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GENETIC DIVERSITY OF DIPLOID AND TETRAPLOID COTTONS DETERMINED BY SSR AND ISSR MARKERS

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ABSTRACT

Cotton as an annual crop is mainly grown for its fiber and oil in the seed. Determining genetic diversity in the germplasm is the first step of plant breeding. This study aimed to determine genetic diversity for diploid and tetraploid cotton genotypes grown in different parts of the world. SSR (Simple Sequence Repeats) and ISSR (Inter Simple Sequence Repeats) markers were used to determine genetic relationships among *Gossypium* species and genotypes. Using 39 SSR and 5 ISSR markers, 173 alleles (averaging 3.93 alleles per locus) were produced from 25 cotton genotypes. Out of 173 alleles, 155 (89.60%) were polymorphic among the genotypes and polymorphic information content (PIC) values were between 0.0040 and 0.9993 averaging 0.4396. Genetic diversity ranged from 0.04 to 0.58 among all the genotypes inspected. This ratio was 0.04-0.23 within *G. hirsutum* L. and 0.07-0.26 within *G. barbadense* L. species. Out of these two species, genetic diversity ranged from 0.23 to 0.57 among other diploid and tetraploid species. Genetic diversity was low within commercial cultivars that are also frequently used in breeding programs (0.08-0.20). It is advisable to use wild type cottons to increase present genetic diversity in germplasm pools to have a better chance for the selection of the desired traits.

Key Words: cotton, *Gossypium*, *g*enetic diversity, molecular markers, diploid, tetraploid

INTRODUCTION

Cotton as an annual crop is mainly grown for its fiber and oil in the seed. It is worth to determine genetic diversity in the germplasm for the success of the breeding process. Cotton has a lot of diseases and pests which affect growth and development of the plant. Especially, diploid cottons have resistance genes for diseases, pests and biotic stress conditions. Their characteristics can be revealed at DNA levels, and the information for these pests, diseases and biotic stress resistant genes can be used in cotton breeding programs.

Information about the degree and distribution of genetic diversity and relationships among breeding materials has a significant effect on crop improvement. Selection of suitable parents is a priority for the most promising crosses and increases the efficiency of breeding programs. Molecular markers increasingly play an important role in crop improvement programs. They have been used to predict genetic variance among inbred lines (Manjarrez-Sandoval et al., 1997), estimate genetic diversity in crops (Iqbal et al., 2001; Gutierrez et al., 2002; Zhang et al., 2005; Chen et al., 2007; Abdurakhmonov et al., 2006; Zhikun et al., 2008; Kantartzi et al., 2009; Nas et al., 2011), protect plant variety rights (Smith and Smith, 1992), classify heterotic groups (Dudley et al., 1991; Senior et al., 1998), study phylogenetic relationships among crops and their wild relatives (Li and et al., 2000), analyze pedigrees (Smith et al., 1997), and select desired traits (Young, 1999).

The hypothesized narrow genetic base of upland cotton germplasm used in breeding has been considered as one of the reasons contributing to the lack of progress in the improvement of cotton growers and industry in the world during the last 15 years (Lewis, 2001). Multivariate analysis of agronomic and fiber traits of ancestral cultivars detected high similarity (Van Esbroeck et al., 1999), supporting the conclusion that modern cotton cultivars have narrow genetic base when evaluated with isozyme and DNA markers (Wendel et al., 1992). This suggested that pedigree analysis may overestimate genetic distance among modern cultivars (Van Esbroeck et al., 1999).

Microsatellites are regions of short, tandemly repeated DNA sequences of 1 to 6 base pairs in eukaryotic genomes. Two different marker strategies have been used based on microsatellites: SSR (simple sequence repeats) and ISSR (inter-simple sequence repeats). SSRs are highly reproducible co-dominant markers, in which the repeated sequences produce polymorphic patterns among alleles, depending on the length of the repeats. Although these markers are generally highly polymorphic, the initial cost of developing them is relatively high (Reddy et al., 2001).

