© Indian Society for Cotton Improvement

RESEARCH ARTICLE

Interspecific Hybridization between *Gossypium hirsutum* and *G. armourianum* : Morphological and Molecular Characterization of Hybrids

S. MANICKAM[1,*] AND A. H. PRAKASH[2]
Central Institute for Cotton Research, Regional Station, Coimbatore-641 003 (India)

Abstract

The interspecific hybrids *G. hirsutum* cv. LRA 5166 × *G. armourianum* (Hybrid 1) and *G. hirsutum* cv. Sumangala × *G. armourianum* (Hybrid 2) were genotyped using Random Amplified Polymorphic DNA (RAPD) markers to confirm hybridity. For hybrid 1, 11 of 30 random primers were informative and 15 of 34 primers were informative for hybrid 2. Based on the presence or absence of DNA bands in the female parent, triploid hybrid and male parent, the molecular markers could be classified into seven Types (I- VII). Results indicated that 48.2% and 55.5% of the RAPD markers had additivity among parents and off-spring in hybrid 1 and hybrid 2, respectively. However, 41.1% and 33.6% of parental markers (Type III, V and VII) were absent in hybrid 1 and hybrid 2, respectively. For both hybrids, 11% of the markers were unique (Type VI). The hybrids having Type IV markers (male specific) clearly showed that DNA based technology such as RAPD can be used to distinguish between true inter-specific hybrids of *Gossypium* and parental selfs. The morphological and cytological characters augmented with RAPD analysis indicated clear distinction between the parents and the resultant hybrids.

Keywords : Cytomorphological studies, Interspecific hybrid, RAPD markers
[Paper Received on : 26/03/2014 and Accepted on: 07/06/2014]

*For correspondence : E-mail : manickam.cicr@gmail.com

Introduction

Cotton is susceptible to biotic and abiotic stresses and genetic variability in the cultivated species for resistance against these parameters is limited. Wild *Gossypium* species are a rich source of resistance genes and interspecific hybridization of cultivated species followed by subsequent breeding will result in genotypes with improved characters (Khadi *et al*., 2002).

The success of a cross, *i.e.* identification of a true hybrid, can be established using morphological, cytological, isozyme and molecular markers. Morphological and cytological characters have often been used for the identification of cotton hybrids. However, these characters may not be significantly distinct and such assessments require laborious experiments. Although isozyme markers are used to identify the hybrids of cultivars (Roxas *et al*., 1993), the paucity of isozyme loci restricts their usefulness in breeding (Helentjaris *et al*., 1986). Molecular marker analysis offers an efficient alternative to this approach as genetic relationships are estimated on the basis of genotype and not on phenotype.

In the present study, crosses were effected between *G. hirsutum* and *G. armourianum* to transfer the characters for insect resistance due to D2 smoothness trait and the caducous bract trait that could reduce trash content of harvested seed cotton to *G. hirsutum.* The F_1 hybrids and the parents were analysed to discriminate true hybrids from accidental selfs using Random Amplified Polymorphic DNA (RAPD) analysis in conjunction with morphological and cytological characterization.

Materials and Methods

Two cultivars of *G. hirsutum* L. (AADD genome) *viz*., LRA 5166 and Sumangala were used as the pistil parents and crossed with pollen of the wild diploid species *G. armourianum* (D genome) following hand emasculation and pollination at the Central Institute for Cotton Research, Regional station, Coimbatore. The resultant hybrids along with *G. hirsutum* parents were raised in the field while the wild diploid species is maintained at the station as a perennial. Observations were taken on five random plants each of *G. hirsutum* parents and the triploid hybrids and the mean of five plants was used for comparison. Data on morphological characters *viz*., plant height (cm), number of nodes/ plant, number of monopodia/ plant, number of sympodia/ plant, internode length (cm), number of leaves, leaf length (cm), leaf breadth (cm), petiole length (cm), stem girth (cm) and number of squares were recorded at 60, 70, 85, and 100 days after sowing (DAS). For cytological study of hybrids, the number of chromosomes was counted in

triploids in metaphase I of pollen mother cells. The cells were pre-fixed in Cornoy's fluid (6:3:1) and stained with 1% aceto carmine (Mehetre *et al*., 1980).

