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An Evaluation of The Utility of Simple Sequence Repeat loci (SSR) ,Expressed Sequence Tags (ESTs) and Expressed Sequence Tag Microsatellites (EST-SSR) as Molecular Markers in Cotton

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Abstract: The present study was undertaken with 21 SSR, 10 EST-SSR and 7 EST primer pairs for genetic diversity estimation in 14 cotton genotypes including 13 *G. barbadense* genotypes, i.e., G45, G70, G75, G76, G77, G80, G83, G85, G86, G87, G88, G89 and G90 and only one genotype (Hindi) belonging to *G. hirsutum*. Analysis of the 14 cotton genotypes as revealed by 7 EST and 31 SSR and EST- SSR primer pairs resulted in 119 alleles among which 79 were polymorphic (66.4%). The number of alleles per primer pair ranged from 1 to 6, except M7 which revealed eleven alleles, with an average of 3.1. While the number of polymorphic alleles ranged from 0 to 5 with an average of 2.1 alleles per primer pair. The allele size varied from 80 to 450 bp and the genetic similarities among all genotypes ranged from 68.9 (between Hindi and G45) to 95.5 (between G70 and G76) in combined data. The classification of the cotton genotypes using the UPGMA grouped cotton genotypes into two clusters. These markers were successful in characterizing nine out of the 14 tested cotton genotypes by unique positive and /or negative markers. PIC values detected by SSR primer pairs ranged from 0.06 in M55 to 0.98 in M23, while it ranged from 0.24 in C7 to 0.92 in C9 in EST-SSR and in EST, the PIC value ranged from 0.14 in EST-E3 to 0.67 in EST-E8.

Keywords: *Gossypium barbadense* L. – Cotton – Genetic diversity – SSR – EST – EST-SSR. *Gossypium hirsutum* L.

INTRODUCTION

In Egypt, cotton is considered one of the most important cash crops. Many of the Egyptian cotton varieties are particularly attractive due to their unique extra long staple dominating all other species. Genome analysis using molecular markers proved to be very useful to breeders in their breeding programs for crop improvement, particularly, when dealing with complex genetic traits. Cotton (*Gossypium barbadense* L.) is one of the two tetraploid species grown commercially for the production of cotton lint. It is cultivated through out temperate and tropical regions, with primary production areas. In 1987, global production of *Gossypium barbadense* exceeded 1.04 billion Kg, with an approximate market value of 2.6 billion dollars[5]. The unique fiber properties (greater length, strength, and fineness) of many *Gossypium barbadense* cultivars have allowed it to command a 50% - 60% price advantage over the other major commercial species, *G. hirsutum*. Microsatellites, or simple sequence repeats (SSRs) as second generation markers are subset of the tandemly repeated DNA family represented by extremely short nucleotide sequence repeats from 1-5 base pairs (bp) that are abundantly present and interspersed in eukaryotic genomes [8]. SSRs are reported to be highly informative in plants providing many different alleles for each marker, even among closely related individuals[29]. SSRs are inherited co-dominantly showing simple Mendelian segregation[4,15]. A new alternative source of microsatellites came from data mining of EST sequence databases of important crops [14, 34]. Several labs initiated large-scale cDNA analyses of the cotton genome, which characterized cDNA clones for identifying expressed sequence tags containing simple sequence repeats (EST-SSRs). Qureshi *et al*.[27] analyzed 739.258 ESTs from the publicly available databases of the major crops species, including cotton and they revealed that SSRs were present in the ESTs at a frequency of 3.4 % and 1.7 % in the sequences of monocots and dicots, respectively[30].

The aims of the present study are: 1) To distinguish cotton genotypes by SSR, EST-SSR and EST markers which will have important impact in breeding programs. 2) To identify allelic diversity among closely related cotton genotypes for each marker 3)To study the genetic diversity of microsatellites derived from genomic library and Genbank sequences in cotton 4) To investigate the utility of SSR and ESTs for determining genetic similarity and relationships in cotton and to measure the informativeness of each marker.

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MATERIALS AND METHODS

Plant Materials: Fourteen Egyptian cotton genotypes (Giza 45, Giza 70, Giza 75, Giza76, Giza 77, Giza 80, Giza 83, Giza 85, Giza 86, Giza 87, Giza 88, Giza 89, Giza 90 and Hindi) were selected from, the Egyptian cotton germplasm available at Cotton Research Institute (CRI), ARC, Egypt and employed in this study.

DNA Extraction and PCR Reactions: Total genomic DNA was isolated from young leaf material following the CTAB procedure of Porebski *et al.*[23] with some modifications as described by Hussein *et al.*[10]. The PCR reaction was conducted in 50 ml reaction volume containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1mM of forward and reverse primers, 2 U Hot start Taq (Bioron) polymerase and 40 ng genomic DNA. Hot start and touchdown PCR temperature profile was used as follows: an initial denaturizing step at 94°C for 5 min, followed by 10 cycles of touchdown annealing temperature 60°C to 50°C for 60 s in which the annealing temperature was decreased by 1°C every cycle. Another 30 cycles were starting then, a final extension step at 72°C for 7 min was performed.

Electrophoresis of SSR, EST-SSR and ESTs: Two different methods were used to resolve the PCR products 1) agarose gel electrophoresis system, where the PCR products were loaded on 2% agarose gel. 2) 8 % acrylamide gel capable to resolving few bases–pair polymorphisms of the SSR, EST-SSR and EST loci. The preparation of acrylamide gels was described in details in Adawy *et al.* [1].

