Identification of Cotton (Gossypium spp) Genotypes by Inter Simple Sequence Repeat (ISSR) Markers

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Abstract- *Cotton (Gossypium spp) is one of the most important commercial fiber and oil yielding crop in India. Genetic variability and relationship between varieties are of important for cotton breeding. ISSR markers were used for identification and genetic diversity analysis of cotton genotypes. 10 cotton genotypes were subjected to ISSR analysis using 10 ISSR primers. PCR products were subjected to 1.0% agarose gel electrophoresis and banding patterns were compared 10 ISSR primers produced 1069 bands, with 141 polymorphic markers, which showed 13.2% polymorphism. The ISSR primer IS-07 produced polymorphic marker a all the genotypes studied as it had generated 7 unique bands which could identify different genotypes. A dendrogram constructed from ISSR data classified 10 cotton genotypes into two major clusters with average similarity of 68% and the similarity between the genotypes varied with a range of 68 to 99%. Among the 10 ISSR primers studied, 8 primers could identify the genotypes individually. ISSR technique was thus found to be efficient method for detecting DNA polymorphism useful for varietal identification in cotton genotypes.*

Keywords- cotton. ISSR-varietal identification

I. INTRODUCTION

Cotton (Gossypium spp.) is considered as hub of textile industry, which provides the world's most important natural textile fibre and is the second most important oil seed crop in the world. It is grown every continent except Antarctica and in over 100 countries around the world. In many countries cotton is one of the primary economic bases, providing employment and income for millions of people involved in its production, processing and marketing (United Nations, 2003). Taxonomically cotton belongs to the family Malvaceae and genus Gossypium. The genus Gossypium includes 49 species (Percival and Kobel, 1990). Four of these are cultivated, 43 are wild diploid and two are wild tetraploid. Of the four cultivated species G. hirusutum and G. barbadense are tetraploid and commonly known as New World Cotton whereas, G. arboreum and G. herbaceum are diploid and known as Old World Cotton (Noormuhammadi et al., 2011).

Cotton is grown globally over 33.14 m ha with a total production of 116.6 million bales of 489 lb and the productivity of 760 kg/ha (Anon., 2013). India is the second largest cotton producer in the world after China with cultivable area of 115.53 lakh ha with production of 375 lakh bales of 170 kg and the productivity of 552 kg/ha (Anon, 2013).

India is the only country in the world cultivating all the four cultivated species of cotton on commercial scale. The varieties belongs to herbaceum group are much finer, longer and is staple than the arboreum varieties and it is cultivated in part of Maharastra, Gujarat, Tamil Nadu, Andhra Pradesh and Karnataka states. The hirsutum group are medium to long stapled and are much finer than those of other groups and are grown in the states of Punjab, Haryana, Tamil Nadu, Andhra Pradesh, Karnataka, Maharastra, Madhya Pradesh, Gujarat and Rajasthan.

Crop improvement programs in India have generated large member of varieties in the last 30 years. With the proliferation of newly developed varieties, identification of these varieties and maintaining their seed lots has become major concern.

Characterization of varieties provides description of the material essential for their identification, conservation, management, utilization in crop improvement programmes, identification of suitable lines for breeding purpose and to avoid duplication of varieties. Characterization of genotype is required for their protection under plant variety protection (PVP) legislation, because varietal testing for Distinctiveness, Uniformity and Stability (DUS) is the basis for grant of protection of new plant varieties under the protection of Plant Varieties and Farmer's Rights Act, 2001 and also provides useful information for understanding the genetic diversity and structure of various cotton gene pools found in different geographic regions.

Varieties can be identified by different methods like morphological, chemical, biochemical and molecular methods. Morphological markers viz., leaf size, leaf colour, leaf hairiness, plant morphology, pollen colour, petal colour and fruit characters etc. has been used for varietal characterization. Though the morphological characterization is simple and

irreplaceable, their expression is influenced by the environment leading to errors in scoring.

Biochemical analysis of seed storage protein show promising results in characterization of genotypes and purity determination of cotton varieties and hybrids. Biochemical markers viz., isozymes and protein may also be biassed since a small portion of genome is represented by these markers and exhibits low polymorphism.