Pollen grain size of the triploid hybrids and their parents were measured using an ocular micrometer after staining with 1% acetocarmine. The maximum diameter of 100 pollen grains was measured in five plants each of the hybrid and parents and the mean data presented.

DNA extraction and RAPD analysis

The DNA was extracted from cotton seeds (Krishna and Jawali, 1997) and leaves (CTAB method of Paterson *et al.,* (1993) modified by Vroh *et al.,* 1996). The DNA was dissolved in Tris EDTA, treated with RNase, purified by chloroform and isoamyl alcohol (24:1) and precipitated with ethanol and sodium acetate and re-suspended in TE. The DNA was quantified by flourimetry after staining with Hoechst 33258. PCR amplification (Williams *et al.,* 1990) was performed with random decamer primers obtained from Operon Technologies Inc., Alameda, CA, USA. Amplification was performed in a 25µl reaction volume and contained 25ng of DNA template; 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) and overlaid with mineral oil. Amplification conditions were maintained at 94°C for 4 min. and 45 cycles of 1min at 94°C for 1 min (denaturation), 36°C for 1min (annealing) and 72°C for 2min (elongation) followed by 10min at 72°C. Amplified products were loaded on 1.5% agarose gel and separated in 1X TBE buffer (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA) at 60V. The gels were stained with 0.5µg/ml ethidium bromide solution and visualized under UV light.

Amplified RAPD markers were scored as present (+) or absent (-) for each sample. Ambiguous bands were not scored. The similarity of samples was calculated as follows: similarity $=2N_{AB} / N_A + N_{B,}$ where N_A and N_B are the numbers of bands in individuals A and B respectively (Chapco *et al.,* 1992; Wilde *et al.,* 1992) and N_{AB} is the number of bands shared by individuals A and B.

Results and Discussion

Interspecific hybridization generally aims at transferring from related species or wild species one or more desirable characters not available in the cultivated species. However, several incompatibility barriers prevent easy transfer of genes across the species. It is essential to establish the hybridity of the cross through morphological, cytological and molecular characterization of the resultant progeny. Hence, the triploid hybrids (2n=3x=39) obtained by crossing cultivated tetraploid *G. hirsutum* with wild diploid *G. armourianum* were characterized both phenotypically and genotypically.

The hybrids were distinguishable morphologically from their respective female parents even at early stage of crop growth itself. Data on various morphological characters studied (Table 1) at different days after sowing clearly indicated differences between tetraploid female parent and the triploid hybrid plant. The hybrids were taller with more number of nodes/plant, monopodia/ plant, sympodia/ plant, internode length, number of leaves, stem girth

Table 1 : Mean morphological characters recorded in maternal parents and hybrids at different days after sowing