ISSR is a different microsatellite-based method that does not need prior knowledge of the genome, cloning or primer design (Zietkiewicz et al., 1994). While the SSR

protocol relies on the amplification of the repeated region using two locus specific primers, in ISSR, a single primer composed of a microsatellite sequence anchored at the 3'or 5' end by 2 to 4 arbitrary, often degenerate nucleotides, is used to amplify the DNA between two opposed microsatellites of the same type. Allelic polymorphisms occur whenever one genome is missing the sequence repeated or have a deletion or insertion. For 5' anchored primers, polymorphisms also occur due to differences in the length of the microsatellite. The sequences of repeats and anchored nucleotides are randomly selected. ISSRs are dominant markers but they have the advantage of analyzing multiple loci in a single reaction.

The goal of this study was to estimate genetic diversity among commercial cultivars, genetic stock materials and wild cotton species using SSR and ISSR markers.

MATERIALS AND METHODS

Twenty-five cotton genotypes were used in the experiment. Of these, 13 were belong to *G. hirsutum* L., 2 to *G. barbadense* L., and 10 were wild accessions (4 diploid and 6 tetraploid species) (Table 1). Genotypes and wild accession were planted in pots in greenhouse to get leaf samples for DNA extraction. Genomic DNA was extracted from approximately 0.5 g of young leaves according to a slight modification of the procedure described by Zhang and Steward (2000).

Table 1. Genotypes used to estimate genetic diversity

Genotypes	Species	Accession number	Origin	Genome
*Çukurova 1518	*G. hirsutum* L.	N/A	Turkey	AD
*Nazilli 84S	*G. hirsutum* L.	N/A	Turkey	AD
*Ersan 92	*G. hirsutum* L.	N/A	Turkey	AD
*Maras 92	*G. hirsutum* L.	N/A	Turkey	AD
*Gürelbey Ms34/1	*G. hirsutum* L.	N/A	Turkey	AD
*Suregrow 125	*G. hirsutum* L.	N/A	USA	AD
*Sayar 314	*G. hirsutum* L.	N/A	Turkey	AD
Ekstrem Okra (Brown)	*G. hirsutum* L.	N/A	Turkey	AD
Albania 6172	*G. hirsutum* L.	N/A	Albania	AD
Lifsiz	*G. hirsutum* L.	N/A	Turkey	AD
Siocra	*G. hirsutum* L.	N/A	Australia	AD
Taşkent 6	*G. hirsutum* L.	N/A	Uzbekistan	AD
Acala Maxa	*G. hirsutum* L.	N/A	USA	AD
Askabat 91	*G. barbadense* L.	N/A	Turkmenistan	AD
Bahar 82	*G. barbadense* L.	N/A	Uzbekistan	AD
G. herbaceum L..	-	A_1-20,PI 408778	Afghanistan	A
G. sturtianum var. *nandewarense*	-	C_{1-n}-3,PI 530754	Australia	C
G. harknesii Brandg	-	D22-8	Mexico	D
G. incanum (schwartz) Hill	-	E_4-4	Pakistan	E
G. mustelinum Miers ex Watt	-	$(AD)_4$-9	Brazil	AD
G. hirsutum var. *yucatanense*	-	$(AD)_1$.1469	USA	AD
G. hirsutum var. *marie galante*	-	$(AD)_1$.1607	USA	AD
G. lanceolatum Tod	-	$(AD)_1$-1 (P)	Mexico	AD
G. darwinii Watt	-	$(AD)_5$-14	Galapagos Island	AD
G. barbadense L.. (GB-4)	-	$(AD)_{2-23}$	Egypt	AD

