 75 µl of isopropanol. The contents of the tube were mixed and centrifuged at room temperature for 10 min. at 14,000 rpm. The pellet obtained was rinsed with 70 per cent ethanol, air-dried and dissolved in 100 µl of 0.01 M Tris-HCl containing 0.001 M EDTA, pH 7.4. The isolated genomic DNA was quantified using fluorometer (Dyna Quant, USA) and then diluted with Millipore sterile water to get a final DNA concentration of 25 ng per µl sample, which is the normally recommended DNA concentration for RAPD analysis. The isolated genomic DNA was also verified for size, intactness, homogeneity and purity by running 0.8 per cent agarose gel electrophoresis.

A total of 23 random primers used for DNA amplification were obtained from Operon Technologies Inc. (Alameda, CA. USA) as listed in Table-2. Genomic DNA was used as a template for PCR amplification. Amplification reactions were done in 20 µl volumes containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl2, 0.1 mM each of dATP, dCTP, dGTP and dTTP, 0.2 µm primer, 25 ng of genomic DNA and 0.5 unit of Taq DNA polymerase.

Amplification was performed in a PTC 100 thermal cycler (MJ Reasearch, USA) for 45 cycles after an initial denaturation of 94°C for 2 Min. Each cycle consisted of a denaturation (1min at 94°C), an annealing (1 min at 36°C), and an extension (2 min at 72°C) steps. The amplification finished with an extension at 72°C for 10 min. followed by maintenance of the reaction mixture at 4°C until they were removed. PCR products were analysed by electrophoresis on 1.5% agarose gels (in Tris-borate-EDTA electrophoresis buffer). The gels were stained with ethidium bromide and visualized under UV light and photographed using Alphaimager Gel Documentation System.

Amplification profiles of 13 cotton genotypes were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The data for all the 23 primers was used to estimate the similarity on the basis of the number of scored amplification products[15]. A dendrogram based on similarity coefficients was constructed by using the unweighted pair group method of arithmetic mean (UPGMA).

Results and Discussion

Amplification of genomic DNA from each of the 13 restorer lines using all 23 primers revealed a varirty of RAPD patterns, with a total of 188 amplified DNA fragments or RAPD markers. Among these 188 RAPD markers, 159 (84.57%) were polymorphic for the 13 genotypes studied (Table 2). All the 23 primers revealed polymorphism. Among the 188 RAPD markers, 5 (2.66%) were unique to *G. barbadense* cultivar Pima (present in Pima but absent in all other cultivars). The degree of polymorphism detected by different primers varied and thus there was considerable variation in the ability of individual primers to detect DNA polymorphism. Only the reproducible electromorphs were scored and considered for further analysis. Example of the level of polymorphism detected is shown in Fig 1. The number of bands resolved per primer ranged from a minimum of 4 (OPF 17) to a maximum of 13) OPF 7 and OPAH 3). Variations in the intensity of the same band in different restorer lines were noticed.

Table 1. *Gossypium* genotypes used for RAPD analysis

S. No.	Genotype	Species	Origin	Remarks
1	BAR7/8-1	*G. hirsutum*	Egyptian Sudan	Blackarm resistant
2	H-105	*G. hirsutum*	Iran	Extra long staple group
3	0737-3	*G. hirsutum*	TNAU, India	Extra long staple group
4	ALBAR-51	*G. hirsutum*	Tanganyka	Long staple group
5	A 618-GF	*G. hirsutum*	Barbados	Long staple group
6	DPL-15	*G. hirsutum*	Arkansas	Fibre length and strength group
7	PK 863	*G. hirsutum*	Periyakulam, India	High ginning group
8	LL 60	*G. hirsutum*	Punjab, India	High ginning group
9	B-50	*G. hirsutum*	France	High ginning group
10	AC-133	*G. hirsutum*	Pakistan	High ginning group
11	Mex 685-9	*G. hirsutum*	USA	Restorer
12	Demeter	*G. hirsutum*	USA	Restorer
13	Pima	*G. barbadense*	USA	Restorer

Table 2. RAPD products generated using 23 random primers in three known restorers and ten identified / newly identified restorers.

