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Molecular and cytomorphological analysis of interspecific F_1 hybrid of CMS *Gossypium hirsutum* L. and *Gossypium anomalum*, Waw. & Peyr.

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Abstract

Random Amplified Polymorphic DNA (RAPD) was employed to characterize the interspecific hybrid of *G. hirsutum* 76 IH × *G. anomalum* and test its hybrid status. Out of 24 random primers screened, 12 were selected which generated 182 amplification products of which 98 amplicons were polymorphic. Based on the presence or absence of DNA bands in the female parent, offspring and male parent, the molecular markers could be classified into seven Groups. Group I markers shared bands in both parents and offspring, Group II markers shared bands in female and offspring, Group III markers shared bands in both parents, Group IV markers shared bands in male parent and offspring; Group VII markers were present only in male parent. Of these, only Group IV markers (because of the presence of true distinct bands present in male parent) are suitable for identifying the hybrid status of the offspring. In this study, 63.3% of the RAPD markers revealed additivity among parents and offspring *G. hirsutum* 76 IH × *G. anomalum*, 34.7% of parental markers (Group III, V and VII) were absent in the offspring while 2% unique markers (Group VI) were present. The hybrid in the present study showed Group IV markers common with male parents, which clearly shows that DNA based technology such as RAPD could be used with greater speed to detect true interspecific hybrids of *Gossypium*. Further the trueness of the hybrid was also confirmed on the basis of cytomorphological observations. The significance of this hybrid in introgression of desirable genes from the wild species *G. anomalum* to cultivated *G. hirsutum* is discussed.

Key words: Cotton, interspecific hybridization, RAPD

Introduction

This genus *Gossypium* consists of 49 species of which 44 are diploid (2n = 2x = 26) and fall into A, B, C, D, E, F, G and K genomes [1] and the remaining are allotetraploids (2n = 4x = 52 AADD). Only four species of *Gossypium* are presently cultivated - two new world tetraploid species *G. hirsutum* L. and *G. barbadense* L. and two old world diploid species *G. arboretum* L. and *G. herbaceum* L. Problems of interspecific and distant hybridization in cotton were discussed [2]. Cotton is susceptible to biotic and abiotic stresses and genetic variability in the cultivated species for these characters is limited. Wild *Gossypium* species are rich source of resistance genes and interspecific hybridization of cultivated *Gossypium* species followed by subsequent breeding will result in genotypes with improved characters. Wild species are also useful in improving technological properties of fibre and verification with resistance.

Morphological and cytological characters have been used for the identification of cotton hybrids. Although isozyme markers are used to identify the hybrids of cultivars, the paucity of isozyme loci restricts their usefulness in breeding. Molecular marker analysis offers an efficient alternative to this approach as genetic relationships are estimated based on genotype not phenotype. Among the variety of molecular marker techniques available RAPD can be used for DNA fingerprinting of genotypes because of its simplicity, requirement of a small quantity of DNA and the ability to generate polymorphisms [3].

African wild species *G. anomalum*, by itself, is resistant to red mites, leaf roller, bud worm, semilooper and other leaf eating caterpillars; it is almost immune to black-arm or angular leaf spot disease caused by *Bacterium malvacearum* and its narrow bracts is a feature that would ensure clean picking in cultivated cotton. The leaves of *G. anomalum* bear profuse growth of epidermal hairs on their lamina and veins are known to contribute to jassid resistance. Earlier reports indicated that fertile derivatives obtained from *G. anomalum* crosses, seed setting was normal in addition to exceptional good fibre qualities like strength, luster and smooth silky feel [4].

The present investigation was undertaken with a view to introgress desirable characters from *G. anomalum* to *G. hirsutum* L., cotton and to test the

trueness of offspring of the cross CMS *G. hirsutum* 76 IH × *G. anomalum* on the basis of RAPD and cytomorphological studies.

Materials and methods

Plant material: *G. hirsutum* 76 IH (AD genome), *G. anomalum* (B genome) and *G. hirsutum* 76 IH × *G. anomalum* were used in the study. These plants were grown and maintained at Cotton Improvement Project, MPKV, Rahuri and Bhabha Atomic Research Centre, (BARC), Mumbai.

