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RAPD markers for genetic diversity study among Indian cotton cultivars

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The present study was undertaken with RAPD markers for genetic diversity estimation in 59 cotton cultivars belonging to four cultivated species of cotton. The selected eighteen RAPD primers produced a total of 251 amplicons, which generated 97.21% polymorphism. The number of amplification products ranged from 7 to 24 for different primers, whereas per cent genetic similarity for the studied primers ranged from 42 to 79. Among 59 cotton cultivars, 36% genetic diversity was observed. In 41 *Gossypium hirsutum* cultivars included in the study, the average genetic similarity was 74%. More genetic diversity was observed in diploid than in tetraploid cotton cultivars. UPGMA cluster analysis placed tetraploid cotton cultivars into two distinct clusters that are in agreement with the traditional taxonomic arrangement of these cultivars into *G. hirsutum* and *G. barbadense*. Analysis of molecular variance in *G. hirsutum* cultivars revealed that most of the variance could be attributed to within breeding-centre variance. All the cultivars could be discriminated from one another based on the combined profiles for eighteen oligonucleotide primers. A negative correlation between average genetic similarity for a primer and the number of cultivars identified by it was observed. Genetically distinct cultivars were identified that could be potentially important sources of germplasm for further cotton improvement in the country.

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THE genus *Gossypium* to which cultivated cottons belong, contains about 45 diploid ($2n = 2x = 26$) and five allotetraploid ($2n = 4x = 52$) species[1], all of which are basically tropical perennials. Two of the diploids, *G. arboreum* L. and *G. herbaceum* L. and two of the allotetraploids, *G. hirsutum* L. and *G. barbadense* L. are cultivated as annuals for their seed and fibre in the tropical and subtropical regions of the world. The allotetraploids are the most commonly grown, making up more than 90% of the world production. Cotton is an important cash crop of India. About 60 million people earn their livelihood through its cultivation or trade and processing. The present lint yield[2] in the country is 308 kg/ha, which was only 151 kg/ha during 1970. All the four cultivated species of cotton grown around the world are grown in India.

Recently, various molecular marker techniques have developed into powerful tools for diversity analysis and establishing relationships between cultivars. Among these, the RAPD technique[3,4] is technically the simplest, less expensive, fast and does not require huge infrastructure to start with.

The RAPD technique has already been used in cotton for genetic diversity studies. In nine Australian cotton cultivars belonging to *G. hirsutum*, 92.1 to 98.9% genetic similarity was observed using this technique[5]. Again, using RAPD markers and morphological traits, the genetic relatedness in 16 elite cotton genotypes was studied and a positive correlation ($r = 0.63$) between the genetic distances derived based on molecular markers and the taxonomic distances calculated on the basis of morphological markers was observed[6]. In yet another study[7] involving 22 *G. hirsutum* varieties, 89.1% polymorphism was observed. Seventeen varieties that could be placed in one cluster had a similarity range of 81.5–93.4%. The only diploid cultivar in this study shared 55.7% genetic similarity with tetraploid counterparts. In diploid cottons belonging to *G. arboreum* and *G. herbaceum* 72% genetic similarity has been reported[8]. Besides these diversity studies, the RAPD technique has also been used for phylogeny[9] and seed purity studies[10] in cotton. Thus the usefulness of this technique for diversity studies and establishing genetic relationships is well documented in cotton.

The experimental material in the present study consisted of fifty-nine cultivars of cotton belonging to four cultivated species (Table 1). These included 11 intra- or inter-specific hybrids that will be referred to as cultivars hereafter. Excluding hybrids, 33 cultivars belong to *G. hirsutum*, whereas five each belong to the three species *G. barbadense*, *G. arboreum* and *G. herbaceum*. These cultivars were pro-

