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Original scientific paper

GENETIC FINGER PRINTING OF COTTON CULTIVARS BY ISSR MOLECULAR MARKERS

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Gossypium hirsutum is one of the main tetraploid cotton species that is cultivated throughout the world. Due to continuous selection of cotton cultivars for specific agronomic traits, the genetic variability within the cultivars decrease that lead to genetic erosion. To tackle the problem of reduced genetic variability, we should track all available genetic diversity within cotton germplasm and use them for inter-specific and intra-specific hybridization and produce new elite cotton cultivars. Therefore, the present study used ISSR molecular markers to illustrate genetic variability in 13 tetraploid cotton genotypes (*Gossypium hirsutum* L.) and to categorize these genotypes based on genetic affinity. 65 cotton plants were studied. The results identified private bands in the studied genotypes, while Network and STRUCTURE analyses of molecular data obtained grouped the genotypes with genetic affinity together. Some of the genotypes differed in their genetic content from the others; therefore, studying the genetic and agronomic variability within available cultivars is very important and produced data to broaden the gene pool for planning further hybridization in cotton.

Keywords: Cotton, genetic variability, ISSR, Network, STRUCTURE analysis

INTRODUCTION

Cotton (*Gossypium* spp.) is very important fiber and oil crop plant that is cultivated throughout the world. Both diploid and tetraploid species of cotton have been used for agronomic purposes. *G. hirsutum* L. (upland cotton, AADD) and *G. barbadense* L. (pima cotton, AADD) are tetraploid (2n = 4x = 52). The most known qualitative trait loci affecting fiber quality were mapped to the D sub-genome of allotetraploid cottons (Nekrutenko and Baker 2003; Sheidai 2008).

Moreover, cotton has a very narrow and low genetic variability (NEKRUTENKO and

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BAKER, 2003) and therefore, using proper molecular markers to investigate genetic variability among and within cotton cultivars is of immediate importance.

Different types of molecular markers were used to study genetic diversity in cotton. These molecular markers were: Random amplified polymorphic DNA (RAPD), allozymes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), and inter-simple sequence repeats (ISSRs) as well as retrotrasposon based molecular marker (REMAP) (see for example, VAN ESBROECK *et al.*, 1998; ABDALLA *et al.*, 2001; SHEIDAI *et al.*, 2007; SHEIDAI *et al.*, 2008; KANTARTZI *et al.*, 2009; NOORMOHAMMADI *et al.*, 2015; NOORMOHAMMADI *et al.*, 2016).

It has been used ISSR markers as they are fast and low-cost molecular markers and revealed a high genetic variability within plant species and populations (SHEIDAI *et al.*, 2016).

MATERIAL AND METHODS

In total, 65 cotton plants of 13 cotton genotypes were used for genetic fingerprint study by ISSR molecular markers. They were cultivated in three rows of 10 m length with 20 cm interplant distance in the experimental field of Gorgan Cotton Research Center of Iran, according to a completely randomized design (CRD) with three replicates. For each genotype, 5 plants were randomly selected and their fresh leaves were used for DNA extraction.

The cotton genotypes of *G. hirsutum* studied are: 1- Super Okra, 2- Sepid, 3- Sealand, 4- Shayan, 5- N2G80, 6- BKW30, 7- Gulorova, 8- Golestan, 9- Khorshid, 10- M13, 11- A95-TS19, 12- ASP-95732, and 13- N25-A2, respectively.

Dna extraction and pcr analysis

Genomic DNA was extracted using the CTAB method based on Krizman et al. (2006).The quality and quantity of extracted DNA was tested by running on 0.8% agarose gel and measurement on Nanodrop spectrophotometer. Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, UBC810, (CA)7AT, (GA)9C, UBC807, UBC811, (GA)9A and (GT)7CA commercialized by UBC (the University of British Columbia) were used.

PCR reactions were performed in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany).

The amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 S at 94°C; 1 min at 60°C and 2 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. The amplification products were visualized by running on 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Genetic diversity and population structure

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). The genetic diversity parameters, Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (FREELAND *et al.*, 2011; WEISING *et al.*, 2005), were determined for each cultivar. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006) and Fst values of

STRUCTURE were used to reveal significant genetic difference among the studied genotypes (SHEIDAI *et al.*, 2014).

Nei's genetic distance was used for clustering (FREELAND *et al.*, 2011). Grouping of the cultivars was done by different clustering methods well as Bayesian based model STRUCTURE analysis (PRITCHARD *et al.*, 2000; SHEIDAI *et al.*, 2014). For STRUCTURE analysis, data were scored as dominant markers (FALUSH *et al.*, 2007), and admixture model was used.

PAST and STRUCTURE programs were used for analyses (HAMMER *et al.*, 2012; PRITCHARD *et al.*, 2000). Delta-k value of STRUCTURE analysis were used based on Evanoo test (EVANNO *et al.*, 2005) to identify the number of genetic groups.

