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Genetic diversity assessment of cotton (*Gossypium hirsutum* L.) genotypes from Pakistan using simple sequence repeat markers

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Abstract

Knowledge of genetic diversity is important for successful genetic improvement programs. Thus, the genetic diversity in 50 representative Pakistani cotton cultivars was studied using 70 simple sequence repeat (SSR) primer pairs. The 70 selected SSR primer pairs generated a total of 241 SSR alleles, of which 147 (60 %) were found to be polymorphic, resulting in 57.5 % polymorphism, and the average number of polymorphic alleles per primer was observed to be 2.10. The pairwise genetic distances between individuals were calculated by the percentage disagreement method, and the resulting matrix showed a mean genetic similarity of 0.315 among the 50 genotypes, revealing a very high level of genetic relatedness. Regarding the pairwise combinations, the genetic similarities between genotypes varied from 0.1 between CIM-446 and CIM-448 to 0.53 between CIM-482 and MNH-6070 and also STAPM-82 and AYT-85094. A UPGMA cluster analysis revealed inconsistencies in the clustering patterns, but did not precisely reflect the origins of the populations, as each cluster consists of cultivars released from different breeding origins. The results suggest that SSR markers are efficient for measuring genetic diversity and relatedness and in identifying varieties of cotton. These results suggest a need for further evaluation of the level of molecular genetic diversity through the application of additional markers for upland cotton genome to improve resolution, as well as effective use of these tools in breeding programs. Such efforts will address the current concerns on the narrowness of the genetic base of widely grown Upland cotton cultivars.

Keywords: Cotton markers; SSRs; Genetic diversity; Microsatellite.
Abbreviations: AFLP, amplified fragment length polymorphism; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat; UPGMA, Unweighted Pair-group Average Percent Disagreement; CTAB, cetyltrimethylammonium bromide.

Introduction

Cotton (*Gossypium spp*.) is the most extensively used natural fiber in textile manufacturing and is one of the most abundantly grown oilseed crops, with a yearly average range from $27 –29 billion worldwide from lint fiber production (Campbell et al., 2010). Cotton is also an important food source for humans and livestock (Sunilkumar et al., 2006). At the global level, cotton production has been relatively stable for the last many years, and, although it is native to the tropics and subtropics, including America, Africa and Asia, it is currently being cultivated in nearly 100 countries. China, USA, India and Pakistan are the top four cotton-growing nations, accounting for approximately 2/3 of the world's cotton acreage (http://www.ers.usda.gov/ Briefing/Cotton/trade.htm). The level of genetic diversity of a crop species is an essential element of sustainable crop production, and the success of any genetic conservation and breeding program depends largely on the identification of the genetic diversity in the gene pool. Thus, information on the genetic diversity and relationships among plant varieties is important to recognize the complexity of the gene pool, to identify gaps in genotype collections and to develop effective preservation and management strategies. In this way, molecular evaluations can provide insight into the genetic structure among varieties of different breeding origins; with this information, breeders can select the appropriate plant material to use in screening and breeding programs, with a goal of introducing new varieties (Russell et al., 1997). Because of advances in molecular marker technology over the past years, there have been extensive efforts to explore the molecular genetic diversity levels in various cotton gene pools and genomic groups, varietals and breeding collections (Iqbal et al., 2001). A narrow genetic base of cultivated cotton genotypes is one of the major factors causing the current declines in cotton yields and quality (Paterson et al., 2004), largely due to a lack of innovative tools to effectively exploit the genetic diversity of Gossypium species. The most effective utilization of the genetic diversity of Gossypium requires modern genomic technologies that help to reveal the molecular basis of agronomically important genetic variations. Efforts have been performed to characterize the cotton germplasm using allozymes, restriction fragment length polymorphism (RFLP) (Wendel and Brubaker, 1993), random amplified polymorphic DNA (RAPD) (Iqbal et al., 1997), amplified fragment length polymorphism (AFLP) (Rana et al., 2005) and simple sequence repeat (SSR) markers (Reddy et al., 2001; Lacape et al., 2007). These characterizations have provided useful knowledge for understanding the genetic diversity and organization of

Table 1. SSR polymorphism revealed by 70 SSR primer pairs.