Table 1. Description of cotton materials used in the study

Serial no.	Name	Species	Breeding place*
1	CPD 428	*Gossypium hirsutum*	DHA
2	DHB 105	*G. hirsutum* × *G. barbadense*	DHA
3	B82-1-1	*G. barbadense*	DHA
4	DS 28	*G. hirsutum*	DHA
5	DCH-32	*G. hirsutum* × *G. barbadense*	DHA
6	SB(YF)425	*G. barbadense*	DHA
7	Laxmi	*G. hirsutum*	DHA
8	Abadhita	*G. hirsutum*	DHA
9	CPD 423	*G. hirsutum*	DHA
10	CPD 420	*G. hirsutum*	DHA
11	Sahana	*G. hirsutum*	DHA
12	DDCC-1	*G. arboreum*	DHA
13	DHH-11	*G. hirsutum* × *G. hirsutum*	DHA
14	Jayadhar	*G. herbaceum*	DHA
15	SB 289E	*G. barbadense*	DHA
16	G 67	*G. hirsutum*	SUR
17	H4	*G. hirsutum* × *G. hirsutum*	SUR
18	G. Cot100	*G. hirsutum*	SUR
19	G. CotHy 6	*G. hirsutum* × *G. hirsutum*	SUR
20	G. Cot 10	*G. hirsutum*	SUR
21	BC68-2	*G. hirsutum*	SUR
22	Sujay	*G. herbaceum*	SUR
23	G 27	*G. arboreum*	SUR
24	Surat Dwarf	*G. hirsutum*	SUR
25	BN	*G. hirsutum*	SUR
26	G. Cot 14	*G. hirsutum*	SUR
27	G. Cot 21	*G. herbaceum*	SUR
28	G. Cot 19	*G. arboreum*	SUR
29	G. Cot 15	*G. arboreum*	SUR
30	G. CotHy 8	*G. hirsutum* × *G. hirsutum*	SUR
31	G. Cot 11	*G. herbaceum*	SUR
32	V 797	*G. herbaceum*	SUR
33	G. Cot 12	*G. hirsutum*	SUR
34	LRA 5166	*G. hirsutum*	COI
35	Surabhi	*G. hirsutum*	COI
36	Anjali	*G. hirsutum*	COI
37	MCU 5 VT	*G. hirsutum*	COI
38	Sruthi	*G. hirsutum* × *G. barbadense*	COI
39	T7	*G. hirsutum*	COI
40	M12	*G. hirsutum*	COI
41	HLS 329	*G. hirsutum*	COI
42	Surya	*G. hirsutum* × *G. hirsutum*	COI
43	RSP-4	*G. barbadense*	COI
44	70E	*G. hirsutum*	COI
45	Savita	*G. hirsutum* × *G. hirsutum*	COI
46	Suvin	*G. barbadense*	COI
47	RS 875	*G. hirsutum*	SGN
48	RST 9	*G. hirsutum*	SGN
49	RS 810	*G. hirsutum*	SGN
50	SGRF 1	*G. hirsutum*	SGN
51	SGRM 1	*G. hirsutum*	SGN
52	RG 8	*G. arboreum*	SGN
53	SH2379	*G. hirsutum*	SIR
54	Om Shankar	*G. hirsutum* × *G. hirsutum*	SIR
55	K 34007	*G. hirsutum*	SIR
56	Pusa 31	*G. hirsutum*	IARI
57	Pusa 8-6	*G. hirsutum*	IARI
58	CP 15/2	*G. hirsutum*	NAG
59	CICRHH-1	*G. hirsutum* × *G. hirsutum*	NAG

*DHA, University of Agricultural Sciences, Dharwad; SUR, Gujarat Agricultural University, Surat; COI, Central Institute for Cotton Research, Regional Station, Coimbatore; SGN, Rajasthan Agricultural University, Sriganganagar; SIR, Central Institute for Cotton Research, Regional Station, Sirsa; IARI, Indian Agricultural Research Institute, New Delhi; NAG, Central Institute for Cotton Research, Nagpur.

cured from seven different places of breeding. Fresh, young leaves (3–4 weeks old) from at least twenty plants from each cultivar were bulked for DNA extraction. Total genomic DNA was extracted following CTAB method[11]. DNA samples were fluorometerically quantified and diluted in TE buffer to a working concentration of approximately 10 ng μl^{-1}.