*: Commercial varieties; N/A: Not Available

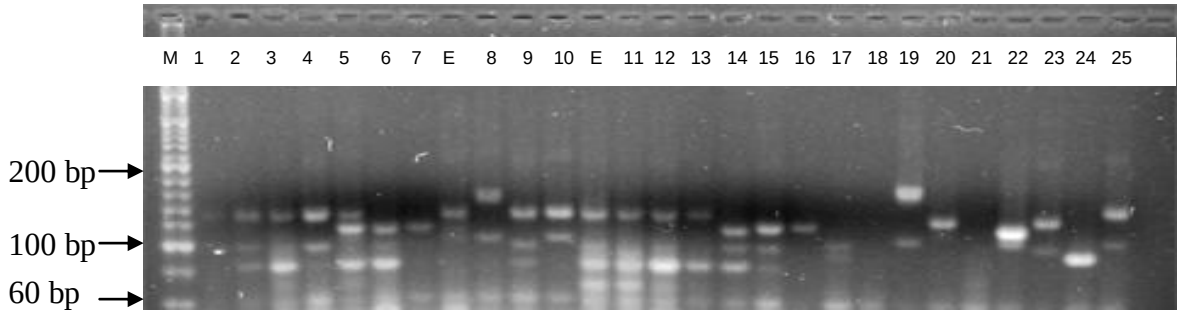

Figure. 1. Microsatellite polymorphsm (locus JESPR 153), M= 20 base pair marker size; 1=Suregrow 125; 2=Cukurova 1518; 3=Nazilli 84S; 4=Gurelbey MS34/1; 5=Ekstrem Okra (Brown); 6=Albania 6172; 7=Ersan 92; 8=Askabat 91; 9=Lifsiz; 10=Bahar 82; 11=Sayar 314; 12=Siocra; 13=Maras 92; 14=Taskent 6; 15=Acala Maxa; 16=G. mustelinum Miers ex Watt; 17=G. harknesii Brandg; 18=G. herbaceum L.; 19=G. barbadence L. (GB-4); 20=G. hirsutum var. yucatanence; 21=G. incanum (Schwartz) Hill; 22=G. lanceolatum Tod; 23=G. hirsutum var. marie galante; 24=G. sturtianum var. nandewarence; 25=G. darwinii Watt; E= Excluded

Extracted genomic DNA was PCR-amplified using 39 SSR and 5 ISSR primer pairs (Table 2). The primers were synthesized by Iontek, Turkey. PCR reactions were performed in a 20 µl volume. The reaction mixture contained 0.25 mM of each dNTP, 2 µl of 10X PCR buffer (Favorgen), 5 µM of each primer, 1 unit of Taq DNA polymerase (Fermentas) and 60 ng of genomic DNA. The PCR-amplification program consisted of one cycle at 95 °C for 3 min, then 35 cycles of [94°C for 1 min, 55 – 60 °C (depending on primer annealing temperatures) for 1 min, 72 °C for 1 min], a final cycle at 72 °C for 5 min. The PCR reactions were carried out in a 96-well block Eppendorf Mastercycler. Amplified PCR products were separated by electrophoresis using 2.5% (w/w) Metaphor agarose (Lonza, USA) + 1.5% (w/w) low melting agarose (Sigma, A5093) gel, stained with

ethidium bromide, visualized and photographed under UV light using an AlphaImager Gel Documentation and Analysis System (Figure 1), and fragment lengths were calculated by molecular weight comparison with 20 bp DNA step ladder mobility (Promega).

The products of SSR and ISSR amplification were recorded as present (1) or absent (0). Polymorphic information content (PIC) values provide an estimate of the discriminatory power of a marker by taking into account not only the number of alleles at a locus, but also the relative frequencies of those alleles in the population under study (Pei et al., 2010). The PIC for each SSR and ISSR locus was determined as described by Kalivas et al. (2011) the frequency of alleles per locus was calculated using the following formula:

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^{2}$$

where P_{ij} is the frequency of the *jth* allele for primer *i* The level of genetic distance (GD) between pair of genotypes was estimated using pairwise comparison (Nei, 1972). GDs were used for cluster analysis with the software Popgen version 3.2, and the dendrogram was drawn with Mega 3.1 software (Kumar et al., 2004) using Neighbor-Joining (NJ) method. The data were also visualized with the principal components analysis module of NTSYSpc.

RESULTS

The 39 SSR and 5 ISSR primer pairs produced a total of 173 unambiguous scorable fragments, and 155 (89.60%) fragments were polymorphic in at least one *Gossypium* species analyzed. The average number of alleles per SSR and ISSR markers was 3.93, ranging from 1 to 8 alleles (Table 2). The mean of the PIC value over the 39 SSR and 5 ISSR markers averaged 0.4396, ranging from 0.0040 for JESPR 135 to 0.9993 for BNL1673.