Primers	Chromosomes	Positions	Total no of alleles	Polymorphic alleles	Percentage polymorphism
BNL1053	-	-	3	1	33
BNL1421	A13	69.4	2	1	50
BNL1672	A9	60.0	2	1	50
BNL3280	D10	107.2	2	1	50
BNL3359	A6	134.6	2	1	50
BNL3590	A2	61.0	3	1	33
BNL409	A13	127.1	3	2	67
BNL448	D4	22.7	2	1	50
BNL946	D10	61.2	5	4	80
JESPR220	D4	102.9	4	3	75
JESPR274	A9	42.8	3	2	67
JESPR291	D8	152.0	4	2	50
NAU1070	D2	13.5	8	6	75
NAU1102	D5	-	4	2	50
NAU1103	D11	7.5	4	3	75
NAU1141	A13	31.2	3	2	67
NAU1156	-	-	3	2	67
NAU1167	A3	105.3	6	5	83
NAU1218	A6	57.6	2	1	50
NAU1230	D5	0.2	2	1	50
NAU1233	A10	40.2	6	4	67
NAU1366	A8	43.8	3	1	33
NAU1366	D11	142.7	3	1	33
NAU1369	A1	27.9	6	4	67
NAU2083	D4	0.0	5	4	80
NAU2162	D8	77.1	3	2	67
NAU2169	D2	24.1	2	1	50
NAU2190	A2	5.9	3	1	33
NAU2265	A10	2.6	6	4	67
NAU2317	D2	54.3	2	1	50
NAU2336	-	-	5	5	100
NAU2355	A1	40.1	3	2	67
NAU2419	D8	70.5	2	1	50
NAU2439	A4	82.2	2	1	50
NAU2477	D5	175.2	3	1	33
NAU2503	D3	81.1	2	1	50
NAU2691	D13	87.4	2	1	50
NAU2697	D13	-	6	4	67
NAU2980	A7	70.7	5	4	80
NAU2995	D13	98.6	2	1	50
NAU3011	D7	30.7	2	1	50
NAU3053	D5	10.0	7	6	86
NAU3092	D5	181.0	5	4	80
NAU3095	D9	25.9	3	2	67
NAU3100	A6	36.4	3	2	67
NAU3206	A8	70.6	5	2	40
NAU3207	D2	104.5	3	2	67
NAU3209	A1	102.9	2	1	50
NAU3254	A1	74.0	3	1	33
NAU3385	A13	83.5	3	2	67
NAU3398	A9	119.2	3	2	67
NAU3414	D7	-	2	1	50
NAU3424	A5	117.0	4	1	25
NAU3529	D7	121.5	2	1	50
NAU3608	A7	23.1	6	4	67
NAU3654	A12	85.0	4	2	50
NAU3860	D1	117.3	3	2	67
NAU3901	D10	30.9	8	5	63
NAU3916	A1	53.3	2	1	50
NAU4073	D9	99.6	4	2	50
NAU418	A2	52.8	2	1	50
NAU453	D10	-	4	1	25
NAU462	A9	117.5	2	1	50
NAU483	A3	28.0	4	3	75
NAU4956	D7	51.0	2	1	50
NAU5005	D5	-	2	1	50
NAU5091	D11	66.5	2	1	50
NAU5189	D9	140.1	3	2	67
NAU6191	-	-	4	3	75
NAU808	A2	-	4	3	75
TOTAL			241	147	4024.9
Avarege			3.44	2.10	57.50