Sixty random decamer primers (Operon Technologies, Inc., USA) were screened on template DNA from three cultivars so as to identify those giving good and scorable amplification products. Eighteen oligonucleotide primers were finally selected for RAPD analysis. Each reaction mixture (25 µl) for PCR amplification consisted of 1X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50 mM K Cl), 3.0 mM $MgCl_2$, 2U *Taq* DNA polymerase; 200 µM each dATP, dTTP, dCTP and dGTP (all reagents from Perkin Elmer), 0.5 µM decamer primer (Operon Technologies, Alameda, USA) and approximately 50 ng genomic DNA template. PCR amplification conditions were as follows: initial extended step of denaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min and elongation at 72°C for 1 min. The 40th cycle was followed by an extended primer extension step at 72°C for 4 min and then being held at 4°C until electrophoresis. PCR was carried out in a Perkin–Elmer (Model 9600) thermocycler. PCR products were mixed with 2.5 µl gel loading dye (6X dye: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and spun briefly in a microfuge before loading[12]. The amplification products were electrophoresed on 1.3% agarose gel at 100 V in 1X TAE buffer. A one kb DNA ladder was used as a molecular size standard. The gels were stained with ethidium bromide and photographed under UV light. For all of the cultivars, bands on RAPD gels were scored as present (1) or absent (0).

Jaccard's similarity coefficient values[13] for each pair-wise comparison between cultivars were calculated and a similarity coefficient matrix was constructed. This matrix was subjected to unweighted pair-group method for arithmetic averages analysis (UPGMA) to generate a dendrogram using average linkage procedure. All these computations were carried out using NTSYS-pc version 1.7 software[14]. Analysis of molecular variance (AMOVA)[15] was applied on the Euclidean distance matrix between individuals to partition the total genetic variation. Statistical significance of variances was tested by random permutation, with the number of permutations set at 1000. AMOVA was done using the computer software Arlequin[16]. The same software was used to calculate the Nei and Li's pair-wise distance matrix between different breeding centres[17]. Robustness of the clustering pattern was tested using 1000 resamplings with Free Tree software[18].

Eighteen primers used in the study generated a total of 251 amplification products, among which 244 were found to be polymorphic (Table 2). This resulted in 97.21% polymorphism. The number of amplification products ranged

Table 2. RAPD primers and their properties

Primer	TB	PP	PP (diploid)	PP (tetraploid)	GS	CI
OPB-01	8	100.00	75.00	75.00	0.72	8
OPB-04	16	100.00	93.75	93.75	0.52	26
OPB-07	20	100.00	85.00	60.00	0.70	20
OPB-11	14	85.71	57.14	78.57	0.72	18
OPB-12	7	85.71	28.57	42.85	0.69	3
OPB-13	14	100.00	64.28	92.85	0.42	27
OPB-17	19	100.00	84.21	78.94	0.64	22
OPB-18	10	100.00	60.00	50.00	0.75	7
OPB-19	9	100.00	66.67	66.67	0.78	14
OPB-20	16	93.75	75.00	75.00	0.70	19
OPC-04	24	95.83	66.67	66.67	0.65	42
OPC-06	12	91.67	91.60	58.30	0.64	15
OPC-11	7	100.00	71.42	85.71	0.79	10
OPC-14	21	95.24	90.47	76.19	0.66	13
OPC-15	21	100.00	42.86	80.95	0.60	29
OPC-18	11	100.00	27.27	81.81	0.63	7
OPC-19	8	100.00	100.00	87.50	0.68	7
OPC-20	14	100.00	78.57	85.71	0.68	15

TB, Total number of bands amplified; PP, Per cent polymorphism; GS, Average Jaccard's genetic similarity; CI, Cultivars identified uniquely.

