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STUDY OF GENETIC VARIABILITY AMONG UPLAND COTTON (*GOSSYPIUM HIRSUTUM* L.) CULTIVARS OF DIVERSE GENETIC ORIGIN

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ABSTRACT

Seventeen genotypes viz., CIM707, 4-F, RH1, NIAB111, MS40, MNH552, CIM443, BH118, CIM1100, CIM473, CIM482, NNH554, CIM448, CIM240, CIM435, NNH93 and NIAB78 of *Gossypium hirsutum* L. were collected from different cotton research organizations of Pakistan. The current study was conducted at Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad during the year 2017. These varieties were grown and used for molecular studies using SSR and RAPD markers to amplify the genomic DNA. In SSR analysis, 45 primers were found polymorphic while in RAPD analysis only 14 primers found polymorphic. Maximum polymorphism was revealed by BNL and NAU primers whereas minimum polymorphism was revealed by JESPER and CIR primers. It was also found that no single DNA primer could deduct polymorphism among all genotypes specifically while several primers amplified polymorphic DNA fragments among few genotypes. It is obvious from similarity matrix that the genetic similarity among seventeen genotypes ranged from 37.21 to 86.05%. The highest similarity was found between CIM1100 and CIM 448 (86.05%) while NIAB111 and RH1 were most dissimilar (37.21%). The dendrogram proved that the majority of the genotypes were clustered into three groups. However, cotton genotypes i.e. CIM707, CIM240, CIM443, CIM473, CIM435 and CIM482 were clustered in group A; CIM 1100, CIM448, MNH 552, and MNH 554 were clustered in group B, whereas NIAB 78 and MNH93 in group C. The four genotypes viz: MS40, 4F, NIAB111 and RH1 remained ungrouped. They do not form any cluster with other genotypes and maintained their individuality. This highlights that mostly cultivated species has relatively narrow genetic base might be due to employing similar parents for the development of new cultivars at a particular breeding institute. Thus there is a prime need for expending the genetic base of most commonly cultivated cotton through hybridization among less commonly cultivated species.

KEYWORDS: cotton germplasm; RADP ; SSR markers; primers; diversity; Pakistan.

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INTRODUCTION

Pakistan has agro based economy largely depends upon cultivation of cotton crop. Cotton (*Gossypium hirsutum* L.) is currently being grown on an area about 3.1 million hectares resulting in output of about 11.7 million bales (Anonymous, 2007a) . China occupies a leading position followed by USA and India with production of 6.07, 4.46 and 4.36 million metric tons respectively (Anonymous, 2007b), while Pakistan ranked at 4th position in production point of view i.e. 2.26 million metric tons. The market price of lint cotton and its fabrics products exported to various countries each year is about Rs. 215 billion (Anonymous, 2007a) and thus, it accounts for 11.5% share in value added products in agriculture sector.

Significant advancement has been made in cotton breeding research regarding improvement in yield, quality, resistance to insects and diseases, earliness and adaptability but still lot of research work is needed in the aspect of yield per unit area. Per unit increase in production is possible by utilizing prevailing genetic information regarding diversity. Evolution of improved varieties with more cotton lint yield per unit area along with satisfactory fibre quality is a continuous prime objective of every cotton breeder. Plant breeders extensively and repeatedly have used similar parents in breeding programs to release new cultivars which have resulted in narrow genetic base in crop plants (Hallauer and Miranda, 1988). Furthermore, the problem was multiplied because of reluctance of breeders to use parents of diverse origins because of unfavourable linkages (Percival and Kohel, 1990) that results in low yield (Robinson *et al.*, 1997). A divergent genetic base of cultivars has been suggested to be an important element in controlling disease (Garrett and Mundt, 1999; Zhu *et al.*, 2000) or insect pests. Moreover, it is widely accepted that future improvements will be based on the information about genetic diversity (Thormann *et al.*, 1994).

