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Research Article

Analysis of genetic diversity among Asiatic cotton (*Gossypium arboreum* L.) cultivars and breeding lines using RAPD and SSR markers

S.K. Verma[2], Muniya Dhanda[1] and Raj Kumar Salar[1*]

[1]Department of Biotechnology, Chaudhary Devi Lal University, Sirsa – 125 055, India
[2]Central Institute of Cotton Research (CICR), Regional Station, Sirsa – 125 055, India
*Corresponding author

Abstract

Two marker system *viz*., RAPD and SSR were used to generate DNA profiles of 18 genotypes of *Gossypium arboreum* L. (diploid). The frequency of polymorphism among selected varieties of cotton for each type of marker was calculated based on the presence or absence of common bands. The results of cluster analysis separated various genotypes of diploid cotton into different groups on the basis of genetic similarity and divergence. The similarity coefficient based on RAPD and SSR markers ranged from 0.3 to 1.0, thus, suggesting a considerable genetic variation among the cotton varieties studied. The data generated may help in association of these markers with quantitative traits/loci that are agronomically important to cotton industry like fiber characteristics, lint yield, resistance to pests *etc*.

Keywords: Gossypium, genetic diversity, RAPD, SSR

Introduction

Cotton (*Gossypium* spp.) is an economically important crop grown throughout the world. Lint, the most economically important product from the cotton plant, provides a source of high quality fiber for the textile industry. The cotton genus *Gossypium* (Malvaceae) comprises four cultivated species: two New World tetraploid species, *Gossypium hirsutum* L. [n = 2x = 26, (AD)1] and *Gossypium barbadense* L. [n = 2x = 26, (AD)2] and two Old World diploid species, *Gossypium arboreum* L. (n = x = 13, A1) and *Gossypium herbaceum* L. (n = x = 13, A2) (Brubaker *et al*. 1999). Tetraploid upland cotton *G. hirsutum* L. is the predominant cultivated cotton with high yield and wide adaptation accounting for more than 90% of the world cotton production. *G. arboreum* in general is characterized with poor fiber quality traits, not fit for processing on modern processing machines and untill very recently not much attention has been paid towards the fiber quality improvement mainly owing to the fact that superior quality *G. hirusutum* was readily available. Currently, enhanced cotton resources are needed to facilitate the improvement of this important crop. Therefore, an important area of cotton genomics is germplasm characterization and utilization. The existence of genetic diversity in an ecosystem or gene pools ensures the adaptation of species to environmental changes and thus its survival. The existence of genetic variability is a prerequisite for the evolution of superior cotton varieties through selection and hybridization. The variety specific DNA markers in a cotton breeding program for variety registration, plant patents, confirmation of the parentage of hybrids, breeder's right protection and early detection of agronomic and economic traits as an aid to marker assisted selection are very much needed and are widely employed (Asif *et al*. 2009). Therefore, it is imperative to locate and utilize the diversity present in cotton.

During the most part of the last century, cotton breeding had made significant contributions to increase cotton lint yield, improve fiber quality and enhance biotic tolerance. However, the desirable and amenable genetic variations for breeders are limited or lacking or difficult to dissect. Due to the narrow genetic base of cotton germplasm that cotton breeders have been utilizing and low efficiency of traditional selection methods, cultivar improvement in cotton has slowed down. During the past few decades, a number of molecular techniques have been recruited to complement traditional methods for the evaluation of biodiversity, estimation of relatedness and genotype identification.