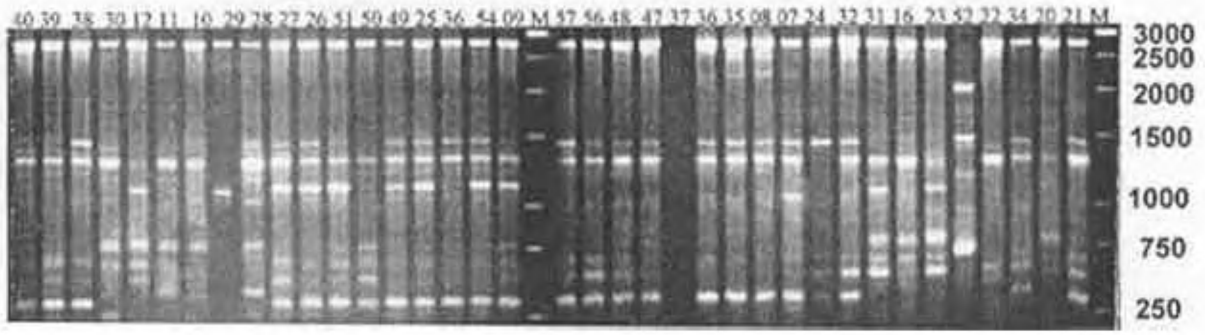

Figure 1. RAPD profiles for 37 cotton cultivars produced using primer OPB-04. Numbers on top are cultivar serial numbers as shown in Table 1. M is a one kb molecular weight standard.

from 7 (OPB-12 and OPC-11) to 24 (OPC-04). All the primers produced polymorphic amplification products, however, the extent of per cent polymorphism varied with each primer (85.71 to 100%). Twelve primers produced bands, all of which were found to be polymorphic. Per cent genetic similarity using RAPD primers ranged from 42 (OPB-13) to 79 (OPC-11). Primer OPC-04 identified uniquely as many as 42 cultivars out of 59, followed by primers OPC-15, OPB-13 and OPB-04, which identified 29, 27 and 26 cultivars respectively. Figure 1 depicts a section of the RAPD profile obtained with primer OPB-04.

We found that all the studied primers produced polymorphic amplification products. Iqbal *et al.*[7] also observed that 98% of the primers in their study produced polymorphic profiles. A high level of polymorphism (97.2%) was observed in our study. Lu and Myers[19] observed a low level of DNA variation among ten varieties of *G. hirsutum*, as they observed only 13.5% polymorphism. Iqbal *et al.*[7] found 89.1% polymorphism among 23 *G. hirsutum* cultivars, wherein one cultivar was of the species *G. arboreum*. Rahman *et al.*[20] observed 66.2% polymorphism in 27 cotton varieties. Conflicting reports on the extent of observed polymorphism in cotton in different studies could be attributed to the nature of the genetic material under investigation. The high degree of polymorphism in our study compared to other reports, is due to the more diverse material which belonged to four different cultivated species of cotton. Moreover, the various cultivars within a species represented different agroclimatic conditions for cotton growth.

Jaccard's pair-wise similarity estimates between genotypes were calculated. The average of these estimates was calculated after grouping the cultivars on the basis of their genomic groups (diploid/tetraploid), species-wise, breeding-centre wise and for hybrids (Table 3). It is evident from Table 3 that there was more diversity in diploid cultivars (51%) than tetraploid cultivars (26%). Furthermore, in tetraploid cultivars, *G. barbadense* possessed more diversity than counterpart tetraploid cultivars of *G. hirsutum*. Among

diploid species, *G. herbaceum* had more diversity (53%) than its cultivars of *G. arboreum* (38%). Center-wise grouping of Jaccard's similarity estimates for upland cotton cultivars suggested that the maximum diversity was in the material from University of Agricultural Sciences, Dharwad (32%), was followed by Gujarat Agricultural University, Surat (21%) and the Central Institute for Cotton Research, Regional Station, Coimbatore (18.5%). Greater genetic similarity was observed in the material from the Central Institute for Cotton Research, Regional Station, Sirsa (93%) followed by Rajasthan Agricultural University, Sriganganagar (91.4%). Hybrids of the tetraploid group had more genetic similarity (77.9%) compared with tetraploid cultivars (74.3%). On the whole, irrespective of the species involved, we found 36% genetic diversity in the material under investigation.