Pollen at anthesis from *G. anomalum* plants were collected and dusted on stigmas of previous day bagged flowers of CMS (A) *G. hirsutum* lines. There was seed set after pollination. Parents and F_1 hybrids were raised and observations on the various morphological characters were recorded (Table 1).

RAPD Analysis

DNA extraction: For DNA extraction, seeds were used in case of parents and young leaves from field grown plants were used in case of offsprings. For isolation of DNA from seeds, procedure of Krishna and Jawali [5] was used. Modified [6] CTAB method [7] using activated charcoal was employed for extraction of DNA from leaves. The DNA was dissolved in TE, treated with RNase, purified by chloroform and isoamyl alcohol (24:1) and precipitated with ethanol and sodium acetate. The DNA was quantified by flourimetry after staining with Hoechst 33258.

DNA amplification: PCR amplification [3] was performed with random decamer primers obtained from Operon Technologies Inc., Alameda, CA, and USA. Amplification was performed in a 25 µl reaction volume and contained 2.5µl 10 × buffer (3Tris hydroxy methyl methylamine propane sulfonic acid, pH 8.8, 1.5 mM $MgCl_2$, 50mM KCl, 0.01% gelatin); 100µM each of dATP, dCTP, dTTP, dGTP; 0.2 µM of primer; 0.3U *Taq* Polymerase (Bangalore Genei Pvt Ltd); 25ng of DNA template and overlaid with paraffin oil. Amplification conditions were maintained at 94°C for 4 min and 45 cycles of 94°C for 1 min (denaturation), 36°C for 1 min (annealing) and 72°C for 2 min (extension) followed by 10 min at 72°C. Amplified products were loaded on 1.5% agarose gel and separated in 1 × TBE buffer (100mM Tris-HCl, pH 8.3. 83mM boric acid, 1mM EDTA) at 60V. The gels were stained with 0.5µg/ml ethidium bromide solution and visualized under UV light.

Data analysis: Amplified RAPD markers were scored as present (+) or absent (–) for each genotype. Ambiguous bands that could not be clearly distinguished were not scored [3]. Genetic similarities of genotypes was calculated as follows: Similarity Index = $\frac{2N_{AB}}{N_A + N_B}$

where
N_{AB} - number of bands shared by individuals A and B
N_A - number of bands in A
N_B - number of bands in B [8,9].

Cytomorphological studies: The various morphological characters were studied in these *G. hirsutum* A line, *G. anomalum* and their F_1 hybrid (Table 4). The length of flower bud of various meiotic stages was determined by studying meiosis in fertile counterpart and haploid. For cytological analysis, young flower buds of all these plants and their respective parents were fixed in Cornoy's fluid (6:3:1) and squashed in 1% aceto-carmine. The analysis of Pollen Mother Cells (PMCs) was made from temporary mounts. Pollen sterility was tested with differential stain. Cytological analysis was made from temporary preparations. Histological studies in the anthers of male sterile lines were undertaken. The cyto-morpho-histological studies were made as per earlier report [10]. Microphotographs were taken on coloured film with the help of Rico 35mm camera mounted on Leica Microscope.

Results and discussion

DNA based polymorphism analysis reveal a pattern of markers that can uniquely identify an individual. RAPD fingerprinting of inbred parental lines and of the resulting hybrids is used for the identification of hybrids plant. An attempt was made to identify the hybrid status of the cross between *G. hirsutum* 76 IH and *G. anomalum* using RAPD. Of the 24 primers (OPB and OPM kits) screened, 12 primers were selected based on distinct banding patterns. The primers produced 182 amplification products with 98 RAPD markers. Of these 98 markers, 79 were polymorphic, 19 were monomorphic (Tables 2 and 3). Monomorphic bands were those present in both parents and offspring, polymorphic were those bands present in at least one, but not all individuals and unique bands were those present in at least one individual and not present in any other. The polymorphisms observed between the parents are used as markers for hybrid analysis. Tracking the inheritance of such markers from parents to the offspring (Fig. 1), one can easily categorize the genuine hybrids from selfed progeny.