Although conventional breeding methods have contributed much to the development of cotton cultivars, however the use of molecular markers has been proposed as an alternate procedure (Toby *et al.,* 1999) for identification of promising parental lines. Moreover, DNA markers can be used for DNA fingerprinting of genotypes to quantify the genetic variations among them and to tag useful genes. In past decade and currently, different genetic markers (plant morphological traits and biochemical contents concentration) are being utilized to find out genotypic diversity but variable results were obtained due to changing environment along with limitation of less number of loci (Tanksley *et al.,* 1989). Thus, it necessitates the utilization of DNA based molecular markers due to increased reliability in finding out genotypic variability and genetic architecture. At present, molecular assisted cotton breeding programs are routine features in developed countries but in Pakistan these are not being utilized due to lack of funds and proper infrastructure.

PCR based techniques are simple and are used routinely. Moreover, RADPs and SSRs marker systems were used in present study to analyze the molecular differences among parents and identification of their hybrids. In general terms, RAPDs and SSRs based markers are very simple, quick and relatively cheaper technique and utilize small quantity of DNA. Hence, in present study, RAPDs and SSR based markers were utilized to estimate genetic variability among diverse cotton varieties useful in cotton breeding programs.

MATERIALS AND METHODS

The research work reported here was carried out in the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad in 2017. The seed of 17 varieties/genotypes were obtained from the Central Cotton Research Institute Multan, Cotton Research Institute Faisalabad and Plant Breeding & Genetics Department, University of Agriculture Faisalabad and grown in the experimental site of Plant Breeding & Genetics Department of University of Agriculture, Faisalabad. Fresh and delicate leaves were transported to the laboratory and stored at -80°C for utilization in DNA extraction. Moreover, DNA was extracted by using CTAB method (Doyle and Doyle, 1990) and spectrophotometer was used for measuring DNA concentration in a sample. Quality of DNA was observed by running 50 ng DNA on 0.8% agarose gel. Confirmation of DNA quantity was also done by equating with Quantification Standards Phage λ DNA (Fermentas) on 0.8% agarose gel. Dilutions of 15 ng/µl were prepared from the stock solutions. First the PCR was started for the parents to find the polymorphic primers. A total of 40 primers were used in PCR. A list of selected RAPD primers and their level of polymorphism is given in Table 1.

Table 1. Summary of polymorphism and RAPD markers

S. No	Primers	Total No. of bands amplified	No. of polymorphic bands	%age of polymorphism
1	GL DecamerA-4	12	5	33
2	GL DecamerA-7	9	5	55
3	GL DecamerA-8	5	2	40
4	GL DecamerA-9	8	2	25
5	GL DecamerA-11	11	8	70
6	GL DecamerA-12	9	4	33
7	GL DecamerA-13	8	4	50
8	GL DecamerA-15	7	2	28
9	GL DecamerA-17	6	2	33
10	GL DecamerA-19	4	3	75
11	GL DecamerC-3	5	4	75
12	GL DecamerC-5	7	2	28
13	GL DecamerC-8	5	2	40
14	GL DecamerC-16	6	4	66

Amplification of products were analyzed by electrophoresis and viewed under ultraviolet tran illuminator and photographed using the Syngene Gel Documentation System. Good quality photographs were used to read the amplification profiles. Bands of less than 20 bp were in some cases difficult to score and were not considered. All visible fragments were counted for each primer and robust polymorphic bands were scored as present (1) or absent (0) for each primer. The data of the primers were used to estimate genetic similarity and genetic distance coefficients on the basis of number of shared amplification products (Nei and Lis, 1979). Similarity coefficients were utilized to generate a dendrogram by means of unweighted pair group method of arithmetic means (UPGMA). The coefficients were calculated by the following statistical equation.

$$F = 2N_{AB} (N_A + N_B)$$

However, 100 microsattellite/STS primers were used in the study to find out the polymorphism among the parents and identification of their hybrids. List of SSR

primers and their sequences, used in the study is given as **Table 1**. Further moer, 15 pairs of SSR primers used in the study were selected from the JESPR series (Gene Link, US), 30 SSR primers from BNL (Brookhaven National Laboratory, New York, USA) series, 15 from CIR series (Cotton International Research, France), 30 from NAU series (Nanjing Agriculture University, China), and 10 STS primers were obtained from Cotton Molecular Breeding Laboratory with the courtesy of Dr. Peng Chee, Associate Professor, Department of Crop and Soil Science, University of Georgia, USA. The primers for the study were selected on the basis of their location to cover the whole cotton genome. For this purpose cotton genomic maps constructed by Nguyen *et al.* (2004); Shen *et al.* (2005); Zhang *et al.* (2005); Han *et al.* (2006), were consulted and followed. The emphasis was to select primers after each 10-20 cM in each chromosomal map. Primer names, source, chromosome number and sequences are also available from the website of cotton micro satellite database (www. cottonmarker.org).