Using RAPD markers greater diversity was observed in diploid than in tetraploid cotton cultivars. This observation has been documented earlier[21]. Prevalence of greater diversity necessitates further collections and conservation of diploid cotton plant genetic resources in the country. In Indian tetraploid *G. hirsutum* cotton cultivars, Vafai-Tabar *et al.*[22] observed 79% average genetic similarity. In 41 *G. hirsutum* cultivars in our study average genetic similarity was 74%. Similarity ranges of 92.1–98.9%[5]; 70–90%[7] and 81.6–94.9%[20] for genetic kinship in elite *G. hirsutum* cultivars have been reported in the exotic materials. Compared to these published reports from outside the country, it can be concluded that Indian *G. hirsutum* cultivars have more genetic diversity within the studied set of cultivars. This might be due to the reason that cultivars included in our study represent a broad range of geographical and climatic adaptations. Furthermore, breeders in different stations are undertaking interspecific hybridizations and some of the cultivars are derivatives of advanced generations of such crosses.

While comparing results of genetic diversity in less commonly cultivated cotton species in India (*G. barbadense* and *G. herbaceum*) to that of the most commonly cultivated ones (*G. hirsutum* and *G. arboreum*), it was observed that the prevalence of genetic diversity is more in the former. This highlights the point that the most commonly cultivated species have a comparatively narrow genetic base, which might be true owing to the fact that common parents are usually employed in the pedigrees for the development of new cultivars. Thus, there is potential for broadening the genetic base of the most commonly cultivated cottons by way of introgression breeding using the less commonly cultivated species. These conclusions, however, may be influenced by the uneven number of cultivars in each group, e.g. diploids were represented by 10 cultivars, whereas tetraploids by 49. Similarly, in tetraploids the number of cultivars sampled was large in *G. hirsutum* (41 genotypes), whereas *G. barbadense* was represented by only five.

Table 3. Jaccard's similarity estimates for different groups

Group	No. of genotypes	Mean (± SD)	Range
Genomic/species group			
Tetraploid	49	0.743 ± 0.112	0.372–0.983
Diploid	10	0.489 ± 0.103	0.296–0.902
G. hirsutum	41	0.773 ± 0.105	0.394–0.983
G. barbadense	5	0.727 ± 0.085	0.662–0.916
G. arboreum	5	0.620 ± 0.119	0.500–0.902
G. herbaceum	5	0.472 ± 0.069	0.382–0.579
Centre-wise groups (*G. hirsutum* and hybrids)			
Dharwad	8	0.679 ± 0.151	0.409–0.918
Surat	11	0.787 ± 0.071	0.638–0.957
Coimbatore	10	0.815 ± 0.067	0.667–0.954
Sriganganagar	5	0.914 ± 0.031	0.886–0.983
Sirsa	3	0.931 ± 0.023	0.896–0.948
Hybrids	11	0.779 ± 0.061	0.664–0.892

The clustering of 59 cotton cultivars using UPGMA cluster analysis is depicted in Figure 2. Among diploid cultivars, Sujay and G.Cot 21 were quite distinct from all other cultivars. All other cultivars could be grouped into two clusters, one belonging to *G. herbaceum* (Cluster I) and the other to *G. arboreum* (Cluster II). *G. arboreum* cultivars G. Cot 19 and G. Cot 15 had maximum similarity with high bootstrap support (100%). Among tetraploid cultivars, all *G. barbadense* cultivars could be grouped in Cluster III. Suvin and SB289E were the cultivars in this group having maximum similarity. Cluster IV contained all the tetraploid cultivars belonging to the cultivated species *G. hirsutum*. *G. hirsutum* cultivars CPD 423, CP15/2, Laxmi and G. Cot12 were found to be the most diverse, as they did not cluster with rest of the *G. hirsutum* cultivars. Using RAPD markers diploid cultivars could be easily separated from tetraploid cultivars, as is evident from Figure 2. UPGMA cluster analysis of genetic similarities based on 251 markers placed tetraploid (AD genome) cotton cultivars into two distinct monophyletic clades, that is in complete agreement with the traditional taxonomic arrangement of these cultivars into *G. barbadense* and *G. hirsutum*. In an earlier report[6] separate clusters have been observed for cultivars of *G. hirsutum* and *G. barbadense*.