Molecular marker is powerful PCR based technique, indicating the differences in the nucleic acid sequences at a particular location or locations in the genome. Molecular markers have high discrimination power, enabling detection of closely related genotypes and they are efficient tool for characterization of the plant species. Various PCR based molecular markers are Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Sequence Tagged Micro Satellite (STMS) etc., are useful in various applications of plant breeding.

Among the various molecular markers, ISSR is the arbitrary marker in which only one primer is used. 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. The polymerase chain reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. Primers can be repeats of di, tri, tetra or penta nucleotides anchored with one or two base sequences at 3 or 5 end. ISSR is the reproducible marker with 92 to 95% efficiency. The present work was designed to study varietal characterization and genetic relationship among the ten genotypes of cotton using ISSR markers.

II. MATERIALS AND METHODS

Plant material

The experimental material consisted of 10 genotypes of cotton (Gossypium spp.) which were collected from Central Institute for Cotton Research (CICR), Nagpur, India.

DNA extraction

The cotton plants were grown in two rows of 6 m length with 45 cm space between the plants in a completely randomized design with three replications. Single plant having fresh and young leaves, was selected randomly from any of the three replicates of each genotype. Fresh leaves (4-5 g) were collected and powdered in the presence of liquid nitrogen in a pre-chilled pestle and morter. DNA extraction buffer containing 2% polyvinylpyrrolidone (PVP) was added to avoid co-isolation of phenolics and polysaccharides in the DNA.

Leaf tissues were cut into small pieces, homogenized and digested with extraction buffer (pH = 8.0): [1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, 2X CTAB, 4% PVP and -mercaptoethanol]. After incubation at 65 C in water bath for an hour, the mixture was emulsified with an equal volume of phenol, chloroform, Isoamyl alcohol (25:24:1). Equal volume of ice-cold iso-propanol was added to precipitate DNA and centrifuged for pelleting. The pellets were washed with 70% alcohol, air-dried and resuspended in 100 l of TE buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8.0) and finally treated with 1 l of RNase. Quantity and quality of extracted DNA was estimated spectrophotometrically and by gel electrophoresis.

A total of ten ISSR primers synthesized from Bangalore Genei Pvt. Ltd. were used. List of ISSR primers used in this study was given in Table 1.

Molecular analysis

ISSR amplification

ISSR amplification was performed in 25 l reaction volume containing 40 ng genomic DNA, 2.5 l reaction buffer (10X Taq polymerase buffer with 15 mM MgCl2), 200 M of each dNTPs, 15 ng of ISSR primers, 1.5 U Tag polymerase. An initial denaturing step of 5 min at 94 C followed by 40 PCR cycles (denaturing at 94 C for 1 min, primer annealing at 60 C for 1 min and primer extension at 72 C for 1 min). A final step of extension at 72 C for 8 min was carried out for polishing the ends of PCR products. The PCR amplification was carried out in a thermal cycler (Thermal cycler gradient, Eppendorf).

Resolution of PCR products

Amplified PCR products of ISSR were separated on 1.0 per cent agarose gel containing 0.1 ig/il of ethidium bromide for about 5 hours at 60 volts. Gels were photographed under UV light with gel documentation system. Fragment size was estimated by using 1 kb molecular size ladder.

Data analysis

Comparison of genotypes was carried out based on the presence and absence of fragment produced by ISSR amplification. The presence of each band was scored as 'I' and it absence as 'O'. The data matrix was read by NTSYS-PC version 2.02 (Numerical Taxonomy and Multivariate Analysis System for Personal Computer) developed by Rohlf (2004) and

analysed by SIMQUAC (similarity for qualitative data) program with Jaccard's similarity coefficient. The SIMQUAC is a program for computing a variety of similarity and dissimilarity coefficients for qualitative data. The qualitative nature of the absence (O) or presence (I) state of ISSR marker was used as the basis for similarity analysis among various cotton genotypes. A matrix of O and 1 act as the input and the output matrix of similarity or dissimilarity coefficients. The resultant similarity matrix was entered into SHAN (sequential, agglomerative, hierarchical and nested clustering method) clustering program, a tree matrix was produced and dendrogram was constructed using UPGMA. Primer banding characteristics such as total number of bands, number of plymorphic bands, number of monomorphic bands and percentage of polymorphic bands were obtained.