With the development of the polymerase chain reaction (PCR) technology, various methods are in practice to locate and quantify the extent of existing genetic diversity but the DNA markers such as RAPD, RFLP, AFLP, SSR and EST-SSR are among the most reliable and sophisticated methods (Ahmad *et al*. 2007). The use of molecular markers is very promising for detailed chromosome mapping, gene cloning and tailoring new plant cultivars (Gostimsky *et al*. 2005). The introduction of DNA markers has offered new possibilities for studying varietal/genotypic relationships (Lu and Myres, 2002; Rana and Bhat, 2004; Dongre and Kharbikar, 2004). DNA markers can also be used for the confirmation of the true to type plants regenerated through tissue cultural techniques (Sun *et al*. 2005; Malabadi *et al*. 2006). SSRs are simple tandemly repeated di- to tetra-nucleotide sequence motifs flanked by unique sequences. PCR primers to the flanking regions have been used to identify chromosomal location of SSRs by genetic mapping (Liu *et al*. 2000b). Once generated, they are useful in cloning specific genes of interest and synteny mapping of functional genes in various related organisms. SSRs are popularly used in full genome sequencing and mapping programmes for a number of organisms and for identifying active genes helping in identification of diagnostics markers as has been demonstrated in many plant species including cotton (Nguyen *et al*. 2004). SSRs are employed in genetic analysis of cotton due to their polymorphic nature (Liu *et al*. 2000a; Reddy *et al*. 2001). EST-derived SSRs have been developed for many plants, such as *Triticum aestivum* L. (Gupta *et al*. 2003; Gao *et al*. 2004), *Hordeum vulgare* L. (Holton *et al*. 2002; Thiel *et al*. 2003) and *Gossypium* L. (Saha *et al*. 2003). The RAPD technique has already been used in cotton for genetic diversity studies. Random Amplified Polymorphic DNA (RAPD) is a DNA polymorphism assay based on the amplification of random DNA segments with single primers nucleotide sequence. RAPD have greatly facilitated linkage mapping in cotton (Khan *et al*. 2000). In genetic analysis of traits, DNA markers have special advantages over morphological or cytological basis because DNA markers (i) are highly polymorphic (ii) have no pleiotropic or epistatic effects (iii) enable employment of nondestructive methods and require small amount of plant tissue (iv) are independent of environmental stresses and (v) provide easy access high reproducibility and high genetic resolution.

The present study focused on the characterization of genetic diversity among cotton genotypes, belonging to *G. arboreum*, using RAPD and SSR DNA markers.

Materials and Methods

Plant Material and Chemicals

The Young leaf material of these cultivars was collected from Central Institute for Cotton Research, Regional Station, Sirsa. All chemicals were obtained from M/s Imperial and M/s HiMedia Laboratories Lt., Mumbai, India unless otherwise indicated.

Biological and geographical region of the varieties under study

Diploid (*Gossypium arboreum*) Cotton cultivars were selected for the molecular polymorphism studies as given in Table 1. The genotypes were being taken from various parts of India representing the most of India. They have been developed independently at each Centre. The genotypes from north India (Rajasthan, Punjab and Haryana) were having low fibre quality but higher seed cotton yield per hectare while the genotypes from central (Maharashtra and Gujarat) and southern (Andhra Pardesh, Karnataka and Tamilnadu) part of India had good fibre quality but low yield.

Table 1 List of cultivars of diploid cotton (*Gossypium arboreum* L) used in diversity analysis.

Varieties	Geographical origin
AKA 9620	PDKV, Akola (Maharashtra)
AKA 9703	DKV, Akola (Maharashtra)
PA 532	MAU, Parbhani (Maharashtra)
PA 528	MAU, Parbhani (Maharashtra)
HD 485	CCS HAU, Hisar (Haryana)
LD 960	PAU, Ludhiana (Punjab)
DLSA 826	UAS, Dharwad (Karnataka)
JLA 505	MPKV, ARS, Jalgaon (Maharashtra)
RG 514	RAU, ARS, Sriganganagar (Rajasthan)
GBAV 105	NAU, RCRS, Bharuch (Gujarat)
GBAV 120	NAU, RCRS, Bharuch (Gujarat)
PA 255	MAU, Parbhani (Maharashtra)
J TAPTI 007	MPKV, ARS, Jalgaon (Maharashtra)
SARVOTAM 16	MPKV, ARS, Jalgaon (Maharashtra)
MDL 2617	ANGRAU, RARS, Mudhol (Andhra Pradesh)
JLA 1600	MPKV, ARS, Jalgaon (Maharashtra)
JLA 2300	MPKV, ARS, Jalgaon (Maharashtra)
TKA 9102/3	TNAU, ARS, Kovilpatti (Tamil Nadu)