S. No.	Primers		Number of polymorphic bands	Number of monomorphic bands	Total number of RAPD fragments
	Name	Sequence (5' to 3')			
1	OPC 10	TGTCTGGGTG	10	-	10
2	OPC 11	AAAGCTGCGG	4	2	6
3	OPC 12	TGTCATCCCC	8	-	8
4	OPC 15	GACGGATCAG	5	2	7
5	OPC 16	CACACTCCAG	3	3	6
6	OPC 17	TTCCCCCCAG	11	1	12
7	OPC 19	GTTGCCAGCC	6	2	8
8	OPC 20	ACTTCGCCAC	11	1	12
9	OPF 4	GGTGATCAGG	5	2	7
10	OPF 7	CCGATATCCC	10	3	13
11	OPF 8	GGGATATCGG	11	1	12
12	OPF 10	GGAAGCTTGG	6	2	8
13	OPF 11	TTGGTACCCC	6	2	8
14	OPF 13	GGCTGCAGAA	3	2	5
15	OPF 17	AACCCGGGAA	3	1	4
16	OPF 18	TTCCCGGGTT	3	1	4
17	OPF 20	GGTCTAGAGG	5	1	6
18	OPAH 3	GGTTACTGCC	12	1	13
19	OPAH 12	TCCAACGGCT	4	2	6
20	OPAH 15	CTACAGCGAG	10	-	10
21	OPAH 16	CAAGGTGGGT	6	-	6
22	OPAH 17	CAGTGGGGAG	9	-	9
23	OPAH 18	GGGCTAGTCA	8	-	8
		Total	159	29	188

Table 3. Similarity indices between the known and newly identified restorers

	Mex 685-9	Demeter	Pima	BAR 7/8-1	H-105	0737-3	ALBAR-51	A 618-GF	DPL-15	PK-863	LL-60	B-50	AC-133
Mex 685-9	1.000												
Demeter	0.463	1.000											
Pima	0.844	0.432	1.000										
BAR 7/8-1	0.675	0.594	0.735	1.000									
H-105	0.705	0.661	0.686	0.784	1.000								
0737-3	0.605	0.785	0.585	0.738	0.774	1.000							
ALBAR-51	0.540	0.881	0.493	0.635	0.704	0.810	1.000						
A 618-GF	0.480	0.953	0.440	0.604	0.680	0.820	0.901	1.000					
DPL-15	0.658	0.772	0.605	0.708	0.860	0.781	0.779	0.760	1.000				
PK-863	0.485	0.954	0.464	0.597	0.672	0.794	0.861	0.940	0.781	1.000			
LL-60	0.696	0.432	0.763	0.667	0.650	0.559	0.503	0.459	0.602	0.437	1.000		
B-50	0.711	0.476	0.745	0.737	0.713	0.622	0.536	0.484	0.674	0.498	0.750	1.000	
AC-133	0.616	0.581	0.618	0.730	0.751	0.680	0.630	0.591	0.751	0.593	0.657	0.777	1.000