RESULTS

In total 38 ISSR bands (loci) were obtained in the studied genotypes. Some of the bands were common in few genotypes, while some other bands were specific band in a single genotype (Table 1). For example, populations 2, 8, 10 and 12 had specific bands. The common bands observed showed shared alleles between the studied genotypes, while specific bands indicated their genetic pecularities (TYAGI *et al.*, 2014). Further analysis of ISSR bands (loci) by Nm (number of migrants) identified these common versus private alleles among the studied cotton genotypes. Result showed that the loci No. 4, 6, 8, 9, 13, 14, 20, 21, 23, 29, 31, 33, and 34, have the lowest Nm value (<0.9) and are the private alleles in the studied genotypes.

Table 1. Details of ISSR bands in the studied cotton genotypes. (The population number as in the text).

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	Pop11	Pop12	Pop13
No .Bands	15	21	17	17	11	15	15	20	18	17	14	26	14
Specific	0	1	0	0	0	0	0	1	0	1	0	4	0
No .Common bands**(%25=>)**	0	1	2	2	0	0	1	0	1	1	1	4	0
No .Comm bands**(%50=>)**	1	5	4	3	1	2	3	5	6	6	6	10	6

Table 2. Genetic diversity parameters determined in the studied cotton genotypes. (Population number as in the text).

Pop	Na	Ne	I	He	uHe	%P
Pop1	0.579	1.158	0.117	0.082	0.091	18.42
Pop2	0.763	1.088	0.089	0.056	0.062	21.05
Pop 3	0.579	1.089	0.072	0.049	0.054	13.16
Pop4	0.553	1.061	0.053	0.035	0.039	10.53
Pop5	0.342	1.046	0.035	0.024	0.027	5.26
Pop6	0.395	1.000	0.000	0.000	0.000	0.00
Pop7	0.526	1.079	0.073	0.049	0.054	13.16
Pop8	0.737	1.088	0.089	0.056	0.062	21.05
Pop9	0.658	1.110	0.095	0.063	0.070	18.42
Pop10	0.763	1.207	0.172	0.116	0.129	31.58
Pop11	0.500	1.109	0.081	0.057	0.063	13.16
Pop12	1.053	1.292	0.227	0.158	0.176	36.84
Pop13	0.474	1.052	0.050	0.032	0.036	10.53

Abbreviations: Na = No. allels, Ne = No. of effective alleles, I = Shanon information index, He = Gene diversity, uHe = Unbiased gene diversity, %P = Genetic polymorphism.

Genetic diversity parameters determined in the studied cotton genotypes are presented in Table 2. Genotype No. 6 did not show genetic variability within its studied samples, while genetic polymorphism percentage varied from 5.26 in genotype No. 5, to 36.84 in genotype No. 12. The genotype No. 10 and 12 had the highest value for Nei gene diversity (≥ 0.10).

Nei genetic distance determined among the studied genotypes revealed that the highest degree of genetic similarity (>095) occurred between genotypes 3 ad 4, 3 and 6, as well as 4 and 6 (Table 3).

Table 3. Nei's genetic distance in the studied cotton genotypes

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1	****	0.9367	0.8682	0.8910	0.8494	0.8538	0.9219	0.7945	0.7721	0.7590	0.6672	0.6199	0.7176
2	0.0653	****	0.8926	0.9207	0.8067	0.8852	0.8607	0.7892	0.7595	0.7376	0.6546	0.6243	0.6552
3	0.1414	0.1136	****	0.9708	0.8918	0.9537	0.8848	0.7511	0.7246	0.6870	0.5705	0.6770	0.5857
4	0.1154	0.0826	0.0296	****	0.9115	0.9638	0.9291	0.7582	0.7215	0.7237	0.5931	0.6537	0.5944
5	0.1632	0.2148	0.1145	0.0927	****	0.8722	0.8838	0.6797	0.6699	0.6958	0.6007	0.5950	0.6325
6	0.1581	0.1220	0.0474	0.0369	0.1368	****	0.8806	0.7555	0.7194	0.6660	0.5391	0.6221	0.5944
7	0.0813	0.1500	0.1224	0.0735	0.1236	0.1272	****	0.7139	0.6670	0.7548	0.6339	0.5978	0.6302
8	0.2301	0.2367	0.2862	0.2768	0.3861	0.2804	0.3370	****	0.9269	0.8417	0.7389	0.6762	0.8026
9	0.2586	0.2751	0.3221	0.3265	0.4006	0.3293	0.4050	0.0759	****	0.8399	0.8176	0.6473	0.8473
10	0.2757	0.3043	0.3755	0.3234	0.3627	0.4065	0.2813	0.1724	0.1745	****	0.8927	0.6718	0.7892
11	0.4047	0.4237	0.5612	0.5224	0.5096	0.6178	0.4559	0.3026	0.2014	0.1135	****	0.5929	0.8400
12	0.4782	0.4711	0.3901	0.4252	0.5191	0.4746	0.5145	0.3913	0.4349	0.3979	0.5228	****	0.6579
13	0.3318	0.4229	0.5350	0.5203	0.4581	0.5202	0.4617	0.2199	0.1657	0.2367	0.1743	0.4187	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