Genomic DNA Isolation

Young leaf material, 4-5 leaves (10-15 g), were harvested and brought to laboratory on ice. The leaves were transferred to pre chilled pestle and mortar. Then 10 ml of liquid nitrogen was added and leaf material was ground to fine powder. Warm (65°C) CTAB extraction buffer 15.0 ml was added (1M tris HCl (pH 8.0), 5M NaCl, 0.5M EDTA (pH8.0), 1.5 ml ß-mercaptoethanol, 2% CTAB and 1% PVP which was prepared fresh. This was added to 50 ml Oakridge tubes containing 10-15 g sample approximately and mixed well by shaking. This was incubated at 65°C for 1h in the water bath. The cells were thus lysed and proteins denatured. Tubes were removed from water bath and cooled to room temperature for 5 min. Then added 15 ml of chloroform: Isoamyl alcohol (24:1) and mixed by turning gently for 5-10 min followed by centrifugation at 8000 rpm for 10 min. The aqueous layer was poured into new 50 ml Oakridge tubes and 10 ml chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 8000 rpm for 10 min. Now the upper aqueous layer was transferred in 50 ml Oakridge tubes and then 10 ml of chilled isopropanol was added and mixed by gentle inversion to precipitate DNA or leave it overnight at -20°C for precipitation. DNA was spooled out and put into 1.5 ml centrifuge tubes. DNA pellet was made by centrifugation at 5000 rpm. The supernatant was poured off and DNA pellet was washed with 70% ethanol twice by leaving for 20 min. Ethanol was then poured off and the DNA pellet was dried at room temperature for 30 minutes. Ethanol washing helps in the removal of salts. The pellet was dissolved in 150 µl of TE. The dissolved DNA was stored at 20°C for further use. Quality and quantity of DNA were estimated spectrophotometrically and by gel electrophoresis.

PCR amplification

SSR and RAPD amplification were performed using CG1-96 thermocycler (Corbett Research Inc., Australia) and personal thermocycler and *Taq* DNA polymerase (Bangalore Genie, India, Sigma Chemicals, Co., U.S.A). The PCR reaction mixture contained 50 ng of genomic DNA, 1x PCR buffer with $MgCl_2$, 250 µM dNTPs, 10 mM of primer and 1 unit *Taq* DNA polymerase. PCR amplifications using different primers were performed at different temperatures as the primers were grouped according to different annealing temperatures. PCR amplifications were performed with initial denaturation at 94°C for 5 min. followed by 40 PCR cycles [denaturing at 94°C for 30 sec, primer annealing at 52°C (46°C for RAPD primer) for 1 min, and primer extension at 72°C for 1min.]. The final extension was carried out at 72° C for 8 min.

Agarose gel electrophoresis

PCR amplified DNA fragments for RAPD and SSR markers were resolved by submerged horizontal electrophoresis in 1 % (w/v) agarose gels. Agarose was melted in 150 ml of 1x TAE depending upon whether the amplifications were checked on minigel or on the bigger gel. To this 2 µl of ethidium bromide was added. Gel solution was then poured into gel casting tray. After setting of gel, sealed tapes were removed from both the ends. Gel plate was placed in the electrophoresis chamber and submerged using 1x TAE buffer and comb removed gently. Samples were prepared by adding 6x agarose gel loading dye and pulsed centrifuged for proper mixing. Samples were loaded in the wells and electrophoresis was carried out at constant voltage.

Allele Scoring

Polymorphic bands for RAPD and SSR analysis were scored based on the presence or absence of bands for each cotton variety. Only clear and unambiguous bands were scored. The sizes (in nucleotide base pairs) of the most intensely amplified bands were determined based on their migration relative to molecular weight size markers (1 kb ladder or 100 bp, Gibco BRL lnc. USA).

Data Analysis

The frequency of polymorphism was calculated based on the presence or absence of common bands between selected varieties of cotton for each type of marker. To estimate the genetic similarities among the eighteen genotypes, a genetic similarity coefficient matrix based on the Jaccard's coefficient was computed for the construction of phylogenetic tree using 'simqual' sub programme of software NTSYS-PC (Numerical Taxonomy System software, version 2.1).

Results

Polymorphism using RAPD and SSR primers

In the present investigation eleven RAPD and ten SSR primers were used to detect polymorphism in 18 genotypes of Asiatic cotton (*Gossypium arboreum* L.). A total of 61 loci were detected for 11 RAPD primers for 18 genotypes (Table 2). Highest no. of alleles were shown by primer RAPD 6 (4) (Fig. 1). Similarly 94 loci were detected for 10 SSR primers for 18 genotypes (Table 3). Highest no. of alleles was shown by primer MGHES 14 (7) (Fig. 1). However, no amplification was shown by the primers in case of certain varieties. Analysis of the banding profile generated by different primers revealed that the amplification products generally ranged from 300 to 1000 bp in SSR primers and 500 to 1000 bp in RAPD primers.