The RAPD markers were classified into seven types (Fig. 1) according to the presence or absence of bands (Table 3). 19.4 % of markers were of Group I - common to female and male parents and offspring, while 26.5% were Group II - common to female parent and offspring. 4.1% of markers were common to male and female parents (Group III), 8.2 % were present only in the female parent (Group V) and 22.4% were

Table 1. Biometrical observations on different morphological characters of parents and hybrids

Sr. No.	Characters	Parents/hybrids		
		G. hirsutum	(A) 2n = 4x = 52	*G. anomalum* 2n = 2x = 26
1.	Plant height (cm)	134.6	1756.52	170.00
2.	Branches/Plant (No.) a) Monopodia	1.2	46	3.2
	b) Sympodia	10.2	31	14.2
3.	Length of branch (cm) a) Monopodia	35.2	114.2	82.6
	b) Sympodia	28.6	38.4	46.8
4.	Leaf area (cm^2)	149.36±9.36	69.36±5.65	190.30±6.819
5.	Leaf length (cm)	10.90±0.962	2.94	9.309±0.51
6.	Leaf breadth (cm)	13.10±0.54	5.0	12.30±0.93
7.	Petiole length (cm)	8.60±1.38	2.98	7.50±1.14
8.	Anthers/flower (no)	114.4±6.46	94.00	134.20±12.61
9.	Size of anthers (mm)	0.97±0.13	1.10	0.942±0.048
10.	Petal length (cm)	3.70±0.33	3.20	4.56±0.28
11.	Petal breadth (cm)	3.58±0.08	2.90	4.20±0.35
12.	Petal spot length (cm)	-	1.50	1.84±0.14
13.	Petal spot breadth (cm)	-	1.70	1.26±0.17
14.	Pedicel length (cm)	1.72±0.22	0.40	1.38±0.36
15.	Calyx length (cm)	1.08±0.19	1.10	1.18±0.075
16.	Calyx breadth (cm)	0.55±0.05	0.60	0.46±0.049
17.	Bractiole teeth (no)	10.45±0.68	2.20	5.25±0.83
18.	Bractiole length (cm)	3.78±0.22	1.50	3.48±0.37
19.	Bractiole breadth (cm)	2.92±0.24	0.50	1.84±0.33
20.	Stomatal size length (µ)	117.32±14.84	101.33	108.26±10.43
21.	Stomatal breadth (µ)	65.06±4.04	71.99	73.33±3.99
22.	Pollen size diameter (µ)		109.33	111.99±7.29

Table 2. Primers selected, their sequence and level of polymorphism detected

Primer	Sequence (5'-3')	Amplified fragments	Total no. of bands	No. of monomorphic bands	No. of polymorphic bands	Percent polymorphism
OPM-05	GGGAACGTGT	9	7	0	7	100.0
OPM-06	CTGGGCAACT	7	4	1	3	75.0
OPM-07	CCGTGACTCA	11	5	1	4	80.0
OPM-09	GTCTTGCGGA	21	11	2	9	81.8
OPM-11	GTCCACTGTG	13	7	1	6	85.7
OPM-13	GGTGGTCAAG	15	9	1	8	88.9
OPM-14	AGGGTCGTTC	21	11	4	7	63.6
OPM-15	GACCTACCAC	17	8	4	4	50.0
OPM-16	GTAACCAGCC	28	14	3	11	78.6
OPM-17	TCAGTCCGGG	9	5	0	5	100.0
OPM-18	CACCATCCGT	15	9	0	9	100.0
OPM-20	AGGTCTTGGG	16	8	2	6	75.0
Total		182	98	19	79	

found only in the male parent (Group VII). 17.3 % of Group IV markers were shared by both the male parent and offspring. These Group IV markers due to the presence of distinct bands present in male parent reveal the true hybrid status of the offspring as it rules out the possibility of self-pollination and fertilization. 2% of markers (Group VI) of the offspring were unique i.e. not observed in either male or female parent and may have originated due to inherent incompatibility between the tetraploid and diploid parents leading to elimination

