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Assessment of genetic diversity in old world and new world cotton cultivars using RAPD and ISSR markers

Jayesh Kahodariya, Pritesh Sabara* and Dinesh Vakharia

Department of Biochemistry, Junagadh Agricultural University, Junagadh 362001, India

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In the present investigation, 15 cotton cultivars (8 old world and 7 new world cotton) were used to assess genetic diversity through dominant PCR based markers (21 RAPD and 9 ISSR primers). RAPD (Random amplified polymorphic DNA) primers produced in total 245 bands, of which 241 were polymorphic with 98.36% polymorphism. While ISSR (Inter simple sequence repeats) primers gave 85 bands, of which 81 were polymorphic constituting 95.29% polymorphism. Dendrogram showed clear grouping between old world and new world cotton cultivars. Principle coordinate analysis (PCA) showed congruence with dendrogram pattern, while high bootstrap values at major nodes in both marker systems as well as in pooled data indicated robustness of cluster pattern in dendrogram construction. Analysis of molecular variance (AMOVA) indicated genetic variation within populations for RAPD and pooled data, while the ISSR marker system showed high variation among population. Mantel's test between similarity and cophenetic coefficient matrices in different combinations showed good to very good correlation between matrices except for ISSR cophenetic coefficient matrices showing slightly poor result. The present study indicates huge variation between old world and new world cotton, which can be utilized for conservation of important cultivars and development of future strategies for crop improvement programme.

Keywords: AMOVA, cotton, genetic diversity, *Gossipium*, ISSR, RAPD

Introduction

Cotton is an economically important fiber crop of world[1-2] and stood second as an oil seeds producing crop after soybean[3-4]. It also produces byproducts like livestock feed (cotton seed cake) and cellulose[2]. It is an important cash crop, traditionally known as "white gold" in India, which provides support to more than 60 million people who earn their livelihood through its cultivation, trade or processing[5-6]. Cotton belongs to family Malvaceae and genus *Gossypium*, which comprises for about 50 species, among which 46 species are wild and 4 species are cultivated. Cultivated species contain two diploids, *G. arboretum* L. (n=13, A_2A_2 genome) and *G. herbaceum* L. (n=13, A_1A_1 genome), known as old world cotton, and two tetraploids, *G. barbadense* L. (n=26, AAD_2D_2) and *G. hirsutum* L. (n=26, AAD_1D_1), known as new world cotton[7-8]. Among the four species of cotton, *G. hirsutum* occupies 50% of the total cotton area under production, followed by *G. arboretum* (29%) and *G. herbaceum* (21%); while *G. barbadense* is cultivated with negligible area. The first hybrid cotton released in India was cv. 'hybrid 4' (an interspecific hybid) and now it is grown upon more than 45% area of total cotton cultivation[9].

The identification of cultivars includes tedious, time consuming and space demanding 'Grow out tests' (GOTs), which depend upon environmentally influenced morphological, physiological and agronomical traits. On the contrary, molecular markers, Random amplified polymorphic DNA (RAPD), Inter simple sequence repeat (ISSR), Simple sequence repeat (SSR) etc. are rapid, relatively cheap and do not influence by environmental factors or growth stages[10-11].

Hussain *et al*[2] assessed the genetic diversity among 11 cotton cultivars (8 *G. barbadense* and 3 *G. hirsutum*) using 15 RAPD primers. Esmail *et al*[12] tested 21 cotton genotype using 23 RAPD primers. While genetic diversity in eight diploid (*G. arboretum*) and eleven tetraploid (*G. hirsutum*) were analysed by Dongre *et al*[13] using 19 ISSR primers. Ali *et al*[14] have identified RAPD primer OPL-09, which amplifies DNA fragment responsible for red colour of cotton leaves found less preferred by boll weevil (*Anthonomus andis* Boheman)[15].

The present study was conducted to study the genetic diversity among cotton cultivars belong to old

*Author for correspondence:
Tel: +91-285-2670280-90
priteshrhsabara@gmail.com

and new world cotton, which will help to preserve the cultivars and in development of future strategies for further study and improvement in cotton cultivars.

Materials and Methods

Plant Material and DNA Extraction

In total 15 cotton cultivars (Table 1) were collected from various agriculture universities of Gujarat and maintained at Junagadh Agricultural University, Junagadh, India. The green portion (100 mg) from three different 4-d-old seedlings was used for DNA isolation with 2% CTAB employed by Zhang and Stewart[16] and originally described by Doyle and Doyle[17].