Table 2 Screening profile of RAPD primers used in diversity analysis of *Gossypium arboreum*

Primer type and number	Primer sequence	Loci amplified	Alleles	Polymorphism (%)
RAPD 2	ACGTAGCGTC	5	1	20
RAPD 3	CTGTTGCTAC	2	1	50
RAPD 4	AAGTCCGCTC	3	1	33
RAPD 5	CCCAGTCACT	6	2	33
RAPD 6	CCACGGGAAG	10	4	40
RAPD7	CAGGACTGAC	7	2	28
RAPD 9	TCCCACGCAA	4	2	50
RAPD 10	TCAGAGCGCC	5	2	40
RAPD 13	GTCAGAGTCC	7	3	42
RAPD 15	TGGCGTCCTT	3	0	0
RAPD 16	TCGGCGGTTC	3	1	33

Table 3 Screening profile of 10 SSR primers used in diversity analysis of *Gossypium arboreum*

Primer type and number	Forward Primer: (5'-3') Sequence	Reverse Primer (5'-3')	Loci amplified	Alleles	Polymorphism (%)
SSR-BNL252	TGAAGAGCTCGTTGTTGC AC	CGAAAGAGACAAGCAATGCA	8	0	0
SSR-BNL1053	AGGGTCTGTCATGGTTGG AG	CATGCATGCGTACGTGTGTA	10	0	0
SSR-BNL1721	TGTCGGAATCGGAAGACC GG	GCGCAGATCCTCTTACCAAA	11	3	27
SSR-BNL2572	GTCCTATTACTAA AATTGTTAATTCAGCC	CGATGTTAAATCAATCAGGTCA	9	2	22
SSR-MGHES6	TCGCTTGACTTTCCATTTC C	AACCCTCGGGATTATCGTCT	7	1	14
SSR-MGHES13	CAGGGGAGCCATTGTTAG AA	CAGGGGTCCTGTGTTTCAGT	13	4	37
SSR-MGHES32	CGTCGCTTCCTTTGCTTAA C	GTCGGGTTAATTGCAAATCG	7	0	0
SSR-MGHES14	GAGGAGGCTGTGGTTGAA GA	ATGGTGACCCTGCTTACACC	14	7	50
SSR-MGHES16	ACCCCAATACAACCCCAT TT	GCAGAGAAAAGGGACAGAGG	8	0	0
SSR-MGHES35	TCGAACGGCTCGTTAAAT CT	CAGCAAAGAGTGGTTCTCTGG	7	4	57

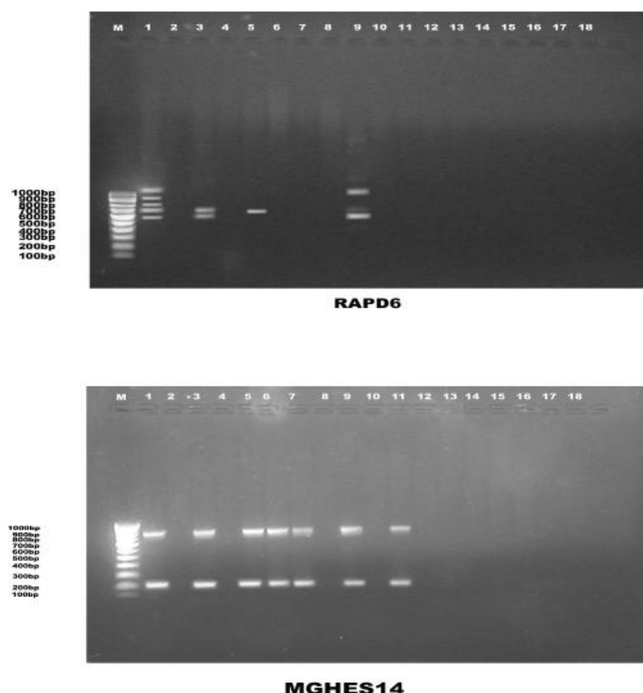

Figure 1 Agarose gel electrophoresis of RAPD 6 and MGHES 14 showing banding pattern among 18 Asiatic cotton varieties.