Table 2. Locus, repeat motif, chromosome, allele size (bp) and number, and polymorphism information content (PIC) for the 39 SSR and 5 ISSR loci used in the analysis of the 25 cotton cultivars

Primer Name*	Repeat Type	Chromosome	No. of Alleles	Allele size (bp)	PIC
BNL448	(CT)13	AD20/AD22	2	100-200	0.4358
BNL852	(CA)13	AD18/19	5	90-110	0.6610
BNL1047	(CA)12	AD25	1	100-220	0.2944
BNL1053	(AC)16	AD21	3	90-220	0.3200
BNL1317	(AG)14	AD9/23	4	100-180	0.8031
BNL1414	(AG)16	AD9/24	6	100-200	0.7059
BNL1611-L1	(AG)12	AD19	3	100-200	0.2470
BNL1611-L2			1		0.2256
BNL1673	(AG)24	A12	3	100-200	0.9994
BNL1679	(AG)17	A12	4	100-200	0.5679
BNL226	(GA)16	AD3	1	100-200	0.0000
BNL2646	(GA)3+G+A2+(AG)4+ (GA)4. (TC)4+(CT)17	AD7/9/15	5	100-200	0.2546
BNL2652	(TC)32	AD13/18	2	100-300	0.5328
BNL2847	(GA)17	AD9	4	150-600	0.3020
BNL285	(GA)12. (GA)3+GC+ (GA)12+A+(AG)2	AD19	4	180-200	0.9576
BNL2960	(GA)10	AD10	4	100-200	0.9010
BNL2986	(AG)10	A7/AD16	5	120-500	0.0389
BNL3103	(GA)13. (TC)14	AD25	1	100-200	0.8976
BNL3171	(GA)26	AD21	2	180-200	0.8640
BNL3511	(AC)11	AD23	4	150-500	0.0044
BNL3816	(TG)15. (TG)5TA(TG)15	AD12/AD26	2	100-200	0.0624
CM13	(AG)23	-	3	80-120	0.9516
CM23	(CTCA)4. (CT)14	AD11	5	80-120	0.4138
CM3	(AG)22	-	2	180-200	0.9872
CM71	(TC)19	AD10	5	70-120	0.9485
CM76	(TC)13(AC)11. (AAAC)4	AD1	1	200	0.9936
CIR084	CA_8TA_7	AD14	1	100-150	0.2944
CIR398	$ACAT_5CA_7$	AD21	3	80-500	0.4838
JESPR135	$(CT)_{11}$	AD11/AD23	3	100-200	0.0040
JESPR153	$(CTA)_{18}$	AD18	8	80-160	0.1594
JESPR169	$(GA)_5(CTT)_{10}$	-	1	100-200	0.0784
JESPR224	$(GA)_{22}$	A6/AD25	7	80-200	0.1896
JESPR232	$(CT)_{18}$	AD8	3	120-180	0.8249
JESPR292	$(CTT)_7$	AD16	2	80-200	0.1536
JESPR292	$(CTT)_7$	AD16	2	80-200	0.6400
JESPR50	$(CAA)_5$	A4/AD5/AD25	3	100-250	0.2316
JESPR56	$(GAA)_{23}$	AD18/AD10/AD1/AD20/AD9	4	80-220	0.9928
Meghes16		-	4	80-180	0.3153
Meghes16		-	1	80-180	0.0784
MUcs0570	(AAT)4	AD17	1	80-300	0.9936
NAU1032	(ATC)6	-	5	100-200	0.0540
NAU1032	(ATC)6	-	1	100-200	0.1536
NAU1369	(AGGCGG)3	AD8/AD24/AD25	2	180-220	0.2240
NAU1369	(AGGCGG)3	AD8/AD24/AD25	1	180-220	0.2256
UDC811**	(GA)8 C	-	4	200-100	0.2203
ISSR12**	(GA)9	-	7	200-1000	0.2962
UDC817 L1**	(CA)8A	-	1	200-1000	0.0784
UDC817 L2**	(CA)8A	-	4	200-1000	0.3409
UDC827 L1**	(AC)8+G	-	1	100-500	0.1536
UDC827 L2**	(AC)8+G	-	5	100-500	0.3936
UDC826**	(AC)8	-	5	200-1000	0.4723

* Microsatellite primers producing multiple loci are indicated by "L".
** ISSR primers

Genetic diversity (GD) ranged from 0.04 to 0.58 among 25 cotton genotypes and wild accessions (Table 3). The lowest GD (0.04) examined was between Siocra and Nazilli 84S genotypes. The highest GD (0.58) examined was between *G. sturtianum* var. *nandewarense* belonging to C genome and Erşan92 (*G. hirsutum*) belonging to AD genome.