Character	Days after sowing	LRA 5166	Sumangala	LRA 5166 × *G. armourianum*	Sumangala × *G. armourianum*
Plant height (cm)	60	47.5	47.7	60.7	44.5
	70	50.7	64.4	100.5	81.6
	85	76.2	74.8	118.7	95.4
	100	86.5	89.6	103.0	111.7
	Mean	**65.2**	**69.1**	**95.7**	**83.3**
No. of nodes/ plant	60	13.2	14.5	15.4	16.6
	70	19.7	18.9	23.7	22.0
	85	26.6	29.7	27.8	28.6
	100	31.2	32.4	28.6	33.8
	Mean	**22.7**	**23.9**	**23.9**	**25.3**
No. of Monopodia	60	0.0	0.5	1.0	1.5
	70	0.0	2.0	3.0	4.5
	85	0.0	2.0	3.0	4.5
	100	0.0	2.0	3.0	4.5
	Mean	**0.0**	**1.6**	**2.5**	**3.8**
No. of Sympodia	60	12.5	12.5	14.0	12.0
	70	17.5	16.5	16.0	14.0
	85	21.5	22.5	18.9	20.0
	100	24.5	26.4	19.0	24.0
	Mean	**19.0**	**19.5**	**17.0**	**17.5**
Internode length (cm)	60	3.5	4.4	4.3	3.0
	70	4.6	4.8	4.9	3.8
	85	4.9	5.1	5.5	4.3
	100	5.4	5.6	5.8	5.1
	Mean	**4.6**	**5.0**	**5.1**	**4.1**
No. of leaves	60	31.0	37.5	56.0	50.0
	70	61.0	66.5	175.0	125.0
	85	74.2	77.6	178.0	135.0
	100	82.6	91.5	278.5	145.0
	Mean	**62.2**	**68.3**	**171.9**	**113.8**
Leaf length (cm)	60	9.4	8.9	8.4	7.5
	70	10.4	11	8.6	7.7
	85	10.8	11.2	8.9	8
	100	11.2	11.5	9.5	8.5
	Mean	**10.5**	**10.7**	**8.9**	**7.9**
Leaf breadth (cm)	60	10.0	11.6	7.9	9.7
	70	10.8	14.5	9.3	10.2
	85	11.4	14.5	9.5	10.3
	100	12.8	14.2	10.8	10.5
	Mean	**11.3**	**13.7**	**9.4**	**10.2**
Stem girth (cm)	60	2.4	2.1	2.0	1.8
	70	2.9	3.7	4.9	4.0
	85	4.2	4.8	6.1	4.5
	100	4.6	5.2	7.3	5.8
	Mean	**3.5**	**4.0**	**5.1**	**4.0**
No. of squares	60	17.5	17.5	17.5	16.0
	70	19.0	21.5	24.5	28.0
	85	26.5	29.5	27.0	34.5
	100	31.0	34.5	73.0	49.5

and number of squares as compared to their tetraploid parent. But the triploids had a smaller leaf area and petiole length than their female parent.

Generally, the leaf morphology of the hybrids was intermediate between the two parents (Plate 1a). Similarly, the floral characteristics were also intermediate in hybrids. The female parents are characterized by prominent bract, cream petal without any petal spot and light yellow pollen, whereas, the male parents have rudimentary bract, dark yellow petal with deep purple petal spot and dark yellow pollen grains. The triploid flowers were characterized by light yellow petal with small petal spot and yellow pollen grains. The bracts were intermediate in size (Plate 1b). Similar observations were recorded in various interspecific hybrids (Patel, 1932; Deshpande *et al*., 1991; Meshram and Tayyab, 1991; Mehetre *et al*., 2003).

Table 2 : Primers selected, their sequence and level of polymorphism detected in hybrid 1 (*G. hirsutum* cv. LRA.5166 × *G. armourianum*)

Primer	Sequence (5'-3')	Amplified fragments	Total no. of bands	No. of polymorphic bands	Percent Polymorphism
OPB-12	CCTTGACGCA	18	8	4	50.0
OPM-05	GGGAACGTGT	10	5	3	60.0
OPM-06	CTGGGCAACT	11	5	2	40.0
OPM-08	TCTGTTCCCC	12	5	3	60.0
OPM-13	GGTGGTCAAG	6	4	4	100.0
OPM-14	AGGGTCGTTC	4	2	1	50.0
OPM-15	GACCTACCAC	11	7	7	100.0
OPM-17	TCAGTCCGGG	10	6	6	100.0
OPM-18	CACCATCCGT	7	6	6	100.0
OPM-19	CCTTCAGGCA	6	3	2	66.7
OPM-20	AGGTCTTGGG	9	5	3	60.0
Total		**104**	**56**	**41**	

Table 3 : Primers selected, their sequence and level of polymorphism detected in hybrid 2 (***G. hirsutum*** cv. Sumangala × ***G. armourianum***)