Table 3. The seven groups of RAPD markers identified from hybrid combinations of *Gossypium*

Group of markers	Property of markers			*G. hirsutum* 76 IH × *G. anomalum*	
	Female	Offspring	Male	(no.)	(%)
I	+	+	+	19	19.4
II	+	+	-	26	26.5
III	+	-	+	4	4.1
IV	-	+	+	17	17.3
V	+	-	-	8	8.2
VI	-	-	+	2	2.0
VII	-	-	+	22	22.4
Total	+	+	+	98	

Table 4. Similarity matrix of the hybrid combination *G. hirsutum* 76 IH × *G. anomalum* and parents

	G. hirsutum 76 IH	*G. hirsutum* 76 IH× *G. anomalum*	*G. anomalum*
G. hirsutum 76 IH	1.0000		
G. hirsutum 76 IH × *G. anomalum*	0.74380	1.0000	
G. anomalum	0.38656	0.57143	1.0000

and rearrangement of chromosomes in the hybrid resulting in rearrangement of DNA sequence. The present observation of the presence of novel bands in the offspring *G. hirsutum* 76 IH × *G. anomalum* is in conformity with that of *Chrysanthemum* hybrids [11].

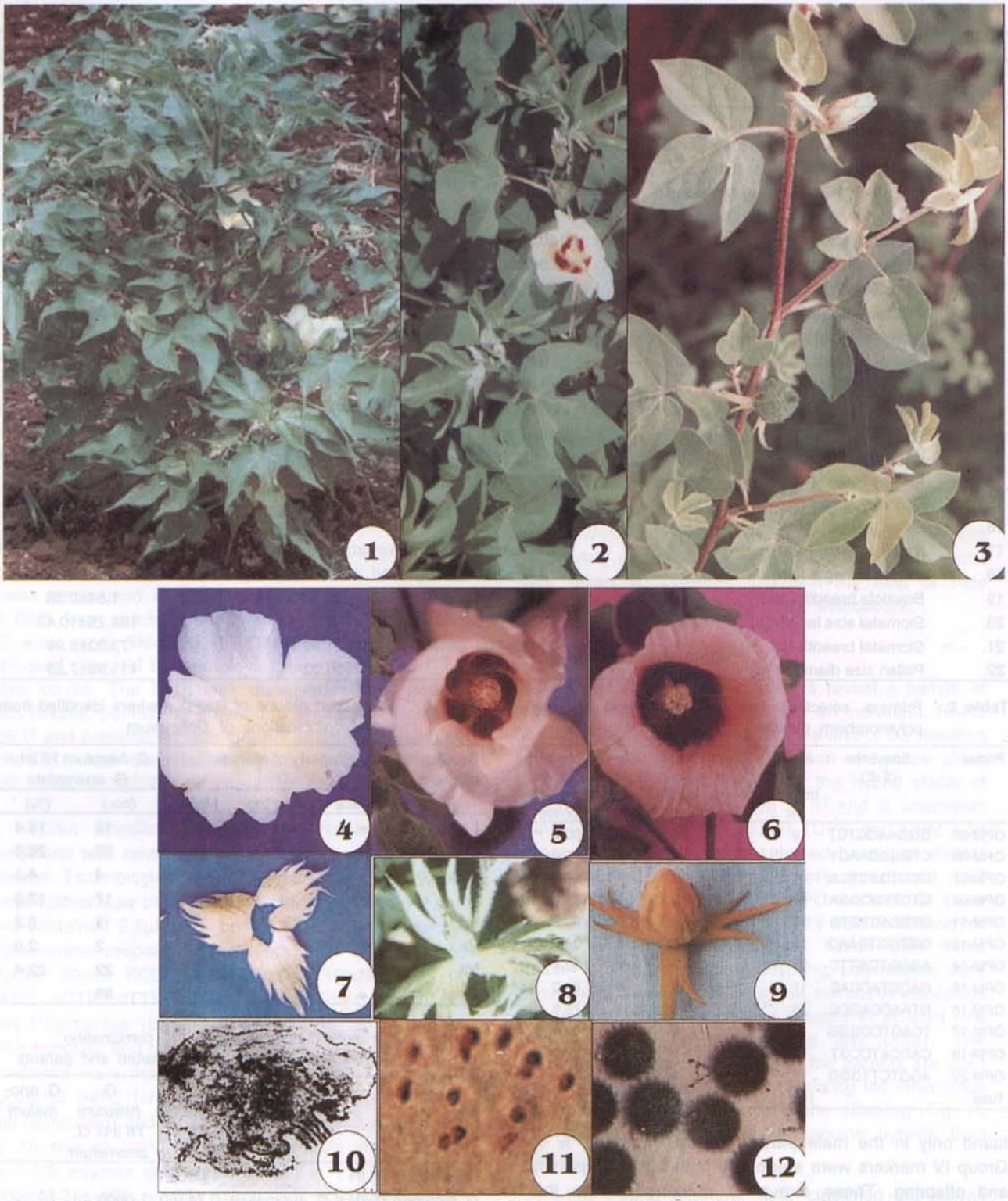

Fig. 1. RAPD molecular marker patterns of hybrid *G. hirsutum* 76 IH x *G. anomalum*. 