

Characterization of cotton (*Gossypium hirsutum*) germplasm by ISSR, RAPD markers and agronomic values

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Using ISSR, RAPD markers and agronomic values, the characterization of 25 cotton (*G. hirsutum*) germplasms was carried out. 45 ISSR primers and 40 RAPD primers were used to amplify the germplasms. 19 scorable ISSR primers generated 90 markers while 21 reproducible RAPD primers generated 150 markers; of which, 49 markers from ISSR and 76 markers from RAPD were scored as polymorphic. Dendrograms were developed for ISSR and RAPD analysis by using NTSYS-pc software. Dendrogram of ISSR analysis showed 3 clusters while that of RAPD showed 4. Six agronomic values were also used for the characterization of germplasms. Based on these agronomic values, non-hierarchical Euclidean cluster analysis was performed to group the 25 cotton germplasms into 6 clusters. Significant similarities were found in the clustering of ISSR and RAPD analysis, however, correlations were not made between the clustering of ISSR, RAPD analysis and agronomic data analysis.

Keywords: ISSR, RAPD, agronomic values, cotton, germplasm

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Introduction

Cotton, the most important commercial crop of India, occupies largest area (around 8 m ha) in the world but produces around 15.0 m bales (one bale=170 kg) of lint with lowest productivity (322 kg lint/ha). India has unique feature in the cotton cultivation in the sense that all the four species of *Gossypium*, *G. hirsutum*, *G. barbadense* (both tetraploid), *G. arboreum* and *G. herbaceum* (both diploid) and their hybrids are cultivated on commercial scale. During the last 50 years, the productivity has increased from 88 kg to over 300 kg lint/ha due to varietal improvement and development in production technology but genotypic improvement has not taken place to the extent it should have been during these years. This could have been done by utilizing the variability available in the germplasm lines (about 8500 of all four species) collected from India and being maintained by CICR. These germplasm lines have been characterized for morphological and yield parameters and to some extent for insect-pests and disease reaction.

For proper utilization of the elite germplasm in varietal improvement programmes, it must be studied

at molecular level. Study of the existing cotton germplasm at molecular level will help in:

- (i) varietal improvement for agronomically important traits,
- (ii) preservation of the intellectual property right of cotton breeders,
- (iii) identification of the individual cultivar by making a molecular passport,
- (iv) prevention of the duplicate entry of different genotypes in cotton gene pool,
- (v) efficient selection of varieties for F1 hybrid development programme, and
- (vi) taxonomic classification of genotypes on the basis of molecular markers. However, the molecular characterization of cotton germplasm lines has not gained momentum so far in India.

DNA markers have proved to be an efficient tool for the molecular characterization of the plant species¹. PCR based molecular markers, inter simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), STS, AFLP, etc are useful in various applications of plant breeding. Among them, RAPD and ISSR are the arbitrary markers in which only one primer is used.

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ISSR is the PCR based molecular marker technique, which involves amplification of the DNA segment present at the amplifiable distance in between two identical microsatellite repeat regions oriented in the opposite strand. This technique uses microsatellites, usually 16 to 25 bp long, as primers in the single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes². Primers used can be repeats of di, tri, tetra or penta nucleotides anchored with one or two base sequences at 3' or 5' end³. Unanchored primers also can be used⁴⁻⁶. ISSR is the reproducible marker with 92-95 % efficiency². By using the ISSR markers, molecular characterization and genetic diversity of tea has been studied⁷.

Reproducible RAPD markers have been used to study genetic variability and evolutionary basis of the wild species of the genus *Gossypium*⁸. It also proved to be an efficient molecular technique for genetic fingerprinting of the cotton cultivars⁹. Using ISSR and RAPD markers, characterization of the cultivated chestnut (*Castanea sativa* Mill.) has been studied¹⁰.

In the present study, using the two marker systems, ISSR and RAPD along with the agronomic values, efforts were made to characterize the working collection of germplasm based on various parameters. Efforts were also made to find duplicates within the selected germplasm.

Materials and Methods

Cotton Germplasm Used

25 germplasms of *G. hirsutum* from germplasm collection centre, CICR, Nagpur has been used for the present study (Table 1).

Genomic DNA Isolation

A modified procedure of Edwards¹¹ was used for DNA isolation, in which 2% polyvinyl pyrrolidone (PVP) was added in DNA extraction buffer to avoid co-isolation of phenolics and polysaccharides in the DNA.