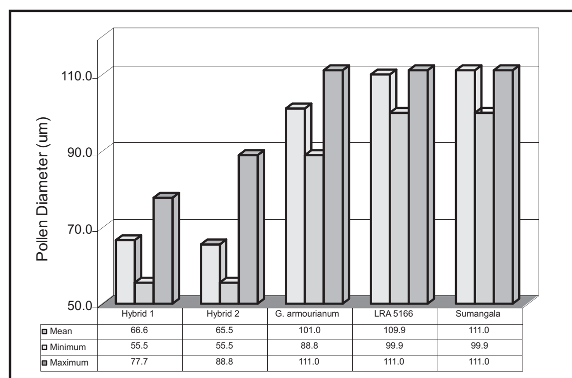
Primer	Sequence (5'-3')	Amplified fragments	Total no. of bands	No. of polymorphic bands	Percent Polymorphism
OPB-01	GTTTCGCTCC	11	6	5	83.3
OPB-02	TGATCCCTGG	10	5	3	60.0
OPB-03	CATCCCCCTG	14	8	7	87.5
OPB-05	TCGGCCCTTC	15	8	5	62.5
OPB-10	CTGCTGGGAC	24	13	12	92.3
OPB-12	CCTTGACGCA	9	5	4	80.0
OPB-18	CCACAGCAGT	24	13	10	76.9
OPB-20	GGACCCTTAC	28	15	10	66.7
OPM-11	GTCCACTGTG	12	6	4	66.7
OPM-14	AGGGTCGTTC	9	6	5	83.3
OPM-15	GACCTACCAC	15	8	7	87.5
OPM-17	TCAGTCCGGG	15	9	8	88.9
OPM-20	AGGTCTTGGG	16	9	7	77.8
OPX-04	CCGCTACCGA	8	4	2	50.0
OPX-10	CCCTAGACTG	6	4	3	75.0
Total		**216**	**119**	**92**	

Meiotic study of chromosomes also confirmed hybridity, wherein 39 chromosomes were counted at metaphase I, whereas, the *G. hirsutum* and *G. armourianum* parents had 52 and 26 chromosomes, respectively. Meshram and Tayyab (1994) while studying the interspecific hybrid between *G. hirsutum* and *G. australe* also observed 39 chromosomes in the hybrids.

It was found that the triploids had the lowest mean diameter of 66.2 µm for pollen grain size as compared to 101.0 µm recorded in *G. armourianum* and 110.5 µm observed in *G. hirsutum* (Plate 2). Wide variability was observed in the pollen grains size of triploids (55.5 µm to 88.8 µm) compared to their parents having more uniform pollen grains. Differential staining (Alexander, 1969) suggested that most of the pollen grains of triploid hybrids were sterile (more than 95%). Stephens (1947) opined that *Gossypium* inter-specific hybrids will exhibit a wide range of fertility from fully fertile to completely sterile, depending upon the parental species.

Random Amplified Polymorphic DNA genotyping was also employed to characterize the interspecific hybrids, *G. hirsutum* cv. LRA 5166 × *G. armourianum* (Hybrid 1) and *G. hirsutum* cv. Sumangala × *G. armourianum* (Hybrid 2). A total of 11 decamer

Fig. 1: Mean and range of pollen diameter (µm) of parents and triploid hybrids

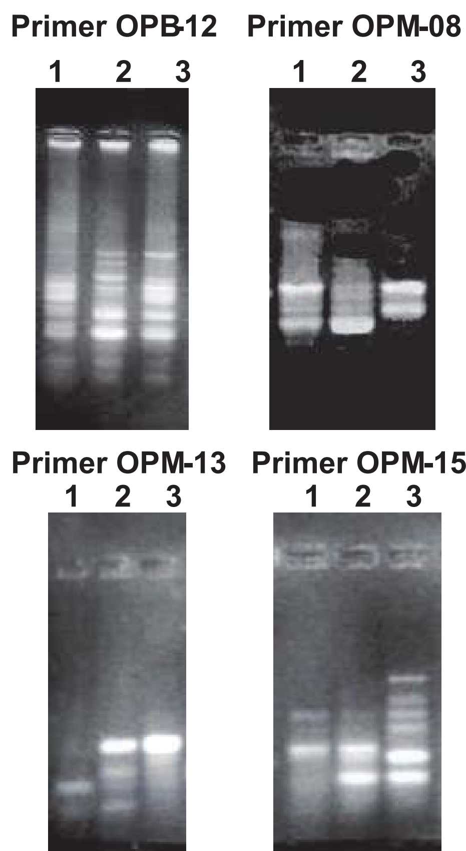


Hybrid 1 = *G. hirsutum* cv. LRA 5166 × *G. armourianum*
Hybrid 2 = *G. hirsutum* cv. Sumangala × *G. armourianum*

Table 4: The seven types of RAPD markers identified from hybrid 1 and 2.