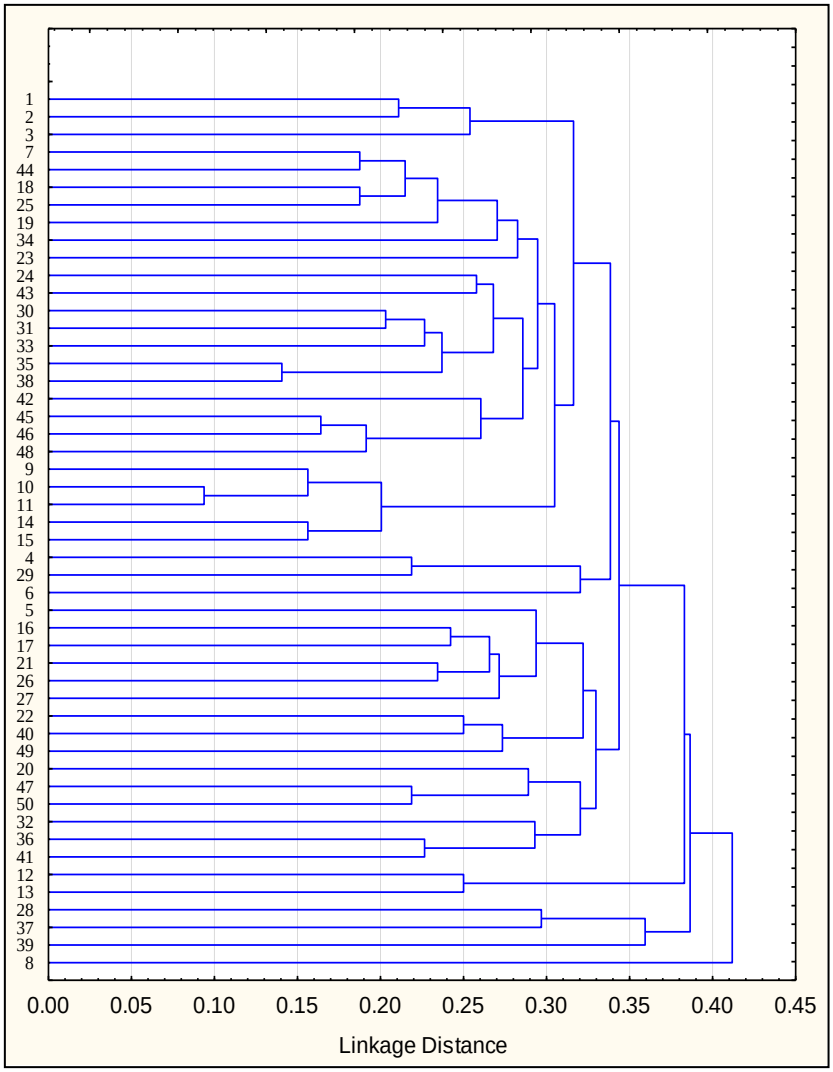


Fig 1. UPGMA tree showing ggenetic relationships of 50 Pakistani cotton cultivars as revealed by 70 SSR markers, the cultivars names from (1 to 50) as mentioned in table 2.

various cotton gene pools found in diverse regions, and the information has been integrated into the effective management of the cotton germplasm in some cotton breeding programs for the control of the genetic diversity. The recent development of plentiful cotton SSR markers has had a more positive effect on the molecular characterization of the cotton germplasm released from specific cotton breeding programs across the world (Zhang et al., 2008). In this study, the main objective was to assess the level of genetic diversity of 50 representative Pakistani cotton cultivars introduced from different origins to specify the primary sources of the germplasm for a variety of improvement programs.