Table 1—Information regarding cotton cultivars used for diversity analysis

No.	Cultivars	Type	Species	Group
1	G.Cot Hyb 8	Hybrid	*G. hirsutum*	New world cotton/American cotton/Exotic cotton (n=26)
2	G.Cot Hyb 10	Hybrid		
3	G.Cot Hyb 12	Hybrid		
4	G.Cot 10	Stable		
5	G.Cot 18	Stable		
6	G.Cot 100	Stable		
7	G.Cot Hyb 102	Interspecific hybrid	*G. hirsutum* × *G. barbadense*	
8	G.Cot 21	Stable	*G. herbaceum*	Old world cotton/Asiatic cotton/Desi cotton (n=13)
9	G.Cot 23	Stable		
10	V797	Stable		
11	G.Cot 15	Stable	*G. arboreum*	
12	G.Cot 19	Stable		
13	G.Cot MDH 11	Hybrid		
14	G.Cot DH 07	Interspecific hybrid	*G. hirsutum* × *G. arboretum*	
15	G.Cot DH 09	Interspecific hybrid		

Primers

Twenty-one RAPD (Table 2) and nine ISSS (Table 3) primers, synthesized from XX-Integrated DNA Technology (XX-IDT), were used for the diversity study. These primers showed good polymorphic bands with reproducibility during PCR amplification.

RAPD Assay

The RAPD PCR reaction mixture (25 µL) contained: 20 mM Tris-HCL pH 8.4, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTPs, 20 pmoles RAPD primers, 100 ng genomic DNA and 1 unit Taq DNA polymerase (Bangalore Genei, Bangalore). The PCR condition for all combinations of cotton cultivars and RAPD primers were: initial denaturation at 94°C for 5 min; 35 cycles of 1 min at 94°C, 45 sec at 36°C as primer annealing temperature, 1 min at 72°C for extension, 7 min at 72°C for final extension of amplicon. Amplified products were loaded on 1.5% agarose gel containing ethidium bromide using 2.5 µL 6× gel loading buffer and electrophoresis were performed at constant 100 V for 2 h in 1× TBE buffer.

Table 2—List of RAPD primers, their GC content, band size, total no. of amplicon, level of polymorphism and resolving power

No.	RAPD primers	Sequence (5′→3′)	GC content (%)	Allele/Band size (bp)	Polymorphic bands/ Total no. of bands	% polymorphism	Total no. of fragments amplified	Resolving power
1	OPA-05	AGGGGTCTTG	60	403-1385	11/11	100	28	3.73
2	OPA-07	GAAACGGGTG	60	457-2018	15/15	100	72	9.6
3	OPA-09	GGGTAACGCC	70	202-1017	12/12	100	54	6.93
4	OPA-10	GTGATCGCAG	60	176-1988	13/13	100	57	6.93
5	OPA-15	TTCCGAACCC	60	253-1799	13/13	100	49	6.13
6	OPA-16	AGCCAGCGAA	60	369-1746	9/9	100	31	4.13
7	OPA-19	CAAACGTCGG	60	157-2392	9/9	100	35	4.67
8	OPB-01	GTTTCGCTCC	60	275-1227	7/7	100	41	5.2
9	OPB-05	TGCGCCCTTC	70	206-629	7/7	100	64	3.47
10	OPB-08	GTCCACACGG	70	230-1566	11/11	100	61	5.87
11	OPB-11	GTAGACCCGT	60	188-1150	9/9	100	30	3.6
12	OPB-15	GGAGGGTGTT	60	227-887	8/9	88.9	112	2.13
13	OPB-17	AGGGAACGG	60	298-2578	13/15	86.7	91	7.47
14	OPC-15	GACGGATCAG	60	182-1686	17/17	100	55	7.33
15	OPC-16	CACACTCCAG	60	127-1757	13/13	100	48	6.0
16	OPC-19	GTTGCCAGCC	70	88-1602	20/20	100	119	11.47
17	OPD-11	AGCGCCATTG	60	178-1279	16/16	100	102	8.13
18	OPD-12	CACCGTATCC	60	148-2007	13/13	100	78	7.33
19	OPM-09	GTCTTGCGGA	60	532-1328	4/5	80	30	2.0
20	OPM-16	GTAACCAGCC	60	286-2635	11/11	100	46	6.1
21	OPP-04	GTTTCGGTCC	60	354-1714	10/10	100	49	6.4
Mean					11.48/11.67	97.89	59.62	5.93
Total					241/245		1252	