Table 3. Pairwise genetic distance coefficients [minimum. maximum and (mean) respectively] within and between cotton species, accessions and genotypes.

Genetic Distance	Wild Type Accessions	Commercial Cultivars	*G. hirsutum*	*G. barbadense*
Wild Type Accessions	0.23 - 0.57 (0.40)			
Commercial Cultivars	0.20 - 0.58 (0.35)	0.08 - 0.20 (0.15)		
G. hirsutum	0.18 - 0.58 (0.34)	0.04 - 0.23 (0.14)	0.04 - 0.23 (0.13)	
G. barbadense	0.23 - 0.49 (0.32)	0.13 - 0.32 (0.23)	0.13 - 0.31 (0.21)	0.07 - 0.26 (0.19)

On the other hand, GD among wild type accessions ranged from 0.23 to 0.57. The lowest GD (0.23) among wild type accessions was between *G. hirsutum* var. *yucatanense* and *G. mustelinum* genotypes while the highest GD (0.57) was among *G. lanceolatum* and *G. sturtianum* var. *nandewarense*.

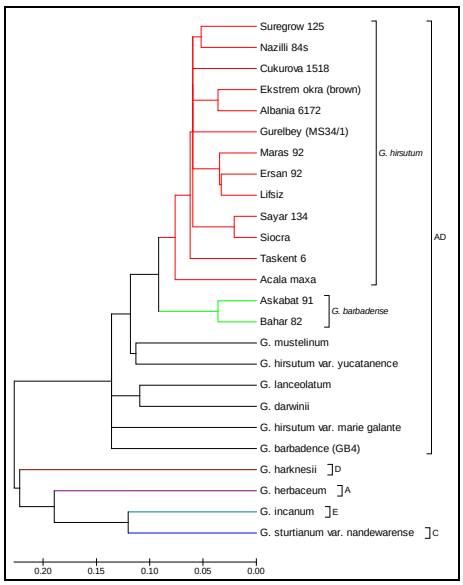

Figure. 2. Dendrogram obtained by Neighbor-Joining (NJ) method cluster analysis based on genetic distances (0.50) among cotton genotypes and wild accessions

The principal components analysis also showed similar relationships between diploid and the other tetraploid cottons as the NJ method shows (Figure 3).

GD among commercial cottons grown in Turkey ranged from 0.08 to 0.20. The lowest GD was between Sayar314 and Erşan92, Sayar314 and Maraş92, and Erşan 92 and Maraş 92 genotypes. The highest GD obtained were between Suregrow 125 and Erşan92 genotypes. GD among genotypes belonging to *G. hirsutum* L. ranged from 0.04 to 0.23. The lowest GD among *G. hirsutum* genotypes were observed between Siocra and Sayar314 genotypes. The highest GD examined between Acala Maxa and Nazilli84S genotypes.

Cluster analysis clearly discriminated diploid wild type cotton from other tetraploid wild types, accessions and genotypes. The dendrogram separated diploid cottons and the other tetraploid cottons into two major clusters. One of the clusters formed by tetraploid cotton was also separated into two groups; one included the *G. hirsutum* genotypes and the other one had the *G. barbadense* genotypes (Figure 2).