Results and discussion

Allelic diversity of SSR markers

A total of 23 (24.7%) of the 93 SSR primer pairs screened revealed monomorphic bands across the 50 cultivars, indicating a considerable homogeneity of the cotton genome; 241 SSR alleles were detected, with an average of 3.44 alleles per primer, of this, only 147 (60 %) alleles were polymorphic across the 50 cultivars (Table 2), resulting in 57.5 % polymorphism, with the average number of polymorphic alleles per primer being 2.10. However, these polymorphic alleles could include some null alleles because it was difficult to separate non-amplification due to the experimental error from null alleles. The number of polymorphic alleles detected per primer pair ranged from one to eight, with an average of 2.10 alleles per primer pair. The maximum number of band fragments was produced by the primers NAU1070 and NAU 3901 (8 bands), the most informative primer with 75% polymorphism, whereas the minimum number of fragments produced (for many primers with various polymorphism) was 2 bands (Table 1). The average sizes of the alleles were found to be in the 50 to 1000 bp range. The SSR markers revealed a considerable amount of variation in the sampled genome, even though the overall polymorphism detected for these cotton cultivars was relatively low. The narrow genetic base of cotton has been mentioned in many studies using such molecular markers as SSRs (Bertini et al., 2006; Zhang et al., 2011; Kalivas et al., 2011), within Upland cultivars generally revealing a low level of genetic variety. There was little variation in the estimation of the molecular diversity among the Upland cultivars (*G. hirsutum*); however, Abdurakhmonov (2007) reported that the genetic distance for the Upland cultivars was in the 0.01 to 0.28 range. The simple sequence repeat (SSR) allelic diversity found in our population for association

analysis is approximately the same as the total diversity presented in more extended studies. The same mean number of alleles per locus as in our study was found in a collection of 106 accessions, with 2.13 SSR alleles (Guo et al. 2006).

Genetic relationships and cluster analysis

The data obtained from the SSR analysis was used to generate a similarity matrix using the Nei (1972) method with data from 70 SSR markers. The resulting matrix showed a mean genetic similarity of 0.315 among the 50 genotypes, revealing a high level of genetic relatedness. With regard to the pairwise combinations, the genetic similarities between the genotypes varied from 0.1 between CIM-446 and CIM-448 to 0.53 between CIM-482 and MNH-6070 and STAPM-82 and AYT-85094 (Table 3). Cultivar CIM-446 showed a high similarity (0.10) with the CIM-448 cultivar developed by the Central of Cotton Research Institute Multan (CCRI). Conversely, cultivar CIM-482 showed a high similarity (0.53) with cultivar MNH-6070 from different institutes. Using the clustering based on the UPGMA analysis, a dendrogram was constructed for the 50 genotypes with 70 microsatellite primers, as presented in Figure 1. Based on the SSR similarity, the 50 cultivars revealed several variation patterns. A single genotype, UA-73, appeared at the base of the dendrogram, with the three independent sister genotype entries first (MNH-802 and AYT-85094) second (CIM-482 and CIM-506) and third (XIAO-VEMIAN, XU-2HOU-142 and STAPM-82) from the top of the dendrogram (Figure 1). There were three major clusters and some sub-clusters distinguished in the modern cultivars. Cluster A consisted of sub-cluster A1 (MNH-6070, 299-F, MNH-814and L-S-S), A2 (UA-7-25/46, FH-125, CIM-1100, MNH-812, MG-66, BT-2009, 841/52, MNH-638). Cluster B consisted 3 cultivars (U-4(5143), U-4 and S-12), and Cluster C consisted of sub-cluster C1 (CIM-109, CIM-446, CIM-448, CIM-534, CIM-473 and M-944-00-0243) and C2 (TH-35/99, CIM-4/99, MNH-552, NIAB-824, MNH-636, NIBGE-4, CRS-2007, FH-901, FH-900, FH-113, MNH-807, MNH-806, SLS-1, MS-40, 4F, GR-156, BH-118, S-11, AM-82, FH-1000 and UA-13-102) (figure 1). It was clear that the clustering was not associated with the breeding origin, as each cluster consisted of cultivars released from different breeding programs. The sister cultivars (CIM-482 and CIM-506) are from same breeding station (Central Cotton Research Institute) from different breeding periods and parentage, as reported by Khan et al. (2009). However, the other sister cultivars (MNH-802 and AYT-85094) were also from different breeding institutes, whereas XIAO-VEMIAN, XU-2HOU-142 and STAPM-82 were exotic lines. The inconsistencies between the cultivar clustering and known parentage of these cotton cultivars in this study revealed a narrow genetic base, in agreement with the results of Iqbal et al. (2001). However, different recent studies have reported a relatively higher genetic diversity, with an average genetic distance of up to ~37-77% in *G. hirsutum*, as based on the analysis of some germplasm resources from Pakistan (Khan et al., 2009), Brazil (Bertini et al., 2006), China (Liu et al., 2011; Zhang et al., 2011) and India (Chaudhary et al., 2010), results obtained from SSRs or a combination of SSR and RAPD markers. Sapkal et al. (2011) reported genetic diversity (up to 57%) for 91 Upland cotton accessions with genetic male sterility maintainer and restorer properties that was similar to the finding in our study (57.5%). These results suggested the presence of useful genetic diversity both in exotic and breeding line resources. There is a need for further evaluation of the molecular genetic diversity through the application of additional markers across upland cotton genomes to improve the resolution and the effective use of these tools in breeding programs. Such efforts will address the current concerns on the narrowness of the genetic base of widely grown Upland cotton cultivars. However, studies of the estimated molecular genetic diversity still offer a useful guide for cotton breeding; as such studies are more informative than selection and traditional pedigree analysis. The characterization of cotton cultivars using SSR markers provided a useful guide for selecting specific germplasm with distinct genetic backgrounds in efforts to diversify cotton breeding programs.