Table 3—List of ISSR primers, their GC content, band size, total no. of amplicon, level of polymorphism and resolving power

No.	ISSR primers	Sequence (5′→3′)	GC content (%)	Tm	Allele/Band size (bp)	Polymorphic bands/ Total no. of bands	% polymorphism	Total no. of fragments amplified	Resolving power
1	IS 4	$(AGC)_5$ GC	70.5	62.8	202-1010	8/8	100	65	4.53
2	IS 7	$(CA)_7$ GT	50.0	49.3	166-1451	11/11	100	61	7.87
3	IS 15	$(GT)_7$ AT	43.7	45.8	245-1013	8/8	100	31	4.13
4	ISSR 5	$(TA)_3$ $(GT)_{15}$	41.6	63.1	209-828	9/9	100	70	6.00
5	ISSR 6	$(GA)_9$ AA $(GA)_5$	46.6	58.6	219-933	10/11	90.91	67	5.07
6	ISSR 7	$(GACA)_4$	50.0	47.4	179-1383	11/13	84.62	87	6.93
7	ISSR 9	$(GAGA)_4$ GAT	47.3	48.8	321-1223	6/7	85.71	53	3.87
8	ISSR 10	$(GAGA)_4$ GAAT	45.0	49.4	187-1187	9/9	100	61	6.00
9	ISSR 12	$(GTGA)_4$	50.0	47.4	214-2272	9/9	100	54	3.2
Mean						9/9.44	95.69	61	5.29
Total						81/85		549	

ISSR Assay

Amplification reactions were carried out in a volume of 25 µL containing 1× PCR reaction buffer (10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$, 50 mM KCl), 200 µM of each dNTPs, 10 pmoles ISSR primers, 100 ng genomic DNA and 1 unit Taq DNA polymerase (Bangalore Genei, Bangalore). PCR amplification was performed as 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturation at 94°C, 45 sec at respective annealing temperature (T_m±2; Table 3) of each ISSR primer and final extension of 5 min at 72°C were carried out for polishing the ends of PCR products. ISSR amplicon were separated on 1.5% agarose gel loaded with 6× loading buffer and electrophoresed at constant 100 V for 2 h in 1× TBE buffer. Separated products stained with ethidium bromide were visualized under UV transelluminator.

Gel Documentation and Scoring

Gels stained with ethidium bromide were documented using Upland and Gene Genius Bios Imaging system, Syngene, UK. The molecular size of amplicons were analysed by using a 100 bp DNA ladder (Bangalore Genei, Bangalore). The banding patterns obtained from both RAPD and ISSR for all the 15 cultivars were scored using binary codes, presence (1) or absent (0), each of which treated as independent characters.

Data Analysis

Pairwise Jaccard's similarity coefficient between all 15 cultivars was calculated[18]. Jaccard's similarity matrix was subjected for cluster analysis using an unweighted pair group method with arithmetic means (UPGMA)[19]. Freetree software was used to generate dendrogram and to test its robustness, bootstrap analysis was carried out using 1000 resamplings[20]. Dendrogram was presented using Treeview software[21]. Number of amplified product, polymorphic band and percentage of polymorphism was calculated for each primer. Average polymorphic information content (PIC) values for particular marker was calculated by taking the mean of PIC value for all the markers produce by particular primer. $PIC=1-(p^2-q^2)$, where p^2 is proportion of accessions having an amplicons and q^2 is proportion of accessions not having the amplicons[22]. Resolving power of primer was calculated using formula $Rp = \sum Ib$, where Ib (band informativeness) = $1-[2 \times (|0.5-p|)$, p being the proportion of cultivars containing the band[23]. PIC and Rp gives idea about the primer which could best distinguish the cultivar. Utility of each marker system was determined by calculating diversity index (DI), effective multiplex ratio (EMR) and marker index (MI) as describe by Powell *et al*[24]. DI for genetic marker was calculated as: $DI=1-\sum pi^2$ (where 'pi' is the allele frequency of the i^{th} allele). DI for polymorphic marker is: $(Di_{av})p=\sum Di_n/n_p$ (where Di_{av} is arithmetic mean of DI for that marker assay, n_p is the number of polymorphic loci and n is the total number of loci). EMR can be described as a product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay, $EMR = n_p (n_p/n)$[11].