Moreover, PCR amplifications of the SSR_s were performed in 67 mM of Tris- HCl (pH 8.8), 16mM of $(NH4)_2$ SO_4, 2.5 mM of $MgCl_2$, 0.2 mM, of dNTPs 0.6µM of primers with 0.5 units of Taq DNA polymerase enzyme and 20 ng genomic DNA per 10 µl reaction (Zhang *et al.*, 2005) . Amplification was performed in Eppendorf DNA Thermal Cycler 9600 programmed for a first denaturation step of 2 minutes at 95°C (intial denaturation) followed by 30 cycles of 45 seconds at 94°C (denaturation), 54°C for 45 seconds (primer annealing) and 72°C for 60 seconds (amplification or extension). The reactions were kept at 72°C for 7 minutes for final extension step. PCR products of SSR were run on 8% polyacrylamide gels, using a Bio Rad gel apparatus. The gel was pre-run for about twenty minutes before loading the sample. About 4 µl of the PCR products were loaded after mixing 3-4 µl gel loading dye (Bromophenol blue) in the reaction of 10µl volume. It was run about 2 hours at 160 volts till the dye went out of the gel. After electrophoresis the gel was separated from the plates and treated for 12 minutes in fixation solution (10% v/v ethanol and 0.5 v/v acetic acid) with gentle shaking followed by incubation for 12-15 minutes in 0.2% w/v silver nitrate solution for staining. After staining, the gel was washed twice with deionized distilled water for two minutes and with 0.0002% w/v sodium thiosulphate for two minutes and then the gel was transferred to developing solution (1.5% sodium hydro oxide and 0.4% formaldehyde) to develop the silver staining DNA bands. The scoring of such markers was done in a way that individual plants having banding pattern were scored as 1 and the plants showing no banding were scored as 0.

Some SSR primer pair's amplified polymorphic fragments of co-dominant nature i.e. amplified the DNA fragments from both parental DNAs but of different molecular weight and hybrid (F_1) had DNA fragments of both molecular weights. The scoring of such markers was done in a way that the F_2 individual plants having banding pattern like parent-1 was scored as 3, like parent-2 as 1 and the plants showing pattern like hybrid were scored as 2 (3 1 2). In SSRs dominant markers the individual plants having banding pattern like parent-1 was scored as 1, like parent-2 as 0 and the plants showing pattern like hybrid were also scored as 1 (1 0 1).

RESULTS AND DISCUSSION

RAPD techniques were used to assess genetic diversity among seventeen cotton varieties/genotypes taken out of 59 originally collected for study. Parentage, year of release and centre of origin of these seventeen cultivars are given in Table 3.

These techniques were also used for identification of parental lines and their hybrids. DNA was extracted from fresh young leaves of the genotype using the procedure given by Doyle and Doyle (1990). It was run on 0.8% agarose gel to check its quantity. DNA samples showing smear on gel or with low concentration were rejected and DNA was extracted again. PCR conditions were optimized with respect to concentration of genomic DNA, 10x PCR buffer, dNTPs, primers and Taq DNA polymerase. Concentration of DNA samples was measured through spectrophotometer. Dilutions of DNA was made as 15ng/µl and tested on 0.8% agarose gel for confirmation of DNA concentration and quality. DNA samples representing unequal concentration on the gel were made equal and again checked by running on gel. Some times DNA samples represent poor fluorescence, concentration of such DNA samples was enhanced to make them equal and then used in PCR for detection of genetic diversity and for identification of parental lines and their hybrids through RAPD and SSR markers. Similarly Taq DNA polymerase and $MgCl_2$ with different concentrations were used to optimize the PCR conditions in order to obtain bright RAPD banding pattern. A total of 40 RAPD primers were screened initially. Fourteen informative primers (Table 1) represent polymorphism among the parents which were selected for further study based on good banding pattern. All the selected random decamer primers produced polymorphic bands which varied with each primer. A polymorphic band was in one or more but not in all individuals and unique bands were present at least one individual and not in any other. Total of 95 RAPD markers were generated

from the 14 selected random decamer primers for assessing genetic variability between the genotypes studied. Forty RAPD markers out of ninty five used for amplification were found polymorphic, resulted in 42% polymorphism. Although 14 primers produced polymorphic amplification products, however the extent of percent polymorphism varied with each primer from 17 to 80%. All genotypes showed a varying range of genetic variability.