Type of markers	Property of markers			Hybrid 1		Hybrid 2	
	Female	Hybrid	Male	(no.)	(%)	(no.)	(%)
I	+	+	+	15	26.8	27	22.7
II	+	+	+	06	10.7	24	20.2
III	+	–	+	04	7.4	04	3.4
IV	–	+	+	06	10.7	15	12.6
V	+	–	–	05	8.9	13	10.9
VI	–	+	–	06	10.7	13	10.9
VII	–	–	+	14	25.0	23	19.3
Total				**56**		**119**	

Fig. 2: RAPD profiles of G. hirsutum cv. LRA 5166 × *G. armourianum* with primers OPB-12, OPM-08,OPM-13 and OPM-15



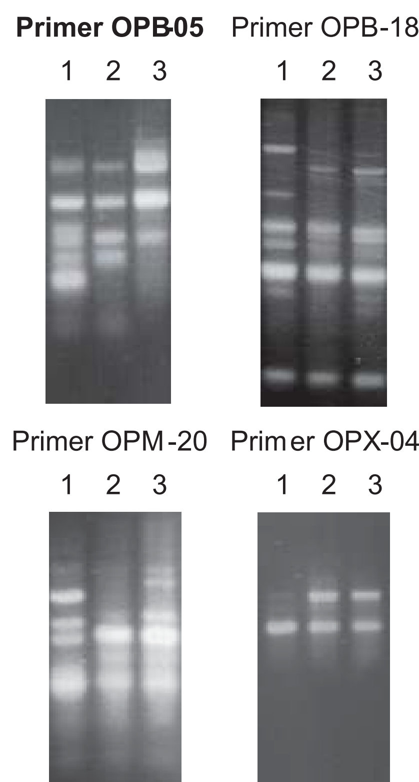
Lane 1 = *G. hirsutum* cv. LRA 5166
Lane 2 = *G. hirsutum* cv. LRA 5166 × *G. armourianum*
Lane 3 = *G. armourianum*

Table 5 : Similarity matrix of the hybrids and parents

Genotype	*G. hirsutum* cv. LRA 5166	*G. armo-urianum*	Hybrid 1	*G. hirsutum* cv. Sumangala	*G. armo-urianum*	Hybrid 2
***G .hirsutum* cv. LRA 5166**	1.000					
G. armourianum	0.550	1.000				
Hybrid 1	0.666	0.583	1.000			
***G. hirsutum* cv. Sumangala**				1.000		
G. armourianum				0.453	1.000	
Hybrid 2				0.694	0.568	1.000

primers were selected from 30 screened primers for hybrid 1 (Table 2) and 15 of 34 primers were selected for hybrid 2 (Table 3). Selection of these primers was done on the basis of multibanded and easily scorable amplification products. From the 11 selected primers in the hybrid 1, 56 clearly interpretable and reproducible

Fig. 3: RAPD profiles of *G. hirsutum* cv Sumangala × *G. armourianum* with primers OPB-05 OPB-18,OPM-20 and OPX-04



Lane 1 = *G. armourianum*
Lane 2 = *G. hirsutum* cv.Sumangala × *G. armourianum*
Lane 3 = *G. hirsutum* cv.Sumangala

RAPD markers were surveyed with 5.1 bands per primer. The 15 selected primers in the hybrid 2 revealed 119 markers with 7.9 bands per primer. The number of bands produced ranged from 2 (OPM-14) to 8 (OPB-12) for hybrid 1 and 4 (OPX-04, OPX-10) to 15 (OPB-20) in hybrid 2.

In hybrid 1, 73.2% of the bands were polymorphic whereas in hybrid 2, 77.3% of the bands were polymorphic. Sources of polymorphism in RAPD assay may include base change within the priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification, deletions or insertions that change the size of DNA fragment which act preventing its amplification (Williams *et al*., 1990).