Analysis of molecular variance (AMOVA), principal coordinated analysis (PCA) and Mantel's test was performed using GenAlex software[25-26]. AMOVA provides information regarding variation between cultivars and within cultivars. The relation between distance matrix elements based on their first two principle coordinates was inferred from PCA analysis and presented graphically. The similarity matrix from RAPD and ISSR marker was subjected to Mantel's test, which provides cooperative analysis

between both markers. Probability of identical matches by chance (P) was calculated as $P=(X_D)^n$ where, X_D is considered to be average similarity index for all pair-wise comparisons and n is number of polymorphic bands[27].

Results

RAPD Data Analysis

Twenty-one RAPD primers gave in total 1252 amplicon from 15 cotton cultivars with band size 127-2635 bp and 5-20 number of bands (OPM-09 & OPC-19, respectively). RAPD primers amplified in total 245 bands, of which 241 were polymorphic with 11.67 bands per primer and 11.48 polymorphic bands per primer, giving 97.88% polymorphism (Table 2). Resolving power for RAPD primer was found to be in the range of 2.00 (OPM-09) to 11.47 (OPC-19) (Table 2), with 5.93 average resolving power for RAPD marker (Table 4). The marker index for RAPD marker was estimated to be 9.93. AMOVA (Table 5) helps in partitioning overall RAPD variation among cultivars and within cultivars, showing 44 and 56% variation, respectively. AMOVA suggests that there is high variation within cultivars and among cultivars, and old cotton cultivars has a higher number of polymorphic loci and percentage of polymorphism. This information helps to preserve valuable germplasm for development of future strategies in crop improvement.

A dendrogram constructed from 15 cotton cultivars based on UPGMA analysis showed eight sub-clusters forming two main clusters of old world cotton (I-IV) and new world cotton (V-VIII) (Fig. 1A) with a Jaccard's similarity coefficient range of 0.184 to 0.753, having an average value of 0.416 (Table 4). In both main clusters, cultivars G.CotDH09, G.CotMDH11, G.Cot18 and G. CotHyb102 of individual sub-cluster I, II, V and VI, respectively, showed more variability as compared to other cultivars of their cluster. Bootstrap values for each node was (>30) showing good robustness of the dendrogram. Cluster analysis of 15 cotton cultivars were compared with PCA analysis and it was found that nearly two third of the total genetic diversity was represented by first three principal component analysis PC1, PC2 and PC3 with Eigen value 0.377 (30.87%), 0.254 (20.82%) and 0.207 (16.99%), respectively.

Table 4—Summery statistics for RAPD and ISSR markers

No.	Parameters studied	RAPD	ISSR	RAPD+ISSR
1	Total no. of bands	245	85	330
2	Total no. of amplified product	1252	549	1801
3	Av. no. of bands per primer	11.67	9.44	11.00
4	Av. no. of polymorphic bands per primer	11.48	9.00	10.73
5	Av. polymorphic information content of primer	0.878	0.853	0.870
6	Av. resolving power of primer	5.93	5.29	5.74
7	Av. diversity index of primer	0.878	0.853	-
8	Av. effective multiplex ration of primer	11.31	8.62	-
9	Av. marker index of primer	9.93	7.35	-
10	Av. Jaccard's similarity coefficient	0.416	0.472	0.401
11	Probability of chance identity between two cultivars	1.59×10^{-92}	3.88×10^{-27}	0.00

ISSR Data Analysis

Nine ISSR primers gave 81 polymorphic bands out of 85 total numbers of amplified bands, comprising 95.69% polymorphic bands. ISSR primers produced in total 549 amplified product with molecular size range 166-2272 bp. The lowest and highest number of bands were produced by ISSR9 (7) and ISSR7 (13). Average number of bands and polymorphic bands for ISSR marker were 9.44 and 9, respectively. Rang of resolving power for ISSR marker was 3.2 to 7.87 with an average of 5.29 per primer (Table 3). AMOVA analysis showed a considerable variation among cultivars (12.51%) as compared to within cultivars (9.48%) (Table 5).