Microsatellite markers are preferred in plant breeding as they are reliable, reproducible, easy to use, cost effective and highly informative. Moreover, 100 SSR primers were used in present study for molecular picturization of parents and identification of their hybrids. The list of SSR primers used in this study along with their sequences is given as Table 1. Forty five percent primers showed polymorphism among the parents (Table 2). Maximum number of polymorphic primers was observed in BNL (Brook Haven National Laboratory, New York) series and NAU (Nanjing Agriculture University, China) series and minimum number was observed in JESPR (Jenkins, S. Saha, A. Pepper from Texas A and M and USDA) and CIR (Cotton International Research, France). BNL and NAU primer series were higher in amplification than the amplification by JESPR and CIR series. NAU series were superior in polymorphism but BNL series were superior in nature of polymorphic DNA fragments as they produced comparatively more co-dominant markers.

Saventeen genotypes of *Gossypium hirsutum* L. viz; CIM-240, CIM-448, CIM-1100, CIM-443, CIM-473, CIM-482, CIM-435, 4-F, MNH-93, MNH-552, MNH-554, MS-40, NIAB-78, NIAB-111, CIM-707, RH-1 and BH-118 were selected from different parts of Pakistan (Table 3).

Table 2. Summary of the polymorphism and SSR markers

S. No	Primer Series	Primers used	No. of polymorphic primers	%age of polymorphic primers	No. of co-dominant markers
1	BNL	30	18	60	12
2	NAU	30	18	60	11
3	CIR	15	3	20	2
4	JESPR	15	3	20	1
5	MUSS	10	4	40	2

Table 3. Centre of development, year of release and parentage of selected cotton genotypes/strains used in molecular studies

S. No.	Cultivar	Parentage	Year of release	Center of origin
1	CIM-240	CIM-70 × W 1106	1992	CCRI, Multan
2	CIM-448	(W 1104 × S12) × CP 15/2 (Sister line of CIM-1100)	1996	CCRI, Multan
3	CIM-1100	(W 1104 × S12) × CP 15/2 (Sister line of CIM-488)	1996	CCRI, Multan
4	CIM-443	CIM-109 × LRA-5166	1998	CCRI, Multan
5	CIM-473	CIM-402 × LRA-5166	2002	CCRI, Multan
6	CIM-482	CIM-229 × CP 15/2	2000	CCRI, Multan
7	CIM-435	CP-15/2 × 491/87 (W1104 × CIM-83)	NA	CCRI, Multan
8	4-F	Introduction from America	1914	CRI, Faisalabad
9	MNH-93	149 F × (MS 39 × MEX 12)	1980	CRS, Multan
10	MNH-552	MS-48 × LRA-5166	NA	CRS, Multan
11	MNH-554	MNH-439 × LRA-5166	2000	CRS, Multan
12	MS-40	Selection of AC 252	1970	CRS, Multan
13	NIAB-78	AC-134 × Delta Pine 16s	1983	NIAB, Faisalabad
14	NIAB-111	NIAB-78 (Pollen radiated 5 Gy “co) × reba p-288	2003	NIAB, Faisalabad
15	CIM-707	CIM-240 × CIM-488	2000	CRI, Faisalabad
16	RH-1	ND	SA	CRS, Rahim Yar Khan
17	BH-118	BS-48 × 829-4/90 (Tx-339 × ST-7A) × (ST-7A × AET-5)	2000	CRS, Bahawalpur