AMOVA revealed significant genetic difference (PhiPT = 0.70, p = 0.001) among cotton genotypes. It showed that 75% of total genetic variation occurred among the studied genotypes, while, 25% of the variance was due to within populations studied. Moreover, paired-sampled AMOVA also revealed significant genetic difference among all pairs of the studied genotypes. The PhiPT value obtained ranged from 0.30 to 0.89, all of which were significant at P= 0.01.

The grouping of the genotypes based on Nei genetic distance (Fig. 1) almost separated individuals of each genotype in distinct clusters. This indicates genetic distinctness of the studied genotypes which is in agreement with AMOVA results. However, in few cases the genotypes were inter-mixed together due to the presence of shared alleles.

Genetic structure of the studied cotton genotypes was investigated by STRUCTURE plot (Fig. 2). The plot obtained revealed genetic similarity between the genotypes No. 1 and 2, and also between genotypes No. 3, 4, and 6. Similarly, genotypes 8 showed genetic similarity to genotype 9, while genotypes 11 and 13 were similar too. The Fst values obtained for STRUCTURE analysis produced high Fst value (>0.70) for genotypes 1, 7, 8, 11, 12, and 13. Therefore, these genotypes differ in their genetic content from the other studied cotton genotypes.

In spite of genetic differences observed in the allele combination of studied genotypes (difference in colored segments), a very limited degree of gene flow or ancestral shared alleles occur among them (similarly colored segments).

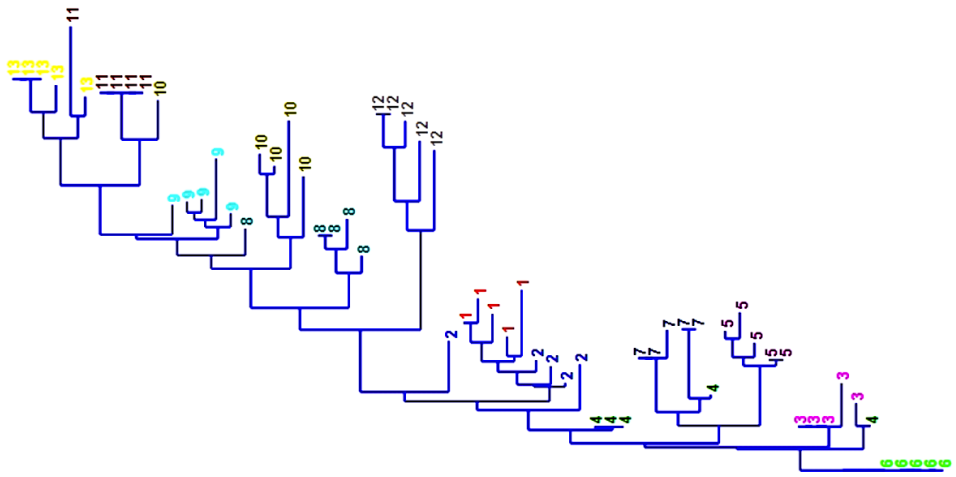

Fig. 1. NJ tree of the studied cotton genotypes based on Nei' genetic distance.

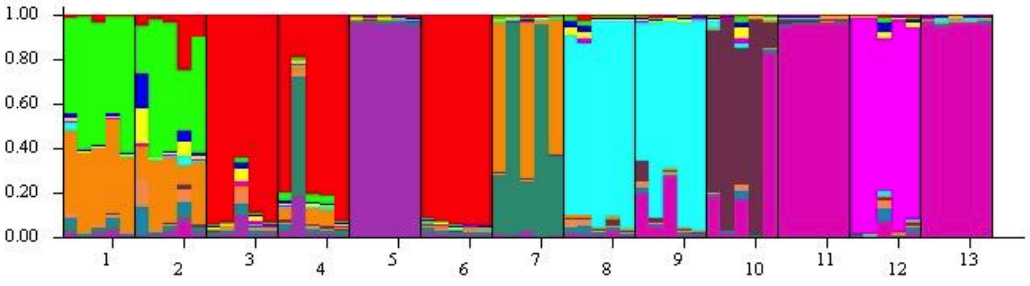

Fig. 2. STRUCTURE plot of the studied cotton genotypes.