Clustering of Cotton species based on 11 RAPD Primers

Figure 2 shows the dendrogram chart of the genetic similarity and diversity among 18 cotton cultivars of *G. arboreum*. The range of dendrogram was 0.22 to 1.00 coefficient. There were two major clusters (A1 & A2) and majority of cultivars (17 out of 18) fall in group A1 and this was further divided into sub clusters and their further sub clusters. Cluster A2 had a single variety RG 514 which was genetically different from cultivars of second cluster A1. There were two major sub clusters of A1 (B1 & B2). AKA 9620 was a single cultivar of sub cluster B1 which was genetically different from B2 sub cluster cultivars. Varieties AKA 9703, LD 960, DLSA 826, TKA 9102/3, JLA 505, JLA 1600, MDL 2617, SARVOTAM 16, J TAPTI 007 and GBAV 105 could not be distinguished among themselves. Other cultivars of this sub cluster (B2) PA 528, PA 255, JLA 2300, PA532, GBAV 120, HD 485 were genetically close to each other.

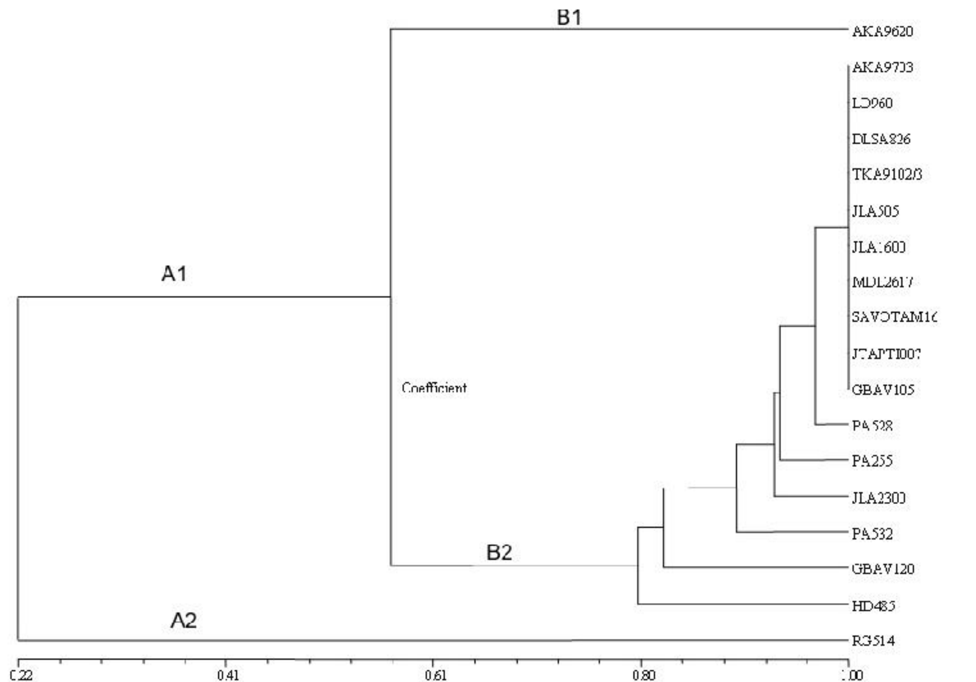

Figure 2 Dendrogram of Cotton cultivars (*Gossypium arboreum*) based on RAPD primers using UPGMA cluster analysis

Clustering of Cotton species based on SSR primers

Dendrogram chart in Fig. 3 exhibit the genetic similarity and diversity among 18 cotton cultivars of Asiatic cotton *G. arboreum* using 10 SSR primers. The range of dendrogram was 0.43 to 1.00. There were two major clusters A1 and A2. These clusters were further divided into sub clusters and further sub clusters. One major sub cluster (B2) of group A1 contained a single variety RG 514 which was genetically different from another sub cluster of this group. Varieties AKA 9620, LD 960 and GBAV 120 showed the great genetic similarity while other varieties of sub cluster B1 (PA 532, HD 485, DLSA 826) were genetically different from each other. Second major cluster was divided into two major sub clusters (C1 & C2) based on genetic diversity. JLA 505 was single variety in major sub cluster C2 which was genetically different from the C1 group varieties. Varieties GBAV 105, PA 255, SARVOTAM 16, TKA 9102/3, JLA 1600, MDL 2617 could not be distinguished among themselves. Other varieties of this sub cluster C1 (AKA 9703, PA 528, J TAPTI 007) were genetically close to each other. But the inter cluster variability was significant and indicated that different groups have originated from different genetic sources and regions. Analysis of SSRs depends on variety and frequency of microsatellite distribution in the genome of a particular variety.