These genotypes were used for molecular analysis to assess genetic diversity and relatedness among them. A total of 40 RAPD and 100 SSR primers were used to amplify the genomic DNA of the 17 genotypes. Out of 40 RAPD markers 16 were monomorphic but remaining 24 primers showed polymorphism among the genotypes. In SSR analysis, 45 primers were found polymorphic among the genotypes. Maximum polymorphism was revealed by BNL and NAU primers, whereas, minimum polymorphism was revealed by JESPR and CIR primers. It was observed that single primer could detect DNA polymorphism among all the genotypes while many primers amplified polymorphic DNA fragments among few genotypes only. Out of the total DNA fragments, 59.17 % were polymorphic. Maximum number of 12 fragments was amplified with primer GLA-04 and a minimum number of four bands were amplified with primer GLA-19. Moreover the size

of DNA fragments was in range of 3.6 to 4.2 kb and the smallest easily recognizable fragment amplified was approximately 0.3 kb. The genomic DNA of the CIM-707 amplified the maximum number of (350) DNA fragments, while the minimum number of (327) DNA bands were amplified from genomic DNA of the Genotype RH-1. It is noticeable from the similarity matrix in Table 4 that range of 37.21 to 86.05% genetic resemblance was observed among 17 genotypes. The highest genetic similarity was found between CIM-1100 and CIM-448 which were 86.05 % similar. NIAB-111 and RH-1 were most dissimilar (37.21%). The dendrogram (Fig. 1) verified that the bulk of the genotypes were clustered in three groups. The first cluster (A) comprised three sub clusters. In first sub cluster, the genotypes CIM-707 and CIM-240 formed a sister group relationship.