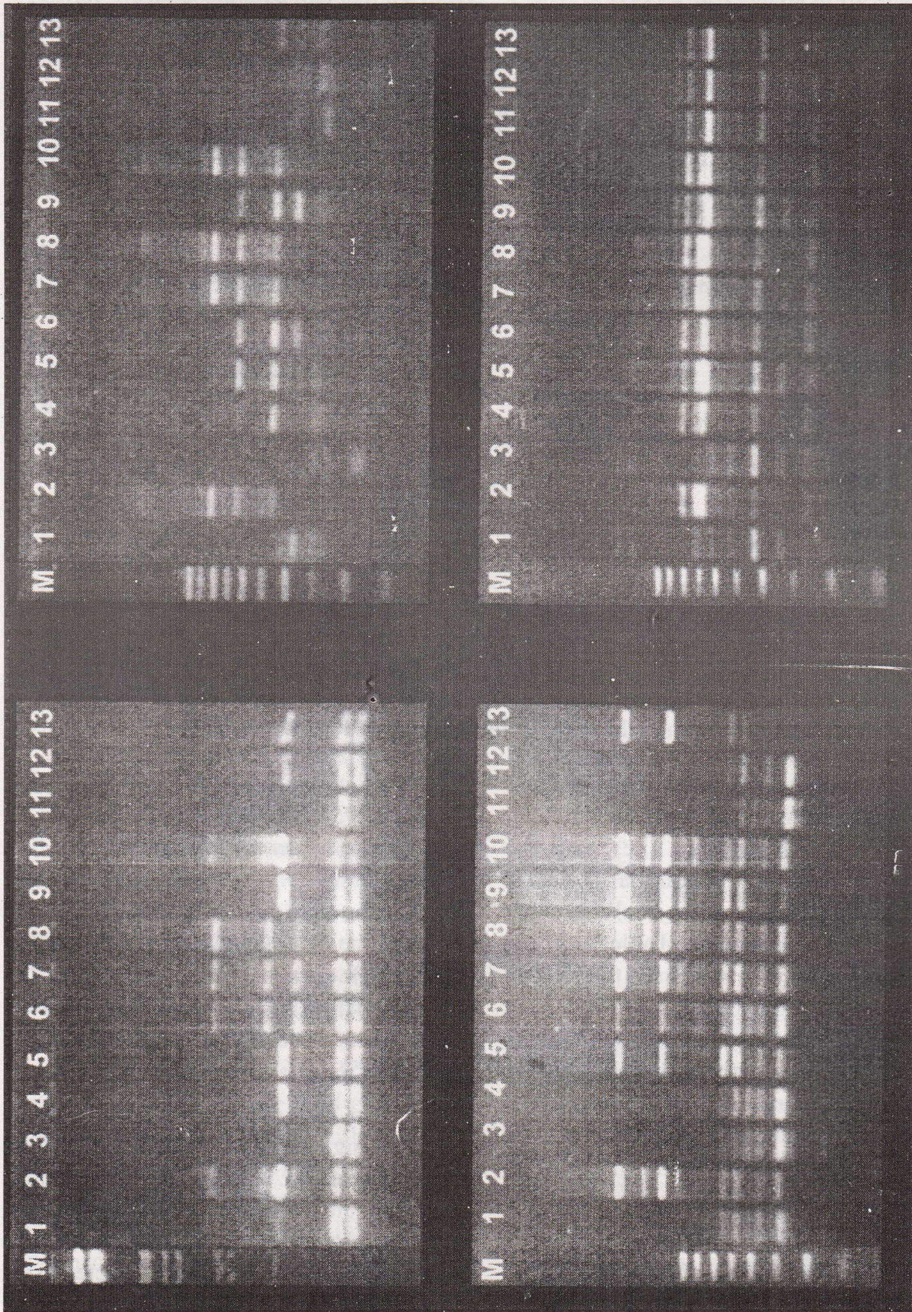

Fig. 1. RAPD polymorphism for OPC 11, OPC 10, OPC 20 & OPF 7.