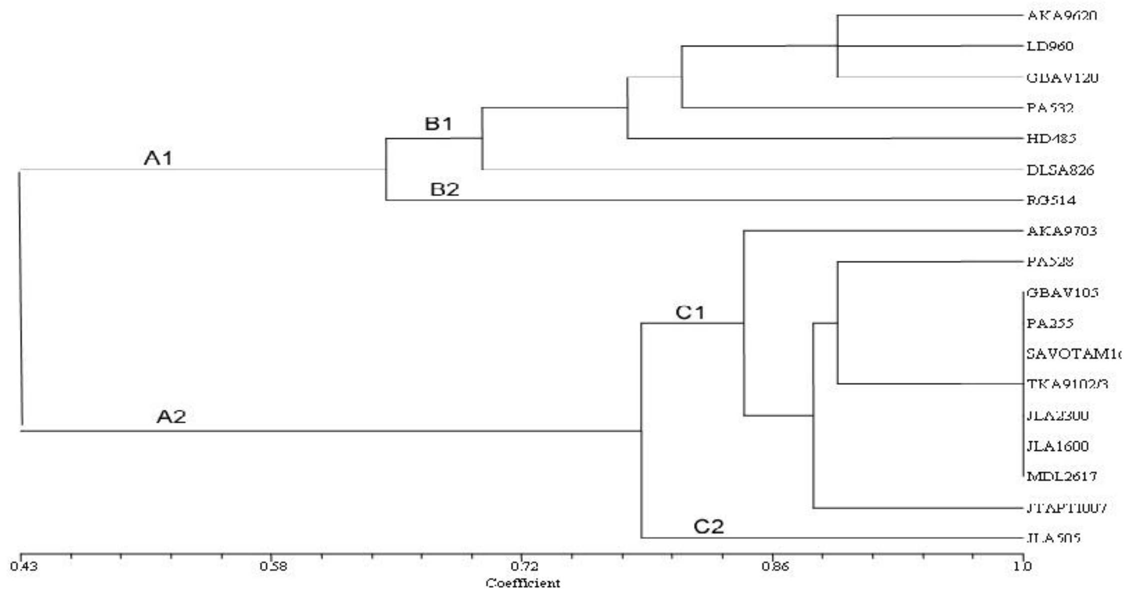

Figure 3 Dendrogram of Cotton cultivars based on SSR primers (*Gossypium arboreum*) using UPGMA cluster analysis

Clustering of Cotton species based on RAPD and SSR primers

Figure 4 depicts the dendrogram chart indicating the genetic diversity and similarity among 18 cotton varieties based on assessment by both RAPD and SSR markers. The range of dendrogram was 0.3 to 1.0. There were two major clusters A1 and A2. Majority of the cultivars (17 out of 18) fell in the group A1 and this was further divided into sub clusters and their further sub clusters. Major sub cluster A2 contained a single cultivar RG 514 which had a great genetic distance from other cultivars. Major cluster A1 was further divided into sub clusters (B1 & B2). In sub cluster B1, the cultivar AKA 9620 and HD 485 were genetically close. Sub cluster B2 was further divided into many sub clusters. Cultivars GBAV 105, SAVOTAM 16, MDL 2617, TKA 9102/3 and JLA 1600 could not be distinguished among themselves. AKA 9703, PA 255, J TAPTI 007, JLA 2300, PA 528 and JLA 505 also fell in the same sub cluster and were genetically close to each other. Cultivars LD 960 and DLSA 826 showed a greater genetic similarity. Other cultivars of this sub group PA 532 and GBAV 120 were also genetically related and fell into the same sub cluster. However, the inter cluster variability was significant and indicated that different group have originated from different genetic sources and regions.