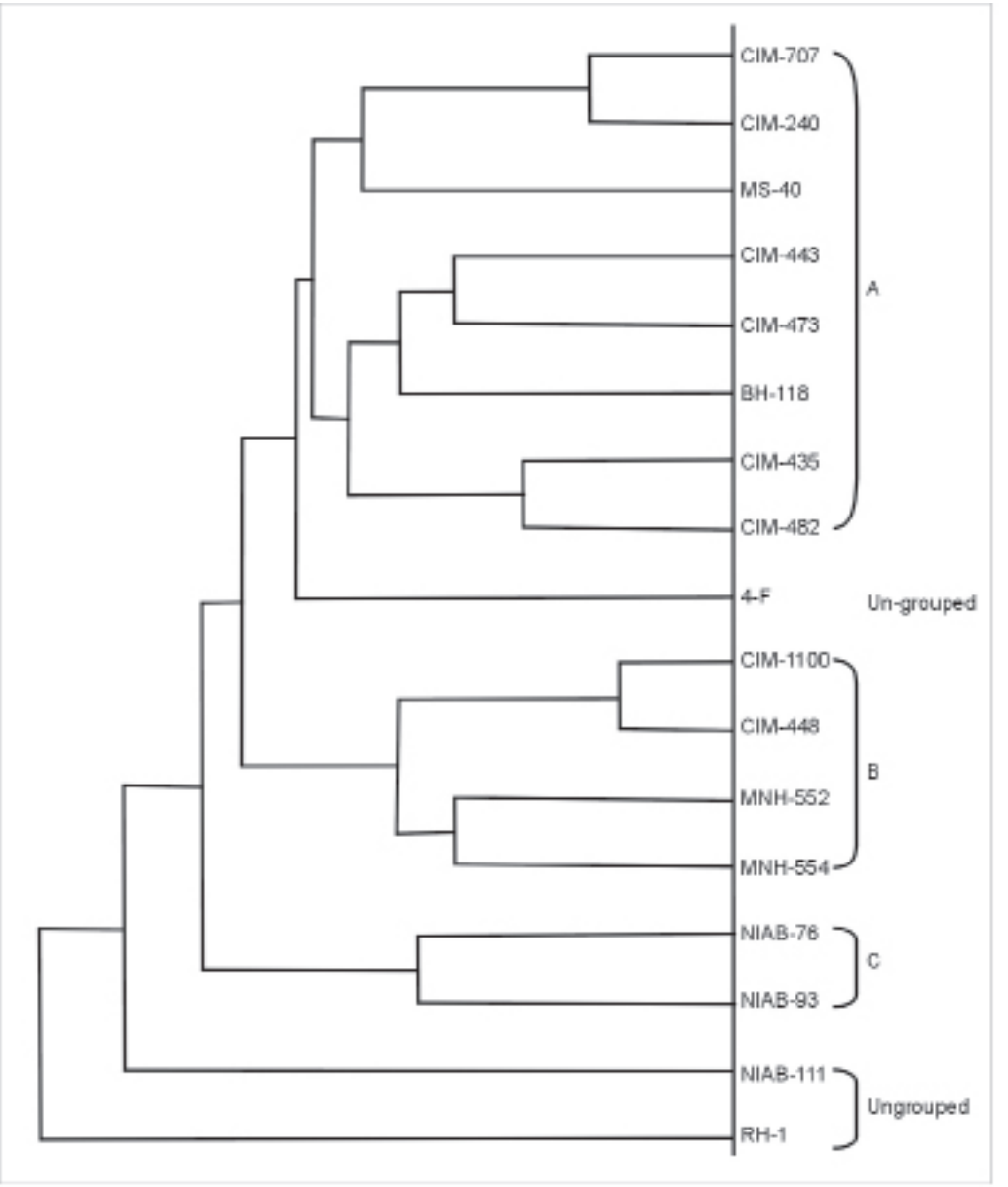

Fig. 1. Dendrogram of seventeen cotton genotypes, showing genetic relationship based on UPGMA method

Similarly, in the second sub cluster group CIM-443 and CIM-473 formed adjoining group relationship. In the third sub cluster CIM-435 and CIM-482 formed a sister group relationship. In the first cluster (A), MS-40 and BH-118 did not show close relationship with any sub group. The second cluster (B) comprised of two sub clusters. In the first sub cluster CIM-1100 and CIM-448 formed a sister group relationship. Similarly in second

sub cluster, MNH-552 and MNH-554 formed a sister group relationship. The third cluster group (C) has only one sub cluster group. In this sub cluster group NIAB-78 and MNH-93 formed a sister group relationship. The four genotypes viz; MS-40, 4-F, NIAB-111 and RH-1 remained ungrouped. They do not form any cluster with other genotypes and maintained their individuality. In this study a high level of average genetic similarity (86.05%) was found among material under study and it ranged from 37.21 to 86.05% among the genotypes. It showed narrow genetic base while in conventional study highly significant differences were observed among genotypes. This high level of average genetic similarity is, might be, due to similar genetic makeup of the genotypes. At maturity, genotypes in the field may differ in traits like number of bolls, seed cotton yield and boll weight etc. because difference in one gene locus may create variability among the genotypes. Rahman *et al.* (2002) reported 89.55% average genetic similarity among 27 cotton genotypes while in other study Vafaie *et al.* (2003) observed 79% average genetic similarity in the Indian tetraploid *Gossypium hirsutum* L. cotton cultivars. Linos *et al.* (2002) found a genetic identity ranging from 61% to 92% in 28 upland cotton cultivars, indicating a relatively narrow genetic base. Rana and Bhat (2005) observed 74% average genetic similarity among 41 *Gossypium hirsutum* L cultivars.

Table 4. Genetic similarity matrix among 17 cotton genotypes of diverse origin using SSRs markers.