The RAPD markers were classified into seven types according to the presence or absence of bands (Table 4, Fig. 2 and Fig. 3). Type I markers shared bands in both parents and hybrid, Type II markers shared bands in female and hybrid, Type III markers shared bands in both parents, Type IV markers shared bands in male parent and hybrid; Type V markers were present only in female parent, Type

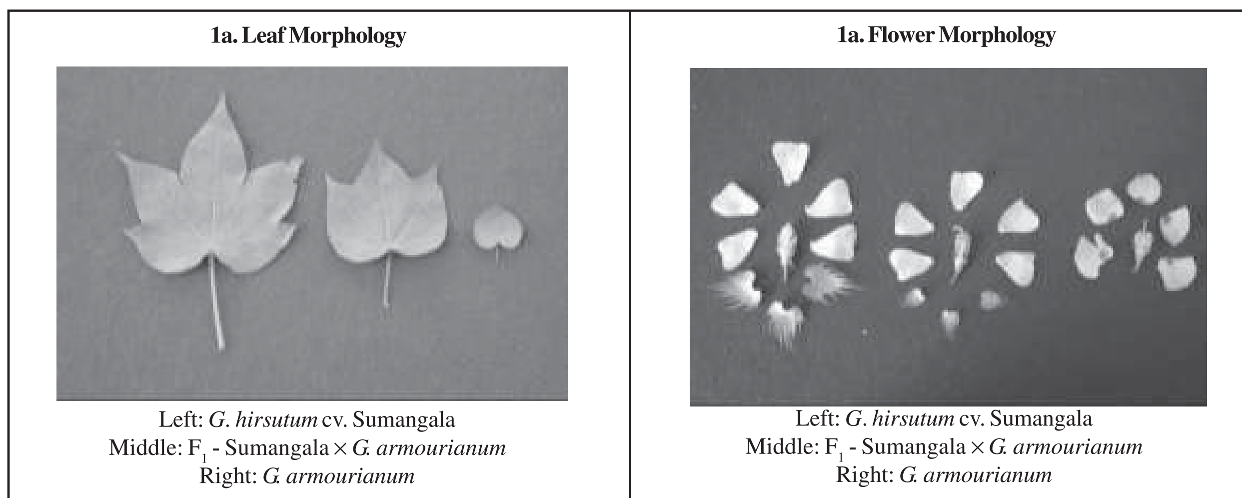


Plate 1: Leaf and flower morphology of parents and hybrid

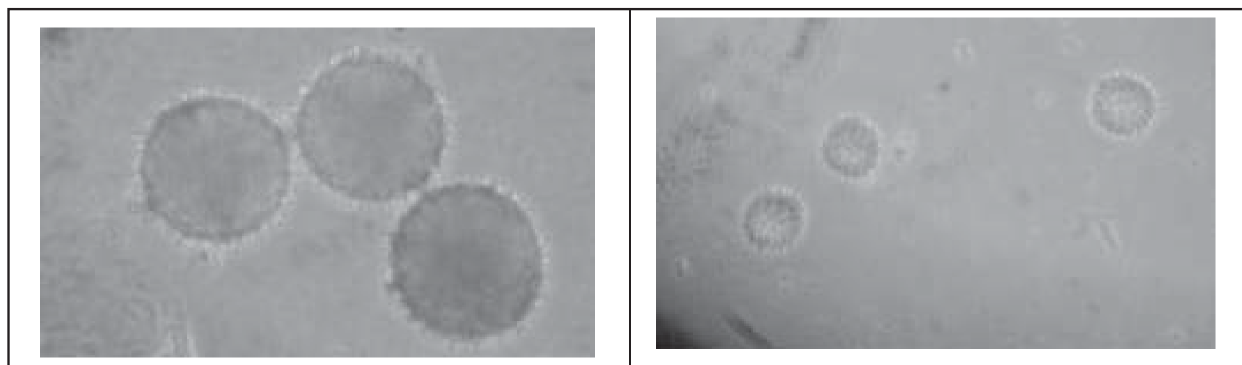


Plate 2: Pollen morphology of *G. hirsutum* parent and hybrid

VI were present in hybrid only and type VII markers were present in male parent. Hybrid 1 exhibited 48.2% bands and hybrid 2 revealed 55.5% bands shared with parents including Type I, II & IV markers. Of these Type IV markers are of special significance as the presence of male specific bands in the hybrid indicates that the off-spring is a true hybrid and rules out the possibility of self pollination and self fertilization.