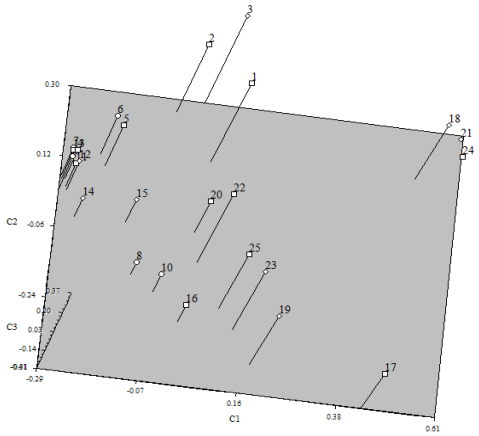

Figure. 3. Principal components analysis of 173 marker (SSR and ISSR) loci for *Gossypium* sp. Genotypes. (1=Suregrow 125; 2=Cukurova 1518; 3=Nazilli 84S; 4=Gurelbey MS34/1; 5=Ekstrem Okra (Brown); 6=Albania 6172; 7=Ersan 92; 8=Askabat 91; 9=Lifsiz; 10=Bahar 82; 11=Sayar 314; 12=Siocra; 13=Maras 92; 14=Taskent 6; 15=Acala Maxa; 16=*G. mustelinum* Miers ex Watt; 17=*G. harknesii* Brandg; 18=*G. herbaceum* L.; 19=*G. barbadence* L. (GB-4); 20=*G. hirsutum* var. *yucatanence*; 21=*G. incanum* (Schwartz) Hill; 22=*G. lanceolatum* Tod; 23=*G. hirsutum* var. *marie galante*; 24=*G. sturtianum* var. *nandewarence*; 25=*G. darwinii* Watt)

DISCUSSION

Primers produced a total of 173 alleles averaging 3.93 per locus (Table 2). Bertini et al. (2006) found 2.13 alleles per locus while Gutierrez et al. (2002) found 139 alleles in a total of 69 loci, averaging 2 alleles per locus. On the

other hand, Liu et al. (2000) used 62 primers that produced total 325 alleles, averaging 5 alleles per locus. Working with 47 wild *Gossypium* species (Lacape et al., 2007) obtained total 1128 alleles for 201 SSR loci and averaged 5.61 alleles per locus. Our results for the number of alleles per locus were higher than Gutierrez et al. (2002) and Bertini et al. (2006) but lower than Liu et al. (2000) and Lacape et al. (2007). This may be due to comparing wild and cultivated cottons together or differences in the amplified regions on the DNA.

PIC values averaged 0.44 and ranged from 0.0040 to 0.9993. Liu et al. (2000) found that PIC values ranged from 0.05 to 0.82 and averaged 0.31 in a work using wild *G. hirsutum* accessions. Different cotton genotypes may yield differences in the PIC values. In the work by Bertini et al. (2006), PIC values ranged from 0.18 to 0.62 and averaged 0.40. The lower PIC values for this experiment were due to the genotypes that came from a breeding program as explained by the author. Average value is very close to what we found but the difference in the range probably comes from the wild cotton accessions used in the present study..

GD values among *G. hirsutum* cotton genotypes were between 0.04 and 0.23. Similar results were previously obtained by Gutierrez et al. (2002) that genetic diversity belonging to *G. hirsutum* L. ranged from 0.06 to 0.34 among 11 parental lines. On the other hand, Zhang et al. (2005) found that GD was between 0.06 and 0.38 among Acala 1517 genotypes with SSR molecular markers. Multani and Lyon (1995) observed that GD ranged from 0.01 to 0.08 among nine Australian cultivars which also showed a lack of genetic diversity. Iqbal et al. (1997) found very high genetic similarity ranging from 0.82 to 0.93 among 17 *G. hirsutum* cultivars on the basis of random amplified polymorphic DNA (RAPD) markers. Khan et al. (2009) used SSR markers to determine genetic differences among *G. hirsutum* genotypes and found GD between 0.19 and 0.36.

Van Esbroeck et al. (1998) have pointed out that the monoculture of some successful cultivars and their extensive use as progenitors in breeding programs has limited the genetic diversity of cultivated cotton cultivars. They found genetic variation as 0.13 among cultivated cotton cultivars.

Comparisons of Aşkabat 91 and Bahar 82 to commercial cultivars yielded genetic diversity as 0.21. Genetic differences among wild cottons ranged from 0.23 to 0.57. Higher genetic variability among wild cotton species shows the usefulness of including them in breeding programs to increase variability within the germplasm pools. Wild type cotton can especially be used to increase biotic and abiotic stresses in cotton and have high fiber quality and yield under stress conditions.

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