III. RESULTS AND DISCUSSION

The results obtained in the present study are given below:

Initially 10 ISSR primers were screened and all the 10 ISSR primers produced scorable bands. All the ten ISSR primers were polymorphic one. A total of 1069 ISSR markers were amplified, out of which 141 were polymorphic markers with 13.2 per cent polymorphism. Average number of polymorphic loci amplified per primer was 14.1. The size of ISSR amplicons was between 250 in HB 807 to 4500 bp in 1S-10.

The number of polymorphic markers generated by each primer was given in Table 2. ISSR allelic profiles are shown in Figs. 1, 2.& 3) The ISSR primers 1S-05 and Cot-1 generated the maximum number (123) of polymorphic markers and the ISSR primer 1S-15 generated the least number (73) of polymorphic markers. The ISSR primers IS-07 and Cot-3 generated highly polymorphic profile. The ISSR primer 807 produced low polymorphic profile. None of the primers individually was so informative as to differentiate all the studied genotypes. The ISSR primer 1S-07 was polymorphic in all the genotypes and produced seven unique bands in genotypes like Kanchana, G.Cot-20, MCU-10, NH-545, JK-4, khandwa-3 and khandwa-2. The primer IS-10 generated one specific band at 2500 bp for the genotype Kanchana. The primer Cot-1 generated a specific band at 1500 bp for the genotype Kanchana. The primer Cot-2 generated one specific band at 3000 bp for JK-4. The ISSR primer Cot-3 generated a specific band at 4000 bp for Kanchana. The ISSR primer HB-8 generated a specific band at 4000 bp for Kanchana. The primer HB-1 generated one specific band at 1700 bp for khandwa-3.

Cluster analysis

Nei and Lei (1979) coefficient method was used to generate similarity matrix (Table 3). 62 to 98% similarity was speckled between genotypes through pair wise combination and 98% similarity has been observed between genotypes Anjalai and G. Cot 20. The genotypes Khandwa-3 and Anjali showed 96% similarity. The genotypes Khandawa-3 and G. cot-20 showed low dissimilarity of 5 percentage.

The genotypes Kanchana and KC-3 were found most dissimilar with the dissimilarity coefficient of 62% followed by Khanchana and G. Cot-20 with the dissimilarity coefficient of 67 percentages.

The dendrogram was constructed for 10 cotton genotypes using UPGMA method Liu et al. (2005). The cotton genotypes were grouped into two main clusters I and II with an average similarity of 68% (Fig. 4). The cluster 1 consisted of Kanchana, which was placed outside the major clusters at one end of dendrogram. The cluster II consisted of 9 genotypes and these were divided into two sub clusters II A and II B. The sub cluster II A consisted of 8 genotypes and these were again further divided into 2 sub subclusters II Ai and II Aii. The sub subcluster II Ai was grouped into 7 genotypes viz., Khandwa-2, Khandwa-3, Anjali, G. cot.-20, NH-545, MCU-10 and JK-4. The sub subcluster II Aii consisted of only one genotype G. cot.-15. The sub subcluster II B also consisted of only one genotype KC-3. The similarity between genotypes varied with a range of 68 to 99%.

IV. DISCUSSION

A number of DNA markers have been used to study the extent of genetic variation in a number of crops and differ mainly in their principles and engender varying amounts of data. The present study was designed to explore genetic diversity among the ten cotton genotypes using ISSR markers.

In the present study, 10 ISSR primers were used to check polymorphism among the 10 genotypes of cotton and all the ten primers were polymorphic one. The 10 ISSR primers generated 1069 markers for the assessment of genetic variability between the genotypes studied. The number of ISSR marker ranged from 73 as produced by the primer 1S-10 to 130 as yielded by primer HB-1 among the 10 genotypes studied. Out of 1069 ISSR markers amplified, only 141 were polymorphic which resulted 13.2% polymorphism. All the ten primers produced polymorphic markers, however the level of polymorphism percentage assorted with each primer range from 02.00 to 23.3%. All the genotypes showed a varying degree of genetic diversity based on their amplification profile. The average percentage of polymorphism was 13.2. A low level of polymorphism was examined among all the genotypes in the study. Different similar and contradictory research findings reported by Chowdhary et al. (2002), Dongre et al. (2007),