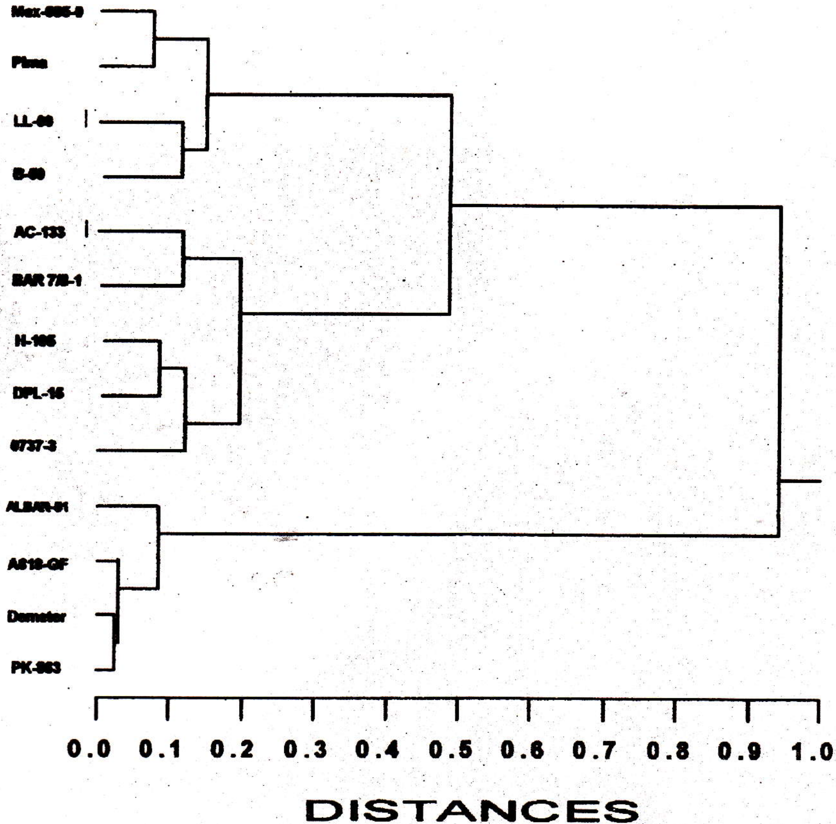

Fig. 2. Cluster dendrogram of 13 restorer lines based on RAPD markers

The main objective of the present study was to estimate the molecular similarity of the identified restorer lines with the known restorer lines. The similarity matrix obtained after multivariate analysis using coefficients[15] is shown in Table 3. These similarity coefficient were used to generate a dendrogram (Fig 2) by UPGMA analysis in order to determine the grouping of different restorer lines. The analysis revealed that the variation in the similarity index ranged from 43.2% to 95.4% between the restorers, the genotypes having the maximum values being similar between them. The genotype Pima belonging to *G. barbadense* L. was grouped along with *G. hirsutum* L. cultivars indicating narrow genetic base. The dendrogram constructed using the similarity index obtained from RAPD clearly grouped the 13 restorers into two major clusters. Nine lines including, two stable restorers and those identified as partial restorers viz., LL-60, B-50, AC-133, BAR 7/8-1, H-105 and 0737-3 were grouped under one major cluster. The first major cluster consisted of two sub-groups, comprising of Mex 685-9, Pima, LL-60 and B-50 in the first and AC-133, BAR 7/8-1, H-105, DPL-15 and 0737-3 in the second. The second major cluster included one known restorer Demeter and three identified restorers ALBAR-51, A 618-GF and PK-863.

The level of polymorphism per prime (6.91) is high and 2.66% of RAPD markers were unique to *G. barbadense* cultivar Pima. Similar inference was reported while studying Pima with other Australian cultivars[16]. The degree of polymorphism was detected using different primers by taking into account the reproducible RAPD markers in order to get reliable conclusions. However, not withstanding reproducibility problems, genetic matrices computed from RAPD bands are sufficiently

reliable in germplasm studies[17]. The number of RAPD markers in this study ranged from 4 to 13. These figures are in agreement with the hypothesis proposed by Waugh and Powell[18], who postulated that in most plants 10-mer primers are able to generate 2 to 10 different amplification products. The variation in the intensity of the same band in different restorer lines may be due to the fact that the specific site chosen by the primer has been found in abundance when compared with other restorer lines. Similarly, Welsh *et al.*[19] reported that the variation in band intensity was due to the abundance of specific sites chosen by the primer in one genotype compared to others. The similarity index between the restorers ranged from 43.2% to 95.4%. Similarity matrix data revealed that the known restorer Demeter and identified restorer DPL-15 are 95.4% similar while Demeter and Pima as well as Demeter and LL-60 are highly distinct (43.2%). A number of Australian Cotton cultivars were studied[16] and reported 92.1 - 98.9% genetic similarity among nine cultivars of *G. hirsutum* L. while *G. barbadense* L. var. Pima showed about 57% similarity with the *G. hirsutum* varieties. Eventhough Pima belongs to *G. barbadense* L., it got grouped into the second cluster with the remaining restorers belonging to *G. hirsutum* L. Pollen fertility, pollen abundancy and selfed seed set percentage of the ten partial restorers revealed the potential of the four lines ALBAR-51, DPL-15, PK-863 and LL-60 to be used as restorers (data not shown). The lines A 618-GF and PK-863 were grouped along with Demeter, the best among the three stable restorers. These lines were identified as the best lines for fertility restoration based on the field experiment. The line H-105, grouped with the other known restorers Mex-685-9 and Pima, also behaves as a good restorer for the *harknessii* based CMS lines. The line DPL-15 was grouped in a separate class which could be confirmed by the presence of distinct bands in different positions when compared to other restorers. These new lines offer scope for developing restorer lines with complete fertility restoration capacity. There is also a possibility that these lines may also restore good fertility in cytoplasms other than *G. harknessii.* Preliminary selection of cotton accessions based on RAPD marker data, may maximize the chance of recovering or identifying superior and stable restorer lines. Combining the results of fertility restoration obtained from field study and the grouping of genotypes based on their molecular similarity, there is much scope for the stable expression of heterosis for economic characters when crossed with the CMS lines. This in turn will result in the identification of the superior restorer lines for *G. harknessii* based CMS lines for commercial exploitation.

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