1-4. Adult plants of: 1. *G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertile B line 76 IH; 2. F_1 hybrid (*G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertility B line 76-IH-20) x *G. anomalum* (2n = 26: B_1, B_1); 3. *G. anomalum* (2n = 26: B_1B_1); 4. F_1 hybrid (*G. hirsutum* L. (2n = 4x = 52; A_hd_h) CMS (A line 76 IH); 5-8. Expression of flower colour petal spot: 5. *G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertile B line 76 IH; 6. F_1 hybrid - (*G. hirsutum* L. (2n = 4x = 52; A_hd_h) CMS A line 76 IH); 7. F_1 hybrid (*G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertile B line 76 IH) x *G. anomalum*; 8. *G. anomalum* (2n = 26: B_1, B_1); 9. Expression of leaf shape in adult plants in flowering stage: (a) F_1 hybrid (*G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertile B line 76 IH) x *G. anomalum*; (b) *G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertile line 76 IH; (c) *G. anomalum* (2n = 26 B_1B_1); 10. Adults of petal colour anther colour and epicalyx in : (a) F_1 hybrid (*G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertile B line 76 IH) x *G. anomalum*; (b) *G. hirsutum* L. (2n = 4x = 52; A_hd_h) normal fertility be line 76 IH; (c) *G. anomalum* (2n = 26: B_1, B_1)