Fresh leaves (4 g) were frozen in liquid nitrogen and ground to fine powder using pestle and mortar. The powder was transferred to 15 ml of DNA extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 1.32% SDS, 600 µl/100 ml β-mercapto ethanol and 2% PVP). The homogenate was incubated at 65°C for 30 min and cooled to room temperature. After adding 3 ml 5 M potassium acetate, the tubes were kept in ice bath for 20 min. Sample tubes were centrifuged at 15,000 rpm

for 15 min in Sorval centrifuge. The supernatant was transferred to freshly autoclaved 50 ml centrifuge tubes and washed twice with equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with chloroform. 0.8th volume of the isopropanol and 250 µl of 5 M NaCl were added to approximately 12 ml of supernatant and the tubes were kept in deep freezer for 1 hr. To pelletize the DNA, the tubes were centrifuged at 15,000 rpm for 15 min in Sorval centrifuge. The precipitated DNA was re-suspended in Tris-EDTA buffer pH 8.0 (10 mM: 1 mM) and 5-10 µg of RNAase was added to it. The quality and quantity were estimated by measuring OD at 260/280 nm and 260 nm, respectively in UV spectrophotometer. Intactness of genomic DNA was checked on 0.8% agarose gel.

Polymerase Chain Reaction (PCR)

ISSR and RAPD with 25 µl reaction volume were carried out in 200 µl PCR tube. Reaction mixture contained 40 ng of genomic DNA, 2.5 µl of reaction buffer (10X Taq polymerase buffer with 15 mM MgCl₂), 200 µM of each dNTPs (Q-Biogene, USA), 20 ng of 10mer RAPD primers (from Operon Technologies, USA), 15 ng of ISSR primers (synthesized from Bangalore Genei, India), 1U Taq polymerase (Q-Biogene, USA). A negative control, without template DNA was included in each round of reactions. A thermocycler Biometra UNO-Thermoblock was programmed as follows:

PCR Programming for ISSR

An initial denaturing step of 5 min at 94°C, 45 cycles consisting each of a denaturing step of 1 min at 94°C, primer annealing step of 45 sec at 49°C and primer extension step of 2 min at 72°C were given. Final step of 5 min at 72°C was given for polishing the ends of PCR products.

PCR Programming for RAPD

An initial denaturing step of 6 min at 94°C, 36 cycles consisting each of a denaturing step of 45 sec at 94°C, primer annealing step of 1 min at 36°C and primer extension step of 1 min at 72°C were given. Final step of 10 min at 72°C was given for polishing the ends of PCR products.

Agarose Gel Electrophoresis

The products of reaction were electrophoresed on 1.6% agarose gel containing 0.1 µg/µl of ethidium

bromide for about 5 hrs at 60 Volts. Gel was photographed under UV light with Tracktel GDS-2 gel documentation.

Primers Used

ISSR

Out of 45 primers used only 19 were scorable on 1.6% agarose gel. Among the 19 primers, 4 were with 3 bp repeats anchored at 3' end, 12 were 2 bp repeats anchored at 3' end and 3 were 2 bp repeats anchored at 5' end. Following were the sequences:

5'(AGC)5 GA 3'
 5'(AGC)5 GG 3'
 5'(AGC)5 GT 3'
 5'(AGC)5 GC 3'
 5'(CA)7 AT 3'
 5'(CA)7 AC 3'
 5'(CA)7 GT 3'
 5'(CA)7 GC 3'
 5'(CA)7 GA 3'
 5'(CA)7 AA 3'
 5'(GT)7 TA 3'
 5'(GT)7 TG 3'
 5'(GT)7 CA 3'
 5'(GT)7 CT 3'
 5'(GT)7 AT3 '
 5'(GT)7 AC 3'
 5'CAG (GA)7 3'
 5'GCT (GA)7 3'
 5'GCA (GA)7 3'

RAPD

Forty primers of OPA and OPB series from Operon Technologies, USA were used for this study.

Data Analysis

DNA fragment size was estimated by comparing the DNA bands with Lambda/Hind III, pUC18/Sau3A-pUC18/TaqI and 100 base pair ladder (Bangalore Genei, India). Amplified DNA bands were scored on gel under UV Trans-illuminator as 1 for the presence and 0 for the absence of bands and assembled in data matrix table. Pairwise comparisons were calculated using the following coefficient, described by Sneath and Sokal¹²: *Simple matching coefficient*: $S_{ij} = (a+d)/(a+b+c+d)$; *Dice's coefficient*: $S_{ij} = 2a/(a+b+c+d)$, equivalent to the coefficient of *Nie* and *Li*; and *Jaccard's coefficient*: $S_{ij} = a/(a+b+c+d)$. Where S_{ij} is the similarity between two individuals, i and j ; a is number of band present in both i and j ; b is the number of bands present in i and absent in j ; c is