Hussain et al. (2006), Sharf et al. (2009) and Parkhiya et al. (2014). Chowdhary et al. (2002) showed low level of intra specific polymorphism in chickpea. Dongre et al. (2007) examined 19 ISSR primers, which generated 90 markers in which 12 ISSR primers were polymorphic which produced 49 markers and average percentage polymorphism was recorded 54.77. Hussain et al. (2006) used 12 ISSR primers to estimate the genetic relationship among 21 cotton genotypes and produced 125 amplicons with 49.6% polymorphism. Sharf et al. (2009) studied 10 ISSR primers, which amplified 70 fragments, in which 62 of them were polymorphic with 88.5 per cent polymorphism. Parkhiya et al. (2014) studied genetic diversity in 15 cotton genotypes by ISSR markers and obtained 86 reproducible bands out of 54 were polymorphic with 62.7% polymorphism. A low level (13.2%) of polymorphism was examined among all the genotypes in the study. This study showed narrow genetic base in the material under investigation, which might be due to almost similar genetic makeup of genotypes.

The amplified ISSR fragments were in the range of 250 bp to 4000 bp. The largest fragment of 4000 bp and the smallest fragment of 250 bp were amplified by Cot-3 and Cot-3. Contradictory and similar findings were reported by Dongre et al. (2007), Noormohammadi et al. (2013) and Salunkhe et al. (2009). Dongre et al. (2007) worked with 19 ISSR primers, which generated 90 markers in which 12 ISSR primers were polymorphic and produced 49 markers in cotton with size ranged between 1000 bp and 1444 bp. Noormohammadi et al. (2013) observed that 17 of 20 HOMO-ISSR-ISSR and hetero-ISSR primers produced 206 reproducible fragments. The sizes of fragments obtained were ranged 250 to 2600 bp. Salunkhe et al. (2009) worked with 55 ISSR primers which generated 101 ISSR markers, in which 83 were polymorphic with size ranged between 100 to 3000 bp.

Similarity index and cluster analysis was done by Jaccard's coefficient and UPGMA using NTSYSpc-2.02e software, respectively. The dendrogram was generated by using Jaccard's coefficient values (Fig. 3) to estimate the genetic similarity among cotton genotypes. Jaccard's coefficient of similarity between 10 cotton genotypes ranged from 62 to 98%. Dongre et al. (2007) reported that Jaccard's coefficient of similarity between 25 cotton germplasms ranged from 60 to 95%, which supported the results obtained in the present study. The ISSR diversity analysis suggested, the genotypes Kanchana, G.Cot-15 and KC-3 were to be most variable genotypes.

The cluster analysis showed 62 to 98% similarity was speckled between genotypes through pair wise combination and 98% similarity has been observed between genotypes Anjalai and G. Cot 20. The genotypes Khandwa-3 and Anjali showed 96% similarity. The genotypes Khandawa-3 and G. cot-20 showed low dissimilarity of 5 percentage.

The conclusion drawn from the present investigation are as under.

1. 10 ISSR primers selected in the present study generated 12.6% polymorphism and all the 10 ISSR primers produced polymorphism. These ISSR primers can be used for further study in different cotton genotype.
2. 10 ISSR primers produced very low level of polymorphism (16.2%) is due to narrow genetic base of genetic material studied.
3. Among the ten ISSR primers tested, the ISSR primer IS-07 generated 7 unique bands in 7 different genotypes. Hence, ISSR primer IS-07 can be used for varietal identification these cotton genotypes.

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Table 1. List of ISSR primers and their sequences

Sl. No.	ISSR primer	Sequence 5'- 3'	Sequence 5'- 3'
1.	IS-05	(CA)6 AT	CACACACACACAAT
2.	IS-07	(CA)6 GT	CACACACACACAGT
3.	IS-10	(CA)6 AA	CACACACACACAAA
4.	IS 15	(GT)6 AT	GTGTGTGTGTGTAT
5.	Cot-1	(GA)7 T	GAGAGAGAGAGAGAT
6.	Cot-2	(GT)7 CA	GTGTGTGTGTGTGTCA
7.	Cot-3	(AGC)5 GA	AGCAGCAGCAGCAGCGA
8.	HB 08	(GA)6GG	GAGAGAGAGAGAGG
9.	HB10	(GA)6CC	GAGAGAGAGAGACC
10.	807	(AG)7GT	AGAGAGAGAGAGAGAGT