About 41.1% and 33.6% markers from parents including Type III, V & VII were absent in the hybrid 1 and 2, respectively. The high number of bands not sharing with parents and hybrid is probably due to segregation of heterozygous chromosomes during meiosis. About 10.7% and 10.9% non-parental bands of Type VI were present in the hybrid 1 and 2, respectively. These unique markers would have originated due to recombination, mutation or random segregation of chromosomes at meiosis during the formation of hybrid (Darnell *et al*., 1990; Huchett *et al*., 1995). Chromosomal crossing over during meiosis may result in loss of priming sites in the hybrid leading to novel RAPD markers in the hybrid (Smith *et al*., 1996). The present observation of the presence of novel bands in both the hybrids is in conformity with that of *Chrysanthemum* hybrids (Huang *et al*., 2000).

Similarity can be used to measure the relatedness between parents, and between parents and off-spring (Nybom and Hall, 1991; Welsh *et al*., 1991). From the similarity matrix (Table 5), it is found that the hybrid 1 is 66.6% similar to its female parent (*G. hirsutum* cv. LRA 5166) and 58.3% similar to its male parent (*G. armourianum*) while the hybrid 2 showed 69.4% similarity to the female parent and 56.8% similarity to the male parent.

The present study using morphological characterization, cytological study and RAPD analysis confirms the true hybrid status of the cross *G. hirsutum* cv. LRA 5166 × *G. armourianum* and *G. hirsutum* cv. Sumangala × *G. armourianum*. It may be concluded that RAPD analysis can be used to test the hybrid

status of a cross in cotton. The triploid hybrids were found to be sterile, since no bolls formed even after effecting back crosses using the triploids as both male as well as female parent. Hence, efforts are being made to double the triploids to make it hexaploid using colchicine to restore fertility of the hybrid. Once colchiploids are developed, it may be used for further back cross with *G. hirsutum* parent to transfer useful characters from *G. armourianum.*

References

Alexander, M. P. (1969) – Differential staining of aborted and non-aborted pollen – *Stain Tech.,* **44:** 117-122.

Chapco, W., Ashton, N. W., Martel R. K. B. and Antonishyn, N. (1992) – A feasibility study of the use of random amplified polymorphic DNA in the population genetics systematics of grasshoppers – *Genome,* **35:** 569-574.

Darnell, J. E., Baltimore, D. and Lodish, H. F. (1990) – Molecular cell biology, Scientific American Books, Inc., pp. 156-159.

Deshpande, L. A., Kokate, R. M., Kulkarni, V. G. and Nerar, Y. S. (1991) – Cytomorphological studies in induced tetraploid G. arboreum and its interspecific hybrid with tetraploid *G. hirsutum* L. – *Indian J. Genet.,* **51:** 194-202.

Endrizzi, J. E., Turecotte, E. L. and Kohel, R. J. (1985) – Genetics, cytology and evolution of Gossypium – *Adv. Genet.,* **23:** 271-327.

Fryxell, P. A. (1992) – A revised taxonomic interpretation of Gossypium L. (Malvaceae) – *Rheedea,* **2:** 108-165.

Gill, M. S. and Bajaj, Y. P. S. (1984) – In vitro production of interspecific hybrid embryos in cotton – *Curr. Sci.,* **53:** 102-104.

Helentjaris, T., Slocum, M. Wright, S., Schaeffer, A. and Nienhuis, J. (1986) - Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphism - *Theor. Appl. Genet.,* **72:** 761-769.

Huang, S. C., Tsai, C. C. and Sheu, C. S. (2000) – Genetic analysis of Chrysanthemum hybrids based on RAPD molecular markers – *Bot. Bull. Acad. Sin.,* **41:** 257-262.