Table 1—Cotton germplasm used for ISSR and RAPD analysis

No.	Accession no.	Germplasm Name/no.	Source
1	JBWR-5	JK285	Dharwad (India)
2	NATP(CLCV) Var-2540	Var-2540	Nagpur (India)
3	JBWR-19	AV3670	MGB Nagpur (India)
4	JBWR-30	ACH-703	Nagpur (India)
5	JBWR-36	G1SC-II-77-4	Surat (India)
6	Acc-163	AR-43	Darsi (India)
7	Acc-166-cyc	AR-46	-do-
8	Acc-167-cy	AR-47	Nandiyal (India)
9	Acc-171	Arkansas 61-28	Arabhavi (India)
10	Acc-173	Arkot	Australia
11	Acc-175	AS 32-59	Karnataka (India)
12	Acc-177	AS-100	Srivilliputtur (India)
13	Acc-181	AS 122	Siruguppa (India)
14	Acc-182	AS 132-59	Gadag (India)
15	Acc-182sp	AS 132-59	-do-
16	Acc-185ccws	Ashmoton	-do-
17	Acc-187	ATH x 444-2	Ivory coast
18	Acc-189	Atlas 59-63	USA
19	Acc-197-B	Austin 64-65	USA
20	Acc-201	B7-955	Indore (India)
21	Acc-206	B55-50	-do-
22	Acc-207	B55-53	-do-
23	Acc-209	B56-33-6	-do-
24	Acc-226	B57-740	-do-
25	Acc-227	B57-819	-do-

the number of bands present in j and absent in i and d is the number of bands absent in both. Jaccard's and Nie & Li's coefficients calculate similarities based on shared presence and not the absence of DNA band. The similarity values found were utilized to group individuals via the Unweighted Pair Group Method (UPGMA). NTSYS-pc¹³ was used to perform all the analysis.

Agronomic Data

Six agronomic values (yield per plant, ginning percentage, boll no. per plant, boll weight, mean hallow length of fibre, and duration of crop) were considered for the characterization of the 25 cotton germplasm. Values of each character from three replications were considered for data analysis. Method for non-hierarchical Euclidean cluster analysis as described by Sneath & Sokal¹² was used for agronomic data analysis.

Results

Out of the 45 ISSR and 40 RAPD primers used, only 19 ISSR and 35 RAPD primers were scorable. All the 19 scorable ISSR primers were reproducible, while out of 35 RAPD primers, only 21 were reproducible; 19 ISSR 21 RAPD primers generated 90 and 150 markers, respectively. Out of them 12 ISSR and 15 RAPD primers were polymorphic and produced 49 and 76 markers, respectively. Size of the amplified products for ISSR and RAPD was 100-1444 and 100-2000 bp, respectively (Figs 1 & 2). The percentage of polymorphic bands for ISSR and RAPD were 54 and 50, respectively (Table 2).

Cluster Analysis

By using UPGMA analysis, dendrogram was obtained for ISSR and RAPD analyses. In ISSR analysis, 25 cotton germplasm were grouped into three major clusters, while 2 germplasms, JBWR-30 (No. 4) and Acc-201 (No. 20) were found placed outside the major clusters and at one end of the dendrogram (Fig. 3). Cluster no. I comprised 10 germplasms, which was highest in number; clusters II and III comprised 5 and 8 germplasms, respectively (from top to bottom in the dendrogram).

The dendrogram of RAPD analysis grouped 25 cotton germplasms into four major clusters. The number of germplasms included in clusters I, II, III and IV were 10, 5, 6 and 4, respectively. Unlike ISSR analysis, the two lines, JBWR-30 (No. 4) and Acc-201, outside the cluster, were found in cluster IV at the bottom of dendrogram in the RAPD analysis.

Major clusters of the ISSR and RAPD analysis were found to be similar, however, arrangement of germplasm within the individual cluster was different. In the cluster I of ISSR dendrogram, the arrangement of germplasm (from top to bottom) was 12, 2, 5, 3, 22, 24, 25, 9, 1, 23, 25. However, in RAPD dendrogram, the pattern was changed. This may be

due to the fact that different marker systems detected different components of DNA variation within the genome.

25 cotton germplasms were also subjected to non-hierarchical Euclidean cluster analysis by taking into account the above mentioned six agronomic values. The analysis showed that 25 cotton germplasms were grouped into 6 major clusters. The cluster IV showed 6 number of germplasms, which were the highest, and cluster III showed 1, the lowest number (Table 3).