Material and methods

Plant material

The seeds of 50 different genotypes of *Gossypium hirsutum* were collected from Central Cotton Research Institute (CCRI) Multan, Pakistan, and Cotton Research Station Multan, Pakistan. These *Gossypium hirsutum* accessions were evaluated for their genetic diversity. The cotton cultivars were grown in a greenhouse. The seeds of all 50 accessions were planted on 12 May 2011 in polyethylene bags, measuring 25 ×5 cm, and filled with approximately 1.15 kg of soil (peat, sand and soil, 1:1:1). All of the bags were saturated to field capacity before planting the seeds. The seeds were soaked overnight, and 4 seeds/bag of each cultivar were sown at a depth of 2 to 3 cm. The seeds started to germinate on 14 May 2011. After germination, only one plant/bag was maintained, and the remaining were uprooted.

Genomic DNA isolation

For extraction of the genomic DNA from each accession group, 4–5 young fully expanded leaves were collected and stored at −80°C. The genomic DNA was isolated from the frozen leaf tissues using the cetyltrimethylammonium bromide (CTAB) method described by (Zhang et al. 2000). The DNA samples were stored at -20 °C until further use. The DNA quality was evaluated with 1% agarose gel electrophoresis prepared using 0.5X TAE buffer, and ethidium bromide (10 ng/100 ml) was added to the gel to stain the DNA bands. The samples were electrophoresed for approximately 30 minutes after which the products were viewed using an ultraviolet transilluminator and photographed using the Syngene Gel Documentation System. The DNA concentration was estimated by the absorbance at 260 nm (Sambrook et al., 1989). The working DNA samples (containing 50 ng/µL) were stored at 4°C for genotyping.

Sources of microsatellite markers

The BNL primers were from Research Genetics Co. (Huntsville, AL, USA, http://www.resgen.com), the JESPR primers were the sequences of Reddy et al. (2001), and the SSR primers of NAU were the EST-SSR sequences of Han et al. (2006).