Huchett, B. I. and Botha, F. C. (1995) – Stability and potential use of RAPD markers in sugarcane genealogy – *Euphytica,* **86:** 117-125

Khadi, B. M., Katageri, I. S., Kachapur, R., Kulkarni V. N. and Vamadevaiah, H. M. (2002) – In vivo and in vitro interspecific cross recovery studies in cotton (Gossypium spp.) - A review – *J. Indian Soc. Cotton Improv.,* **27:** 57-72.

Krishna, T. G. and Jawali, N. (1997) – DNA isolation from single or half seeds suitable for Random Amplified Polymorphic DNA analysis – *Annal Biochem.*, **250:** 125-127.

Mehetre, S. S., Thombre, M. V. and Tayyab, M. A. (1980) – Cyotmorphological studies in an intergeneric hybrid between Gossypium hirsutum L. (2n = 52) and Hibiscus panduraeformis Burm – *Euphytica,* **29:** 323-330.

Mehetre, S. S., Aher, A. R., Gawande, V. L., Patil, V. R. and Mokate, A. S. (2003) – Induced polyploidy in Gossypium: A tool to overcome interspecific incompatibility of cultivated tetraploid and diploid cottons – *Curr. Sci.,* **84:** 1510-1512.

Meshram, L. D. and Tayyab, M. A. (1991) – A new interspecific hybrid between Gossypium hirsutum L. and Gossypium thurberi Fryn – *J. Indian Soc. Cotton Improv.,* **16:** 28-30.

Meshram, L. D. and Tayyab, M. A. (1994) – Interspecific hybrids between G. hirsutum × G. australe F. Muell – *Panjabrao Krishi Vidyapeeth Res. J.,* **18:** 124-126.

Nybom, H. and Hall, H. K. (1991) – Mini- satellite 'DNA fingerprints' can distinguish Rubus cultivars and estimate their degree of relatedness – *Euphytica,* **53:** 107-114

Patel, S. J. (1932) – The F_1 generation of a hybrid between an American and an Asiatic cotton – *Indian J. Agric. Sci.,* **2:** 127-130.

Paterson, A. H., Brubaker, C. L. and Wendel, J. F. (1993) – A rapid method for extraction of cotton (Gossypium spp) genomic DNA suitable for RFLP or PCR analysis – *Plant Mol. Rep.,* **11:** 122-127.

Roxas, N. J. L., Tashiro, Y., Miyazaki, S., Takeshita, A. and Oshima, T. (1993) – Isozyme analysis in higo Chrysanthemum (Dendrathema grandiflora Tzvelvev) – *J. Japan Soc. Hort. Sci.,* **61:** 919-924.

Smith, J. F., Burke, C. C. and Wagner, W. L. (1996) – Interspecific hybridization in natural populations of Cyrtandra (Generiaceae) on the Hawaiian Islands: evidence from RAPD markers – *Pl. Syst. Evol.,* **200:** 61-77.

Stephens, S. G. (1947) – Cytogenetics of Gossypium and the problem of the origin of New World cotton – *Adv. Genet.,* **1:** 431-432.

Stewart, J. Mc D. (1995) – Potential for crop improvement with exotic germplasm and genetic engineering. In: *Proceedings of the World Cotton Research Conference,* (Eds., Constable, G.A. and Forrester, N.W.) CSIRO, Melbourne, Australia, pp. 313-327.

Vroh, B. I., Harvengt, L., Chandelier, A., Mergeai, G. and du Jardin, P. (1996) – Improved RAPD amplification of recalcitrant plant DNA by use of activated charcoal during DNA extraction – *Plant Breeding,* **155:** 205-206.

Welsh, J., Honeycutt, R. J., Mcclelland, M. and Sobral, B. W. S. (1991) – Parentage determination in maize hybrids using the arbitrary primed polymerase chain reaction (AP-PCR) – *Theor. Appl. Genet.,* **82:** 473-476.

Wilde, J., Waugh, R. and Powell, W. (1992) – Genetic fingerprinting of Theobroma clones using randomly amplified polymorphic DNA markers – *Theor. Appl. Genet.,* **83:** 871-877.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S.V. (1990) – DNA polymorphism amplified by arbitrary primers are useful genetic markers – *Nucleic Acids Res.,* **18:** 6531-6535.