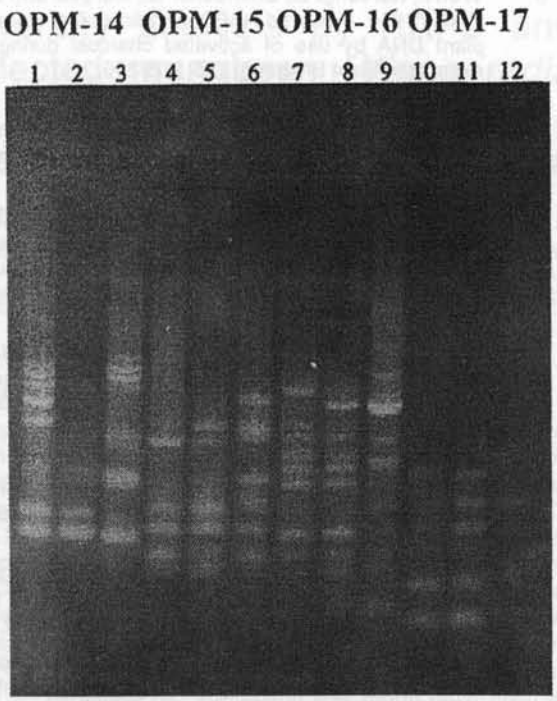

Fig. 1. RAPD molecular marker patterns of hybrid GMrsutum 76 IH × *G. anomalum*: (1-4) Adult plants of 1. *G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertile B line 76-IH-20; 2. F_1 hybrid (*G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertility B line 76-IH-20) × *G. anomalum* (2n=26: B_1, B1); 3. *G. anamalum* (2n=26: B_1B_1); 4. F_1 hybrid (*G. hirsutum* L. (2n=4x=52; A_hd_h) CMS (A line 76-IH-20), (5-8) Expression of flower colour petal spot 5. *G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertile B line 76-IH-20; 6. F_1 hybrid - (*G. hirsutum*, L. (2n=4x=52; A_hd_h) CMS A line 76-IH-20); 7. F_1 hybrid (*G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertile B line 76-IH-20) × *G. anomalum*; 8. *G. anomalum* (2n=26: B_1,B_1); 9. Expression of leaf shape in adult plants in flowering stage (a) F_1 hybrid (*G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertile B line 76-IH-20) × *G. anomalum* (b) *G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertile line 76-IH-20 (c) *G. anomalum* (2n=26 B_1B_1); 10. Adults of petal colour anther colour and epicalyx in (a) F_1 hybrid (*G. hirsutum* L. (2n=4x=52 A_hd_h) normal fertile B line 76-IH-20) × *G anomalum* (b) *G. hirsutum* L. (2n=4x=52; A_hd_h) normal fertility be line 76-IH-20 (c) *G. anomalum* (2n=26: B_1,B_1)

Differences in markers from parents to offspring may have originated due to recombination, mutation or random segregation of chromosomes at meiosis during the formation of hybrid [12]. Markers present in the parents but not in the offspring may have resulted due to the loss of priming sites as a result of chromosomal crossing over during meiosis [13].

Similarity can be used to measure the relatedness between parents, and between parents and offspring [14]. Furthermore, by offering a molecular tool to verify the degree of dissimilarity between the parental lines, RAPD analysis may also be used to search for new parental lines. From the similarity matrix (Table 4) it is found that the offspring *G. hirsutum* 76 IH × *G. anomalum*, is 57.1% similar to the male parent, *G. anomalum* and 74.4% similar to the female parent, *G. hirsutum* 76 IH. The higher degree of similarity between the female parent and offspring, as compared to the male parent and offspring is an indication that the hybrid resembles the female parent *G. hirsutum* 76 IH.

Morphological characters: Growth habit, branching pattern (Figs. 1-3) were intermediate. Leaf hairiness, leaf shape of *G. anomalum* (Fig. 3) was found dominant in F_1 hybrid (Fig. 2) over *G. hirsutum* leaf shape (Fig. 1). Further petal spot of *G. anomalum* (Fig. 6) was found dominant in F_1 (Fig. 5) while flower colour of *G. anomalum* (Fig. 6) was found incompletely dominant (Fig. 5) over flower colour of female lines (Fig. 4) of *G. hirsutum* cotton. As per our earlier report [15] red spot and leaf shape (LA) of *G. anomalum* is dominant, which is confirmed, in present studies. The bract shape of *G. anomalum* (Fig. 9) is incompletely dominant in F_1 (Fig. 8) over *G. hirsutum* (Fig. 7).

Cytological observations: The anther smear studies in various stages of bud development of male sterile line and F_1 indicated lack of PMC formation and unique feature of degeneration of PMCs prior to meiosis, hence, chromosome behavior during meiosis could not be studied. Coalescence of sporogenous tissue occurred during pre-meiotic stages. Disorganization of tapetum

was found due to coalescence of PMCs resulting in complete male sterility (Fig. 10) and (Table 4 Fig. 12) in *G. anomalum* and normal chromosome behavior during meiosis (Fig. 11) was observed.

As the B genome is partly homologous with both the A and D genomes, exchange of genes can occur between all these genomes in the hexaploid (*hirsutum* × *anomalum*) by backcrossing such a hexaploid to *hirsutum*, [16] obtained alien (chromosome) substitution and addition races. These races are useful for transferring *anomalum* genes to *hirsutum* because they enable the breeder to avoid most of the economically undesirable wild characters except those located in the transferred chromosomes.

The present study clearly indicated that *G. hirsutum* 76 IH × *G. anomalum* is a true hybrid due to the presence of distinct bands present in male parent. This RAPD analysis is found to be useful for establishing hybrid status of the offspring.

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