	Varieties	1	2	3	4	5	6	7	8	9
1.	CIM-707	****	0.7209	0.6047	0.6977	0.7209	0.6977	0.7442	0.6744	0.6744
2.	CIM-443	0.3272	****	0.6977	0.6977	0.7209	0.7442	0.6512	0.6279	0.6279
3.	CIM-435	0.5031	0.3600	****	0.5814	0.7907	0.6744	0.5814	0.6047	0.6512
4.	CIM-1100	0.3600	0.3600	0.5423	****	0.6047	0.6279	0.8605	0.6512	0.6047
5.	CIM-482	0.3272	0.3272	0.2348	0.5031	****	0.6512	0.6512	0.5814	0.5814
6.	CIM-473	0.3600	0.2955	0.3939	0.4654	0.4290	****	0.6744	0.6047	0.6977
7.	CIM-448	0.2955	0.4290	0.5423	0.1503	0.4290	0.3939	****	0.6512	0.6047
8.	NIAB-78	0.3939	0.4654	0.5031	0.4290	0.5423	0.5031	0.4290	****	0.6279
9.	MS-40	0.3939	0.4654	0.4290	0.5031	0.5423	0.3600	0.5031	0.4654	****
10.	NIAB-111	0.4654	0.5423	0.5831	0.5031	0.4654	0.5831	0.5031	0.7167	0.8168
11.	4-F	0.4654	0.3039	0.4290	0.5831	0.3939	0.5031	0.6702	0.6257	0.654
12.	MNH-93	0.4654	0.7176	0.5831	0.4290	0.6257	0.7655	0.3600	0.3272	0.3939
13.	MNH-552	0.6257	0.5423	0.5831	0.3600	0.7167	0.6702	0.4290	0.5423	0.7167
14.	MNH-554	0.2955	0.3600	0.3939	0.2647	0.3600	0.3272	0.3272	0.5831	0.5031
15.	RH-1	0.6702	0.5831	0.8168	0.4654	0.7655	0.7167	0.5423	0.7655	0.4290
16.	BH-118	0.3272	0.3272	0.4290	0.4290	0.3939	0.3600	0.4290	0.4654	0.6257
17.	CIM-240	0.1777	0.2955	0.3939	0.3939	0.2955	0.4654	0.4654	0.4290	0.3600
	Varieties		**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1.	CIM-707		0.6279	0.6279	0.6279	0.5349	0.7442	0.5116	0.7209	0.8372
2.	CIM-443		0.5814	0.6744	0.4884	0.5814	0.6977	0.5581	0.7209	0.7442
3.	CIM-435		0.5581	0.6512	0.5581	0.5581	0.6744	0.4419	0.6512	0.6744
4.	CIM-1100		0.6047	0.5581	0.6512	0.6977	0.7674	0.6279	0.6512	0.6744
5.	CIM-482		0.6279	0.6744	0.5349	0.4884	0.6977	0.4651	0.6744	0.7442
6.	CIM-473		0.5581	0.6047	0.4651	0.5116	0.7209	0.4884	0.6977	0.6279
7.	CIM-448		0.6047	0.5116	0.6977	0.6512	0.7209	0.5814	0.6512	0.6279
8.	NIAB-78		0.4884	0.5349	0.7209	0.5814	0.5581	0.4651	0.6279	0.6512
9.	MS-40		0.4419	0.6279	0.6744	0.4884	0.6047	0.6512	0.534	0.6977
10.	NIAB-111		****	0.5349	0.5349	0.4884	0.6047	0.3721	0.5349	0.6047
11.	4-F		0.6257	****	0.5814	0.5814	0.047	0.5581	0.6279	0.6977
12.	MNH-93		0.6257	0.5423	****	0.5814	0.5581	0.6047	0.6279	0.5581
13.	MNH-552		0.7167	0.5423	0.5423	****	0.0.7442	0.5116	0.5814	0.5116
14.	MNH-554		0.5031	0.5031	0.5831	0.2955	****	0.5349	0.6977	0.7209
15.	RH-1		0.9886	0.5831	0.5031	0.6702	0.6257	****	0.4186	0.5814
16.	BH-118		0.6257	0.4654	0.4654	0.5423	0.3600	0.8708	****	0.5581
17.	CIM-240		0.5031	0.3600	0.5831	0.6702	0.3272	0.5423	0.5831	****

CONCLUSION

The research work presented in this manuscript indicated that generally most of present cultivated species have a relatively slight genetic base due to continuously utilization of common varieties for pedigree creation helpful in evolution of new cotton cultivars. Thus, there is a great desire and need of the time to extend the genetic base of cotton varieties through hybridization breeding employing less commonly cultivated genotypes and even species.

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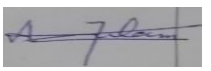
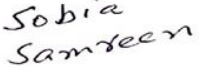
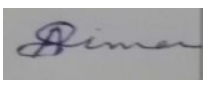
1.	Khalid Hussain	Planned and conducted the research work	
2.	Iftikhar Ahmad Khan	Supervisor	
3.	Ghulam Abbas	Planned and contributed in research methodology	
4.	Sobia Samreen	Assisted in material and method section	
5.	Asima Batool	Critically reviewed the manuscript	
6.	Nasira Perveen	Cooperated in review of literature citation