Table 2. ISSR bands and their characteristics generated by 10 ISSR PRIMERS

Sl. No.	*1) Primers*	Total number of bands	Number of polymorphic bands	Percentage polymorphism	Band size
1.	IS-05	123	3	2.50	550 - 2500
2.	IS-07	89	20	23.00	500 - 2000
3.	IS-10	90	11	12.20	280 - 2500
4.	IS-15	73	3	4.10	600 - 2000
5.	Cot-1	123	24	20.00	350 - 3000
6.	Cot-2	119	10	8.50	400 - 3000
7.	Cot-3	120	28	23.30	250 - 3000
8.	HB-1	120	20	16.70	300 - 2000
9.	HB-8	100	20	20.00	400 - 1800
10.	807	112	2	2.00	250 - 2000

Table 3. Genetic similarity matrix

	Kanchana	Khandwa2	Khandwa3	JK-4	Anjali	G.Cot-20	G.Cot-15	KC-3	NH-545	MCU-10
Kanchana	1.0000									
Khandwa2	0.7500	1.0000								
Khandwa3	0.6875	0.8889	1.0000							
JK-4	0.6667	0.8714	0.9000	1.0000						
Anjali	0.6790	0.8767	0.9577	0.9143	1.0000					
G.Cot-20	0.6707	0.8649	0.9444	0.9014	0.9859	1.0000				
G.Cot-15	0.6711	0.8028	0.8571	0.8382	0.8714	0.8592	1.0000			
KC-3	0.6234	0.7746	0.8286	0.8636	0.8429	0.8310	0.8182	1.0000		
NH-545	0.6914	0.8649	0.9178	0.9014	0.9583	0.9452	0.8333	0.8310	1.0000	
MCU-10	0.6988	0.8442	0.8947	0.8533	0.9333	0.9211	0.8133	0.7867	0.9467	1.0000

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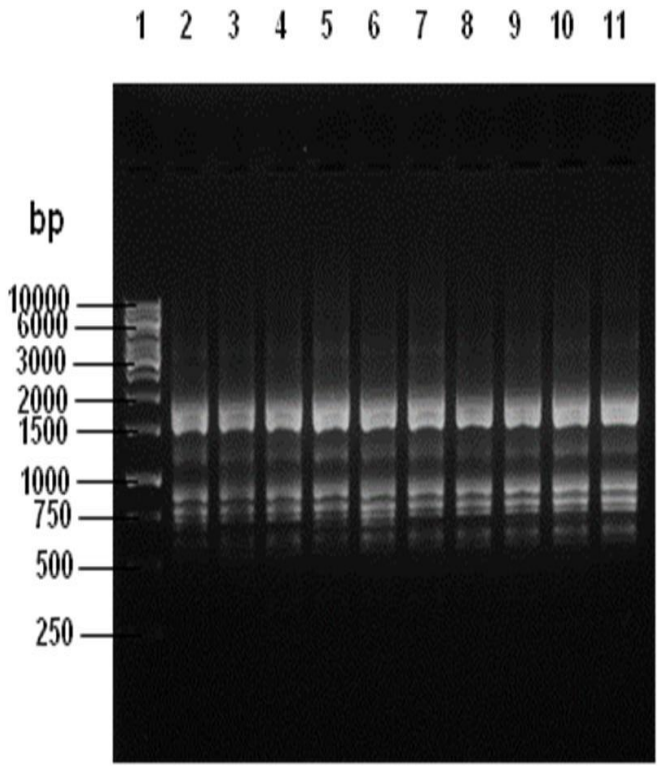
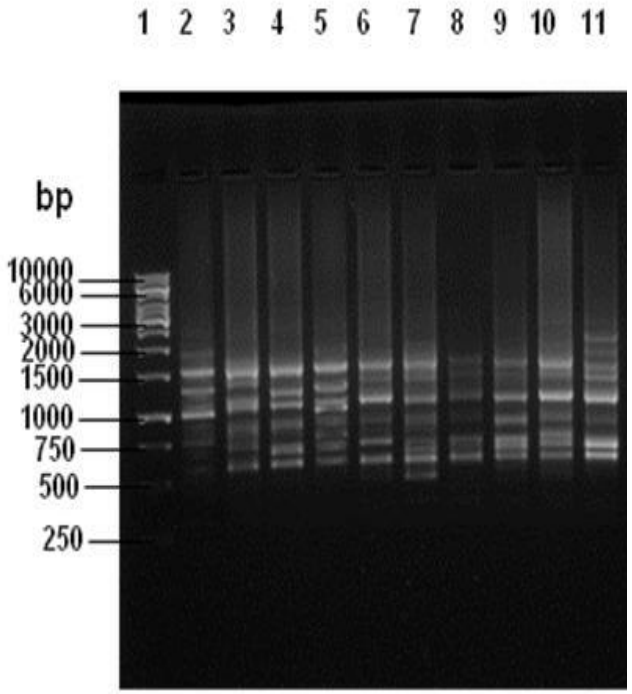