Table 5—Summary of AMOVA based on RAPD and ISSR individually, and in combination, among the cotton cultivars

Source of variation	Among cultivars			Within cultivars		
d.f.	1			13		
Marker	RAPD	ISSR	RAPD + ISSR	RAPD	ISSR	RAPD + ISSR
S.S.D	220.61	102.87	323.47	417.39	123.27	540.66
Variance component	25.25	12.51	37.75	32.11	9.48	41.59
Percentage	44	57	48	56	43	52
P-value	<0.002	<0.001	<0.001	<0.002	<0.001	<0.001

Level of significance is based on 1000 iteration steps

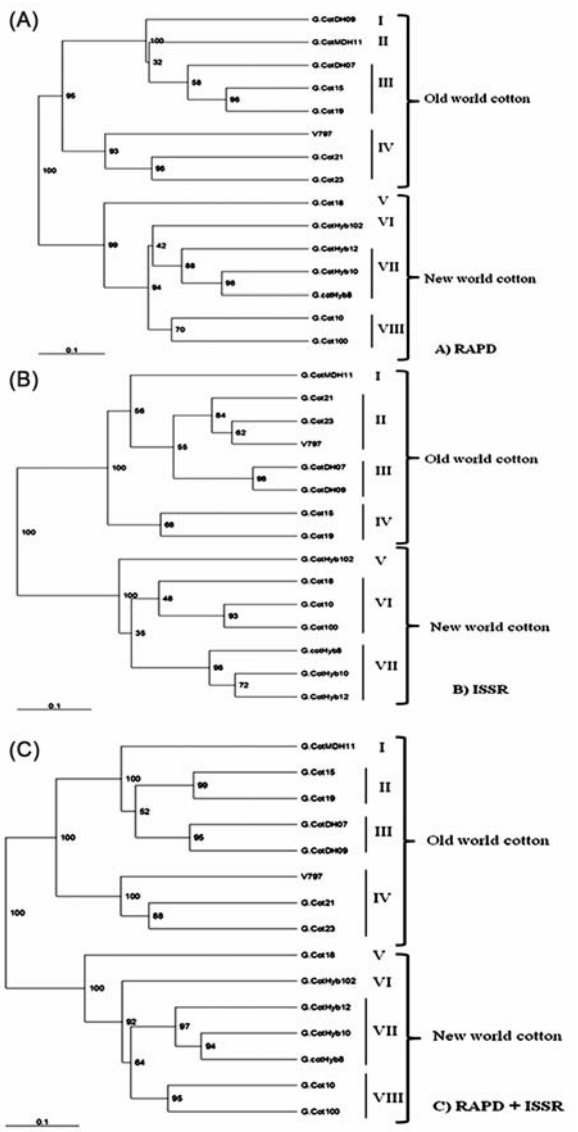

Fig. 1 (A-C)—Dendograms generated using UPGMA for data generated by RAPD (A), ISSR (B) (primers) and RAPD+ISSR (C) in 15 cotton cultivars.

A dendogram based on ISSR data generated seven sub-cluster, which produce two main clusters showing a clear group of old world (I-IV) and new world (V-VII) cotton (Fig. 1B). Sub-cluster I (G.CotMDH11) and V (G. CotHyb102) contain single cultivar showing more diversity as compared to other cultivars. Jaccard's similarity coefficient range for ISSR marker was 0.185 to 0.881 with mean value of 0.472 (Table 4). Bootstrap analysis showed very high value (100) at major nodes depicting the robustness of the dendrogram. Comparison between dendrogram and PCA analysis showed that nearly 80% of diversity is covered by first three component analysis PC1, PC2,

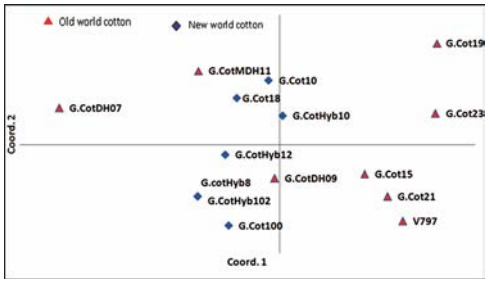

Fig. 2—Two dimension plot of principal coordinates analysis (PCA) of two cotton cultivar groups

PC3 with 35.18% (0.523), 27.55% (0.409) and 14.46% (0.215), respectively.

Combined Analysis of RAPD and ISSR Data

The dendrogram constructed based on combined RAPD and ISSR data shows in total eight sub-clusters forming two main groups as shown in case of RAPD and ISSR (Fig. 1c). Sub-cluster I to IV belong to old world cotton, in which G.CotMDH11 (I) show more diversity as compared to others. In case of new world cotton, G.Cot18 (V) and G.CotHyb102 (VI) have shown more diversity as compared to other new world cotton. The average Jaccard's similarity coefficient for combined data was 0.401, indicating robustness of dendrogram as major nodes have high bootstrap value. Comparative analysis between dendrogram and PCA (Fig. 2) shows similar result as shown in RAPD cluster analysis. This may be because number of primers used and total number of bands generated by RAPD markers are higher as compared to ISSR marker. Genetic diversity and AMOVA analysis data are shown in Table 5.