Genotyping with SSR markers

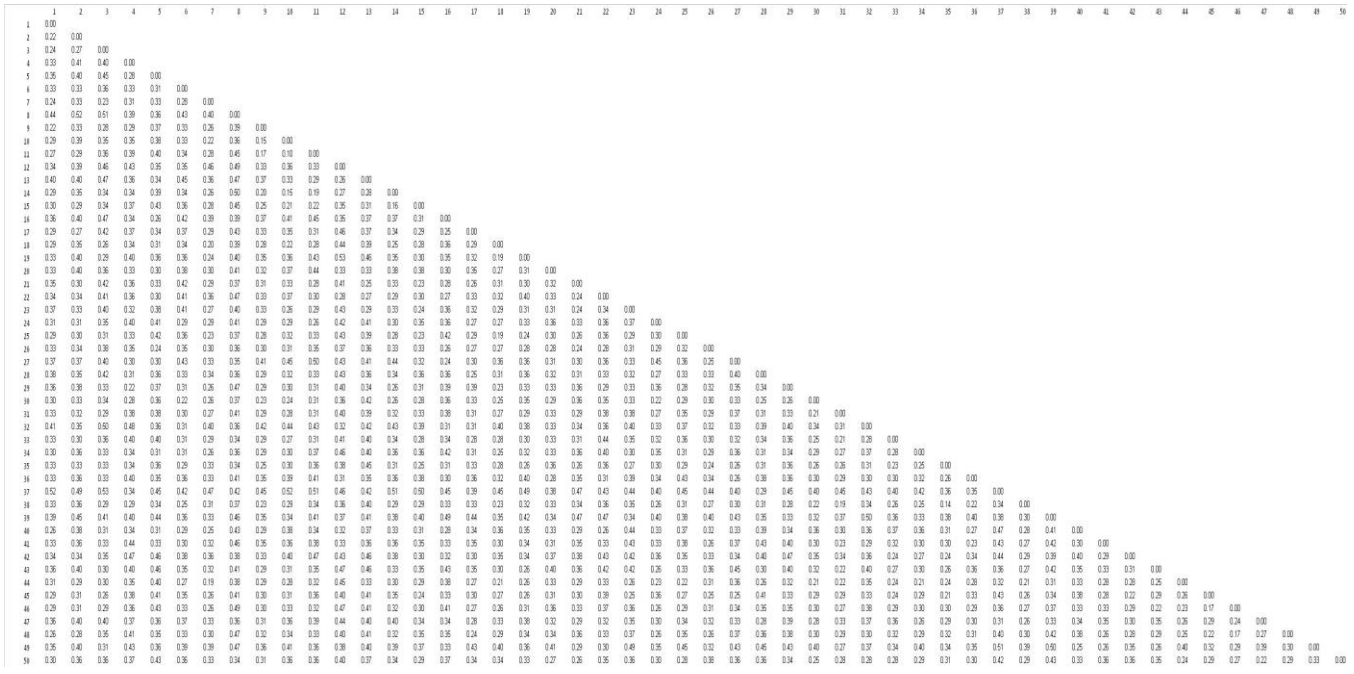
All 50 accessions were genotyped using a 70 core set of SSR marker primers. These chromosome-specific primer pairs

Table 2. Fifty Pakistani cotton cultivars with origin of release.

Sr. No	Accession Name	Center Name	Sr. No	Accession Name	Center Name
1	XIAO-VEMIAN	Exotic lines	26	FH-125	CRS, FSD
2	XU-2HOU-142	Exotic lines	27	MNH-812	CRS, Multan
3	STAPM-82	Exotic lines	28	AM-82	CCRI, Multan
4	U-4(5143)	Exotic lines	29	S-12	CRS, Multan
5	UA-7-25/46	Exotic lines	30	4 F	CRS, FSD
6	U-4	Exotic lines	31	GR-156	CRS, Multan
7	UA-13-102	Exotic lines	32	299-F	CCRI, Multan
8	UA-73	Exotic lines	33	MS-40	CRS, Multan
9	CIM-109	CCRI, Multan	34	SLS-1	CRS, Multan
10	CIM-446	CCRI, Multan	35	MNH-806	CRS, Multan
11	CIM-448	CCRI, Multan	36	MNH-814	CRS, Multan
12	CIM-482	CCRI, Multan	37	AYT-85094	CCRI, Multan
13	CIM-506	CCRI, Multan	38	MNH-807	CRS, Multan
14	CIM-534	CCRI, Multan	39	MNH-802	CRS, Multan
15	CIM-473	CCRI, Multan	40	841/52	CCRI, Multan
16	CIM-1100	CCRI, Multan	41	L-S-S	CRS, FSD
17	CIM-4/99	CCRI, Multan	42	FH-901	CRS, FSD
18	TH-35/99	Exotic lines	43	FH-900	CRS, FSD
19	BH-118	CRS, BWP	44	FH-1000	CRS, FSD
20	MNH-6070	CRS, Multan	45	CRS-2007	CRS, Multan
21	MG-66	CRS, Multan	46	NIBGE-4	NIBGE, FSD
22	BT-2009	-	47	NIAB-824	NIAB, FSD
23	M-944-00-0243	Exotic lines	48	MNH-636	CRS, Multan
24	FH-113	CRS, FSD	49	MNH-638	CRS, Multan
25	S-11	CRS, Multan	50	MNH-552	CRS, Multan