Out of three inter-specific hybrids included in the study, two hybrids (Sruthi and DHB-105) grouped in the *G. hirsutum* cluster, whereas the third hybrid (DCH-32) clustered with *G. barbadense*. Further analysis of the *G. barbadense* cluster suggested that the female *G. hirsutum* parent of the latter hybrid (DS28) had more average genetic similarity (68%) with the *G. barbadense* cultivars compared with the female parents of the other inter-specific hybrids. Cultivar CPD 423, in the tetraploid cluster, is one of the oldest cultivars grown in the country. The lowest value of genetic similarity (0.394) among *G. hirsutum* cultivars was between this cultivar and RS810. Within the diploid group, separation of *G. arboreum* cultivars from *G. herbaceum* is not pronounced, although these diploid cultivars have a slight tendency to be clustered within their

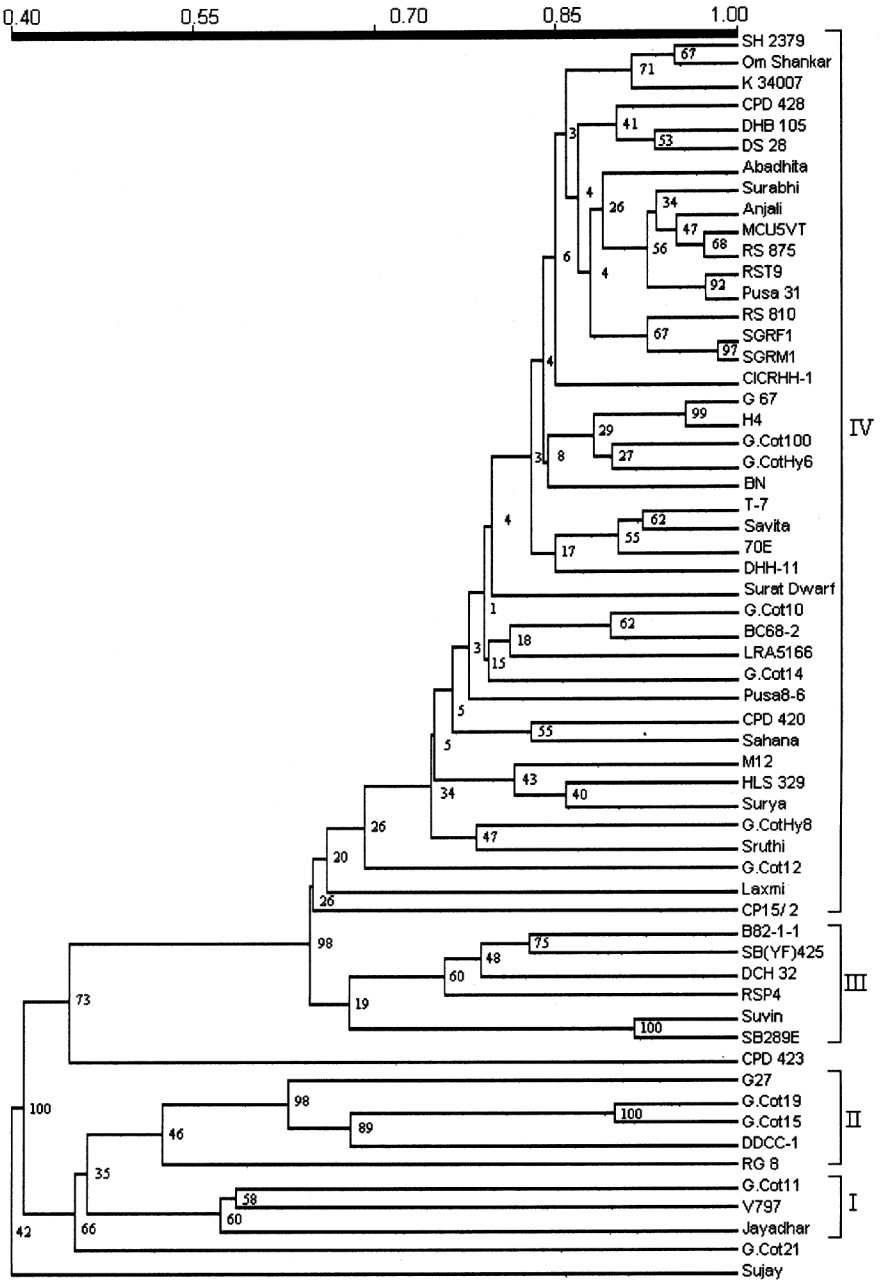

Figure 2. UPGMA cluster analysis-based dendrogram depicting genetic relationships among cotton cultivars. Per cent bootstrap values are depicted inside the figure. The scale on top shows Jaccard's similarity values.

respective taxonomic group, with poor bootstrap support. In an earlier report[8] these diploid cultivars could be easily separated into two distinct clusters, each representing different dipoid cultivated species. This discrepancy might be due to different parts of the cotton genome being scanned, since the oligonucleotides employed in the two studies were unique except for two primers.

G. hirsutum cultivars from different breeding centres were found to be more or less interspersed in the dendrogram and no pronounced centre-wise grouping of the different cultivars was discernible. All the cultivars from Sirsa were in one of the sub-clusters of Cluster IV. Similarly, some cultivars from Surat (G67, H4, G. Cot100, G. Cot Hy 6 and BN), Coimbatore (Surabhi, Anjali and MCU5VT; and M12, HLS 329 and Surya), Dharwad (CPD 428, DHB105 and DS28) and Sriganganagar (RS810, SGRF1 and SGRM1) also formed sub-clusters in this cluster, but with poor bootstrap support. AMOVA was performed using breeding centres as the grouping criterion (Table 4). This analysis revealed that most of the variation could be attributed to within breeding-centre variance, while variance among breeding centres was little. It indicates that breeders have not been working in isolation, and that breeding material for the development of these cultivars has been

Table 4. Analysis of molecular variance for different cotton breeding centres

Source	Degrees of freedom	Sum of squares	Variance component	Percentage of variation
Among breeding centres	4	97.62	1.599	10.91
Within breeding centres	32	417.79	13.056	89.09
Total	36	515.40	14.655	

Table 5. Pairwise distance matrix between different cotton breeding centers

	SIR	DHA	SUR	COI	SGN
SIR	0.000				
DHA	0.103	0.000			
SUR	0.108	0.799*	0.000		
COI	0.219*	0.117*	0.095	0.000	
SGN	0.352*	0.620	0.104	0.129*	0.000

*Significant at $P = 0.05$.

shared between the breeding stations and cotton improvement programmes. Pairwise genetic distances between the breeding centres revealed that cultivars from breeding stations Surat and Coimbatore were the closest (Table 5), whereas those from Dharwad and Surat were the least close.

From the above study it can be concluded that RAPD markers were found to reveal sufficient genetic diversity and a high level of genetic polymorphism. Diploid cultivars had more genetic diversity than tetraploid cultivars. UPGMA cluster analysis placed all the tetraploid cultivars within their respective known taxonomic groups, while breeding centre-wise grouping of cultivars was not discernible. Species-specific markers can be identified that would be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred. Genetically distinct cultivars were identified that could be potentially important sources of germplasm for cotton improvement.

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