Clusters of the agronomic value analysis were found to be completely different from ISSR and RAPD analysis, while the results of ISSR and RAPD analysis showed commonality to a larger extent.

Discussion

Three parameters, ISSR, RAPD analysis and data analysis of agronomic values were used for the

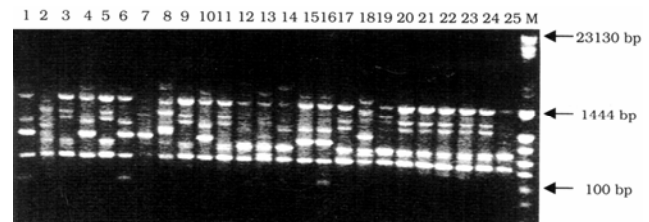


Fig. 1—ISSR amplification of the 25 cotton germplasm (Nos 1-25 listed in Table 1), with primer ISSR-4, M- λ /Hind II, pUC 18/Sau 3A/Taq I

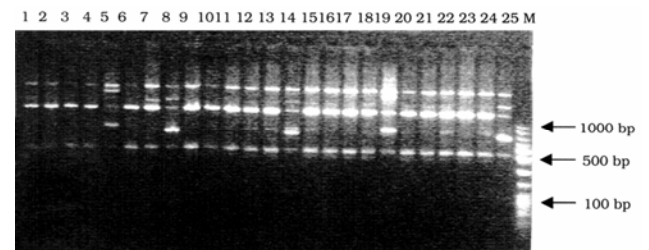


Fig. 2—RAPD amplification of the 25 cotton germplasm (Nos 1-25 listed in Table 1), with primer OPA-8, M-100 bp ladder

Table 2—ISSR and RAPD analysis

No.	Observations	ISSR	RAPD
1	Total no. of primers used	45	40
2	Total no. of scorable primers	19	35
3	No. of reproducible primers	19	21
4	No. of polymorphic primers	12	15
5	Total no. of bands amplified from reproducible primers	90	150
6	Total no. of polymorphic bands identified	49	76
7	Average no. of polymorphic bands per polymorphic primer	4.08	5.06
8	Percentage of polymorphic bands		
9	Size range of amplified products	54.44 % 100-1444bp	50.66 % 100-2000bp