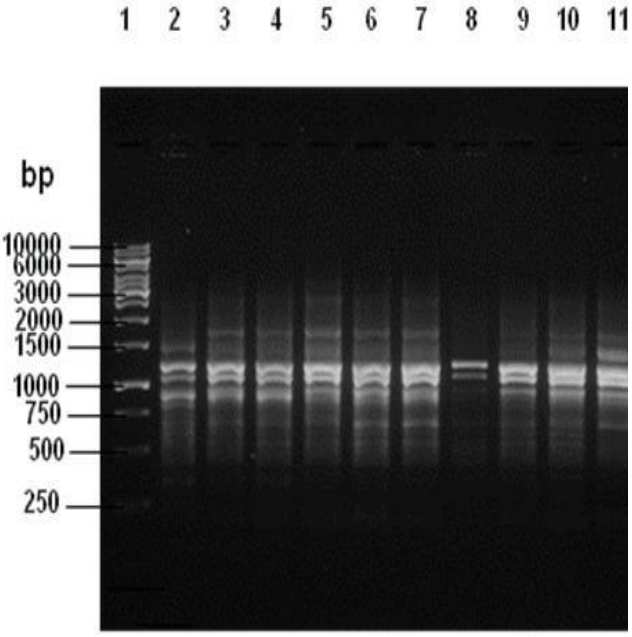
L1 - 1Kb DNA Ladder; L2 – Khanchana; L3 - Khandwa-2; L4 - Khandwa – 3; L5 - JK4; L6 – Anjali; L7 - GCot20; L8 - GCot15; L9 - KC3; L10 - NH545; L11 - MCU -10

Fig. 1. ISSR profile analysis of ten cotton genotypes with ISSR primer IS – 05



L1 - 1Kb DNA Ladder; L2 – Khanchana; L3 - Khandwa-2; L4 - Khandwa – 3; L5 - JK4; L6 – Anjali; L7 - GCot20; L8 - GCot15; L9 - KC3; L10 - NH545; L11 - MCU -10

Fig. 2. ISSR profile analysis of ten cotton genotypes with ISSR primer IS – 07



L1 - 1Kb DNA Ladder; L2 – Khanchana; L3 - Khandwa-2; L4 - Khandwa – 3; L5 - JK4; L6 – Anjali; L7 - GCot20; L8 - GCot15; L9 - KC3; L10 - NH545; L11 - MCU -10

Fig. 3. ISSR profile analysis of 10 cotton genotypes with ISSR primer Cot-1

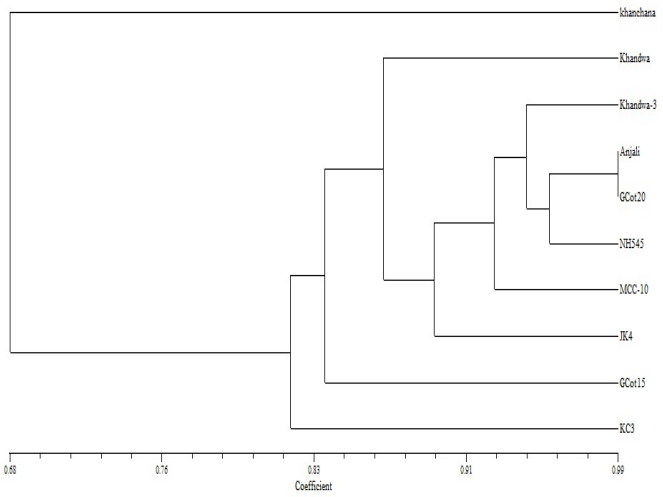


Fig. 4. Dendrogram of 10 cotton genotypes developed from the ISSR data using UPGMA