CCRI=Central Cotton Research Institute; NIAB=Nuclear Institute for Agriculture and Biology; CRS=Cotton Research Station; FSD=Cotton Research Institute, Faisalabad;, BWP=Cotton Research Station, Bahawalpur

Table 3 . Matrix of genetic distances based on percentage disagreements, the cultivars names from (1 to 50) as mentioned in table 2.



were selected using the results of different laboratories, published papers (Siu et al. 2000, Han et al. 2004; Shen et al. 2005; Abdurakhmonov 2007), and based on their informativeness relative to important QTLs and chromosome distribution. The PCR amplifications were performed in a 10 µl reaction mix containing 1 µl 10× PCR buffer, 0.2 µl dNTPs (5 mM each), 0.1 µl 25 mM $MgCl_2$, 0.1 µl Taq DNA polymerase, and 1 µl (50 ng) genomic DNA. The microsatellites were amplified using the standard PCR procedures described by Zhang et al. (2000).

Silver staining and development of bands

After a specific migration of the band on the gel, the gels were placed in fixing solution (40 % ethanol and 10 % glacial acetic acid) for 20 minutes and then washed three times with distilled water and stained with silver staining solution (0.2 % $AgNO_3$) for 20 minutes. After staining, the gels were again washed three times with distilled water for 20 seconds, and the developing solution (3 % NaOH and 0.05 % formaldehyde) was applied for 3-5 minutes.

Data analysis

A comparison of the fifty core accessions of *G. hirsutum* L. was performed on the basis of the presence or absence of bands generated by the SSR primers. The number of bands produced for each primer was scored manually for presence (1) or absence (0), and a binary matrix was generated and then used for the analysis. The pairwise genetic distances between individuals were calculated by the percentage disagreement method. To assess the genetic relationships of the cotton cultivars, the data were used in a cluster analysis with the unweighted pair-group method with arithmetic averages (UPGMA) in which the samples were grouped based on their similarity using the statistical software package STATISTCA- ver.6 (StatSoft, Inc., 2001).

Conclusion

The current study showed that microsatellite markers are efficient for measuring the genetic diversity and relatedness and for identifying varieties of cotton. The results revealed relatively low genetic diversity of Pakistani cotton cultivars and also generate information about levels of genetic relatedness. We suggest the further evaluation of the molecular genetic diversity in efforts to conserve the maximum diversity level by applying additional markers to improve the resolution of these tools for use in breeding programs. This is because the narrowness of the genetic diversity in the germplasm was associated with the recent and potentially future declines in *G. hirsutum* L. production and its quality, serving as a timely warning to increase the speed of the efforts to widen the genetic base of the germplasm resource by mobilizing new genetic variations from the gene pool. The wide ranges of the genetic diversity of the genus, particularly the diversity of exotic wild cotton germplasm, are key resources to improve cotton cultivars and address the various essential problems associated with the resistance to insects and pathogens, tolerance to abiotic stresses and fiber quality.

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