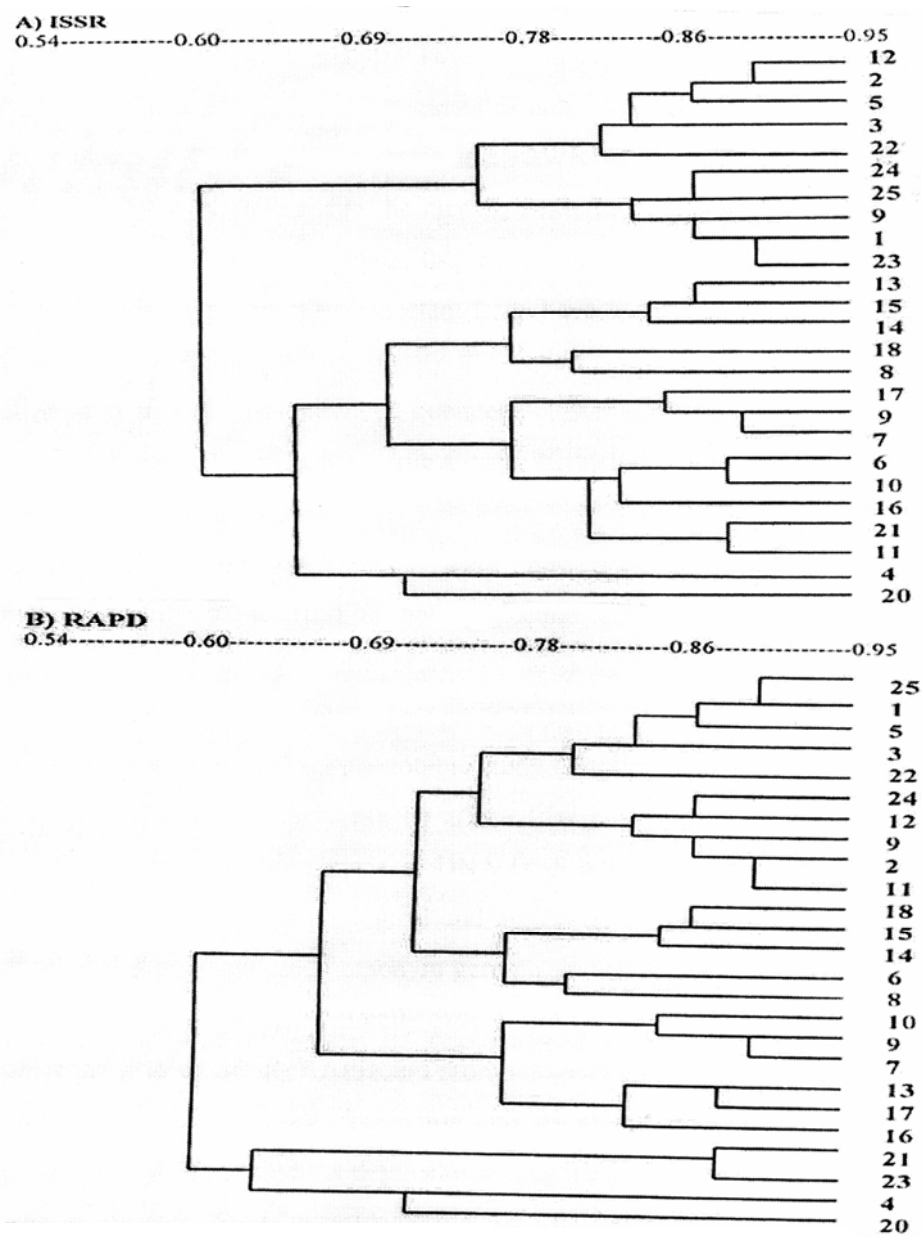


Fig. 3—UPGMA dendrogram obtained by cluster analysis based on A) ISSR and B) RAPD amplified fragments, for twenty five cotton germplasm studied

Table 3—Non hierarchical Euclidean cluster analysis of 25 cotton germplasms

Cluster	I	II	III	IV	V	VI
Germplasm (No.)	Acc-163 (7)	JBWR-5 (1)	NATP	JBWR-19 (3)	JBWR-36 (5)	Acc -171 (9)
		Acc-181 (13)	(CLCV)-2540 (2)	JBWR-30 (4)	Acc-175 (11)	Acc-182 (14)
	Acc-166-cyc (8)	Acc-187 (17)		Acc-163 (6)	Acc-185ccws (16)	Acc-182sp (15)
	Acc-209 (23)	Acc-197-B (19)		Acc-173 (10)	Acc-201 (20)	Acc-189 (18)
		Acc-206 (21)		Acc-177 (12)	Acc-207 (22)	Acc-226 (24)
				Acc-227 (25)		

characterization of 25 cotton germplasms. A dendrogram obtained based on UPGMA analysis showed significant similarity between clustering pattern of ISSR and RAPD analysis, although position of the germplasm within clusters was not found similar as discussed in the result. This may be due to the fact that different markers detected different components of DNA variation within the genome. This lack of consistency between different marker techniques was also observed in soybean¹⁴.

Germplasms, JBWR-30 (No. 4) and Acc-201 (No. 20) did not find place in any cluster of dendrogram obtained from ISSR analysis but they were placed in the cluster IV of dendrogram obtained from RAPD analysis. In both the analyses, however, these two germplasms were placed at one end of the dendrogram, revealing the fact that the genetic distance between two lines was very narrow.

Duplicate entries were the major problem in the germplasm collection and their maintenance. One of the objectives of the present study was to observe duplicates in the germplasms; however, no duplicates were observed. The supposed homonym germplasm accession Acc-182 (No. 14) and Acc-182sp (No. 15), which were very close to each other phenotypically, showed differences at molecular level when analyzed with RAPD and ISSR. The variation at DNA level was shown in genetically close cultivars of tea [*Camellia sinensis* (L.) O. Kuntze] by using ISSR marker⁷. Thus, two marker systems are relevant in the molecular marker studies for detecting polymorphism in the genetically closest individuals.

In comparison to RAPD, Yang *et al*¹⁵ found lower cost and time for ISSR. Moreover, because of the length of the primer and higher annealing temperature, reproducibility was high. RAPD costs are more due to its lower reproducibility and more time consumption and, therefore, not preferred in isolation.

In conclusion, the characterization based on two molecular marker systems (ISSR and RAPD) was faster, less expensive and more reliable as well as more useful in the elimination of duplicates in the germplasm collections. The characterizations based on the agronomic values could be useful in cases when it is necessary to obtain an agronomic description of the germplasms used. The mapped molecular markers closely associated to agronomic values (both single and quantitative trait loci) can,

however, provide the best solution for characterization studies.

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