The Evanno test for the number of k (genetic groups), produced k = 3 as the optimum number. As it is evident in STRUCTURE plot based on k = 3, the genotypes 1-7 comprised the first genetic group (all have red-colored segments in general). Similarly, the genotypes No. 8-11 and 13 comprise the second genetic group (they contain mostly green colored segments). However, genotype No. 12 alone formed the third genetic group (blue colored). Therefore, we can choose parents from these 3 genetic groups for inter-cultivar hybridization.

DISCUSSION

Obtaining new and elite cotton cultivars with good agronomic and yield characteristics is an important task. The cotton breeders rely on available genetic variability between parents to

create novel gene combinations that may lead to obtain such elite and superior genotypes. Therefore, studying the genetic and agronomic variability within available cultivars is very important and produced data to broaden the gene pool for planning further hybridization in cotton.

The present study revealed that ISSR molecular markers are efficient markers to illustrate genetic variability in cotton genotypes and can discriminate cotton genotypes present in germplasm. Cluster analysis separated the studied genotypes in distinct groups revealing their genetic difference.

Some of the genotypes had specific bands, which can be correlated with their agronomic characteristics. Identification of specific bands is very important for both genetic fingerprinting and cultivar discrimination in cotton as well as for developing association mapping populations in cotton (TYAGI *et al.*, 2014).

Use of efficient, low-cost and fast genetic screening method is a must for breeders to identify diverse parental genotypes when dealing with large cotton germplasm (VAN ESBROECK *et al.*, 1998; SHEIDAI *et al.*, 2007; SHEIDAI *et al.*, 2008; TYAGI *et al.*, 2014; NOORMOHAMMADI *et al.*, 2015).

The present study identified the genotypes that differ from the others We also identified the genotypes with high degree of genetic affinity. Therefore, we can suggest different crossing combinations between these groups based on genetic distance and desirable agronomic characteristics. Genetic finger printing characterizes the individuals and accessions for the choice of parental genotypes in breeding programs. For any meaningful plant-breeding program, accurate determination of genetic diversity and portioning within and between gene pools is an essential step (ULLOA *et al.*, 2007; ZHANG *et al.*, 2011).

Cotton is an important cash crop worldwide. Although it is classified as one of the most salt-tolerant major crops and considered a pioneer crop in reclamation of saline soils, its growth and development as well as yield and fiber quality are negatively affected by excessive salts in the soil. The use of cotton varieties tolerant to higher soil salinity levels is one of the approaches to control salinity stress effects on cotton. Therefore, identification and screening of salt-tolerant cotton cultivars or germplasm is of great importance (MA *et al.*, 2011).

The results of present study may be used in further association and QTL studies, both common as well as private bands could be checked for association with salt- or drought-tolerance in elite genotypes. They can be sequenced and those sequences may be used in future cotton genotype finger printing or barcoding (ZHENG *et al.*, 2016).

The earlier studies in cotton reported low degree of genetic variability in these plants (LIU *et al.*, 2006; MURTAZA, 2006; WANG *et al.*, 2007; TYAGI *et al.*, 2014). This is mainly due to continuous artificial selection for higher productivity in cotton farming led (IQBAL *et al.*, 1997). This in turn is hindering breeding programs worldwide. The present investigation revealed almost moderate to high level of genetic variability within each cotton genotype studied, therefore, we suggest first to perform multiple crossing between genotypes to combine different genes in resulting offspring. But we should also consider both genetic distance as well as desirable agronomic characteristics of the presumed parental genotypes.

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GENETIČA ANALIZA KULTIVARA PAMUKA ISSR MOLEKULARNIM MARKERIMA

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Izvod

Gossypium hirsutum je jedna od glavnih tetraploidnih vrsta pamuka koja se gaji u širom sveta. Zbog kontinuirane selekcije sorti pamuka za specifične agronomske osobine, smanjuje se genetička varijabilnost što dovodi do genetske erozije. Da bismo se suočili sa problemom smanjene genetičke varijabilnosti, trebalo bi da pratimo dostupnu genetičku raznovrsnost germplazme pamuka i da je koristimo za hibridizaciju unutar I između vrste i proizvodnju novih sorti pamuka. Stoga je ova studija koristila ISSR molekularne markere za ilustraciju genetičke varijabilnosti kod 13 tetraploidnih pamučnih genotipova (*Gossipium hirsutum* L.) i kategorizaciju ovih genotipova. Rezultati su identifikovali posebne trake u proučavanim genotipovima, dok su Network i STRUCTURE analize grupisale genotipove na osnovu dobijenih molekularnih podataka. Neki od genotipova su se razlikovali u genetičkom sadržaju od drugih; stoga, proučavanje genetičke i agronomske varijabilnosti unutar dostupnih sorti je veoma važno i dobijeni su podaci kako bi se proširio genski *pool* za planiranje dalje hibridizacije kod pamuka.

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