Discussion

Assessment of genetic diversity in cotton was studied earlier using RAPD[4,6] and ISSR primers[28]. Rana and Bhat[6] used 18 RAPD primers and found 97.21% polymorphism, amplifying 244 polymorphic bands out of 251 amplicons. While Chaudhary *et al*[7] discovered 12.3 bands per primer with 91.6% polymorphic bands out of 370 bands. Dongre *et al*[28] assessed wild *Gossypium* species using ISSR marker and found 88% polymorphism with 9.3 polymorphic bands per ISSR primer. ISSR primers containing $(GA)_n$, $(CA)_n$, $(AGC)_n$, $(GACA)_n$ and $(GTGA)_n$ were also found more effective and informative in Tetraploid potato[29], Passion fruit[30] and Papaya[31]. In the present study, RAPD markers were found to be best as compared to ISSR marker due to its ability to produce more number of band, amplified product,

average number of bands per primer and average number of polymorphic band per primer. The RAPD markers were also found better in case of average polymorphic information content, resolving power and marker index as compared to ISSR markers. In contrast, Acampora *et al*[32], Sarwat *et al*[33] and Behera *et al*[34] in their studies found ISSR primers comparatively more informative than RAPD primers. The probability of chance identity between two cultivars also indicated more reliability of RAPD markers in differentiating between close cultivars as compared to ISSR marker (Table 4). This was also supported by Tiwari *et al*[35] by their work on Brinjal. It is probably because both the marker systems target the different portion of the genome and RAPD primers mostly produce a large number of band compared to ISSR primers.

Cluster analysis based on RAPD, ISSR and RAPD+ISSR marker systems clearly separated the old world and new world cotton and put them into two separate clusters (Fig. 1). Similar results were obtained in apricot[11] and *Rhodiola imbricate*[36] when they studied the populations from different locations. To validate the clustering pattern of dendrogram, pattern finding method of PCA was used (Fig. 2), which indicated good congruence with the clustering pattern, as found in Jatropha[37]. To check the precise genetic relationship between cotton cultivars, the bootstrap resampling method was used, which showed high bootstrap support (>90) at major nodes in all the dendrogram, further validating the marker analysis. Babu *et al*[38] found similar boot strap support (>70) at each major node in the dendrogram of RAPD, ISSR and pooled data during study of Napier Grass. Similar high result of bootstrapping was also obtained in Jatropha[39]. When overall genetic data compared between marker systems, RAPD marker showed high value for DI, EMR and MI compared to ISSR (Table 4). AMOVA was performed to test the variation among and within the cotton cultivars (Table 5) and found that in case of RAPD and RAPD+ISSR, variation existed within the cultivars, while ISSR markers showed variation among the cultivars. Comparable results were obtained in Jatropha[37], Apricot[11] and *Rhodiola imbricate*[36].

Correlation through Mantel's test between RAPD, ISSR and RAPD+ISSR marker systems was carried out using similarity, cophenetic and similarity/ cophenetic matrices (Table 6). The result indicated good ($0.8 \leq r < 0.9$) to very good ($0.9 \leq r$) fitness for RAPD, ISSR and pooled data, while ISSR marker showed marginally poor relation ($0.7 \leq r < 0.8$) with RAPD marker in case of cophenetic matrices, showing some distortion between these two matrices[39]. However, contrasting results were found in case of scarlet runner bean[32].

Table 6—Correlations between matrices (Mantel test)[a]

	RAPD	ISSR	RAPD+ISSR
RAPD	0.937	0.873	0.999
ISSR	0.771	0.958	0.874
RAPD+ISSR	0.973	0.896	0.969

[a]Diagonal: Similarity/cophenetic; Above diagonal: Cophenetic; Below diagonal: Similarity

The present study clearly shows that dominant molecular markers RAPD and ISSR were extremely useful in detecting genetic diversity of old and new world cotton, which have a different ploidy level. Diversity analysis indicated more variation in old world cotton as compared to new world cotton, so it would be helpful to conserve old world cotton for the development of future strategies for cotton improvement.

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