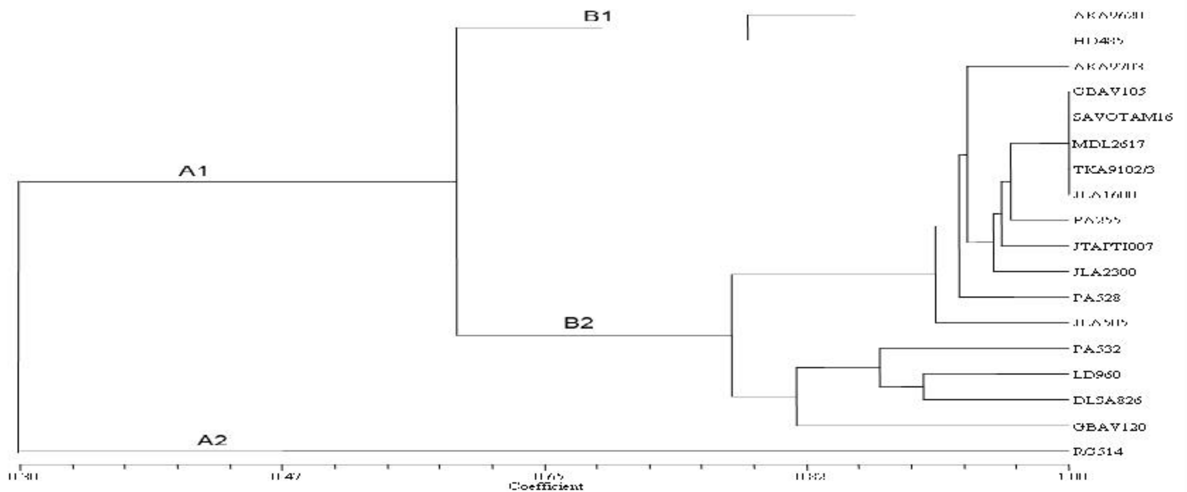

Figure 4 Dendrogram of Cotton cultivars (*Gossypium arboreum*) based on RAPD and SSR primer using UPGMA cluster analysis

The dendrogram constructed from the fingerprint data obtained showed considerable diversity among 18 cotton cultivars of *G. arboreum*. The cultivars AKA 9620 & HD 485 showed 80% similarity. The cultivars PA 255, J TAPTI 007, JLA 2300, PA 528 & JLA 505 showed 82% similarity. GBAV 105, SAVOTAM16, MDL2617, TKA 9102/3 & JLA 1600 were genetically same and could not be distinguished among them. PA 532, LD 960, DLSA 826, GBAV 120 showed 82% genetic similarity. Cultivar RG 514 showed only 30% genetic similarity with other cultivars.

Discussion

Molecular markers are becoming increasingly important in theoretical and applied genetic research (Park *et al*. 2005). In the present investigation, microsatellite markers analysis showed 0-57 % polymorphism indicating high genetic diversity in 18 accessions studied. A novel segregation distortion in intraspecific population of Asian cotton (*G. arboretum* L.) has been detected using SSR and SRAP markers (Li *et al*. 2007). The use of SSR and RAPD markers for cotton genomic research has been reported earlier by Reddy *et al*. (2001) and Rana and Bhat (2004). Recently, Yu *et al*. (2011) developed 3177 new EST-SSR markers for constructing a high genetic linkage map in *G. hirsutum* and *G. barbandense*. High genetic maps can be used for further genetic analysis of quantitative traits, marker-assisted selection and genome organization architecture in cotton as well as for comparative genomics between cotton and other species. Cotton SSR and RAPD fingerprints which have been generated can be considered as a base line towards assessment of genetic diversity. Fingerprint of cotton varieties generated in the study can be used for conclusive identification of specific hybrids as the information of diversity obtained may be used for future varietals development efforts targeted for improved cotton yields.

Genetic mapping is an important tool for understanding the cotton genome and facilitates genomic research. Further investigations with additional SSR and RAPD would help in increasing the working knowledge of genetics of cotton. This will help in association of these markers with quantitative traits/ loci that are agronomically important to cotton industry like fiber characteristics, lint yield, resistance to pest *etc*. The data generated in the present investigation has direct application for seed service agencies and can help the agricultural community towards higher economic gains.

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