Data Analysis: PCR gel profiles for each primer set were visually scored by assigning a number to each band according to its migration distance. For primer sets that revealed polymorphisms, the PCR reaction was repeated to verify results. The presence or absence of a band in a variety was coded 1 or 0, respectively. Estimation of Genetic Similarity (GS) was calculated for all pairs of varieties using Dice coefficients where GS is the number of fragments in both varieties. UPGMA was performed with matrix of GS estimates, to measure the informativeness of each marker. The polymorphism information content (PIC), was calculated using the following formula: PIC=1-åp2i where pi is the frequency of the i th SSR allele[33].

RESULTS AND DISCUSSIONS

EST, EST –SSR and SSR Primers: A computer search has been performed through the cotton database to identify the Published ESTs. Primers have been designed using the computer program primer 3 and DNA Star. Out of twenty EST primer pairs derived from published sequences [26], a set of 10 EST-SSR primers were successful. In addition 21 SSR and 7 EST primer pairs from cotton database were also used. These primers were synthesized at (AGERI) on an ABI 392 DNA / RNA synthesizer (Applied Biosystems). The code and accession numbers of the forward and reverse primer pairs is given in Tables (1 and 2). The length of primers ranged from 18 to 25 bp.

Genetic Diversity: The genetic divergence was estimated by 21 SSR, 10 EST-SSR and 7 EST primer pairs. These markers succeeded to produce polymorphic and monomorphic alleles when applied on the 14 cotton genotypes. The amplified products were separated on 8% acrylamide gels because the resolution power is higher than agarose gels, and sequences differing by 2 bp could not be resolved using agarose gels[1,2,9,12,31]. In this respect, Perez *et al.*,[21] studied the utilization of three electrophoresis separation methods for the analysis of each SSR polymorphism in almond and its implications in the assessment of genetic diversity and relatedness. They found that automated SSR detection and polyacrylamide gel electrophoresis were the most efficient methods and able to resolve allelic variation at a finer scale than electrophoresis in metaphor agarose. In addition, Smith *et al.,*[32] stated that SSR detection using polyacrylamide gel electrophoresis showed similar results as automated sequencing, although it is more time consuming than in agarose gels .

In the present study, SSR exhibited 78 major SSR alleles and the number of polymorphic alleles was 47, representing a level of polymorphism of (60.3%). While, EST-SSR showed 27 alleles and the number of polymorphic alleles was 21 detecting a level of polymorphism of (77.8%). In addition EST exhibited 14 alleles, 11 alleles of which were polymorphic, detecting a level of polymorphism of (78.6%). Moreover, combined data exhibited (66.4%) level of polymorphism. The number of polymorphic alleles generated by individual primer pairs ranged from one in (E8, E9, C4, C9, M14, M16, M22, M23 and M24) to 5 in (M19, M20 and C10). The primers (M19, M20 and C10) produced the greatest number of polymorphic products. In this respect, Prasad *et al.*[25] reported that SSR primers were locus specific and microsatellites were multi allelic exhibiting 1-13 alleles per locus in wheat. Bandopadhyay *et al.*[6] designed 78 EST-SSRs and tested for functionality on 18 triticeae species of common wheat. The number of alleles ranged from 2 to 14 with an average of 6.8 alleles per locus. A number of EST-SSR primers also proved useful in discriminating different genera. Moreover, Hussein *et al.*[12] employed twenty-four microsatellite flanking primer pairs to investigate the genetic polymorphism. The number of alleles per primer ranged from 1 to 5, while the number of polymorphic alleles varied from 0 to 5 and the average level of polymorphism was 53.7%.

Table 1: SSR primer pairs, total numbers of alleles, polymorphic alleles , percentage of polymorphism , observed allele size and (frequency) ,banding patterns and PIC value detected by different primer pairs for the 14 cotton varieties.

Code No.	SSR loci	Repeat unit	No. of alleles	Poly. alleles	% poly.	Observed poly. allele size & (Freq.)	PIC value	Banding pattern
C4	BNL- 3258 R BNL- 3258 F		2	2	100	(0.21) 210 , (0.79) 220	0.34	2
C6	BNL- 1597 R BNL- 1597 F		1	0	0	------	0.00	1
C7	BNL- 3359 R BNL- 3359 F		2	2	100	(0.71) 220 , (0.93) 230	0..36	3
C8	BNL- 1434 R BNL- 1434 F		4	4	100	(0.07) 220 , (0.07) 230 (0.86) 260, (0.14) 300	0.23	3
C9	BNL- 3482 R BNL- 3482 F		2	2	100	(0.71) 130 , (0.29) 150	0.42	2
C10	BNL- 3103 R BNL- 3103 F		3	3	100	(0.71) 190 , (0.29) 220 (0.07) 300	0.41	3
M7	JESPR - 7 R	(GAA)14	11	4	36.36	(0.14) 80 , (0.50) 100 (0.50) 150 , (0.21) 250	0.44	3
M8	JESPR - 8R	(GAA)12	2	2	100	(0.29) 280 , (0.71) 300	0.42	2
M11	JESPR - 11R	(GAA)10	4	3	75	(0.21) 400 , (0.14) 410 (0.21) 500	0.90	5
M12	JESPR-12 R	(CTT)7	3	3	100	(0.93) 280 , (0.07) 290 (0.14) 275	0.11	4
M13	JESPR- 13 R	(CTT)19	4	3	75	(0.07) 200 , (0.07) 220 (0.71) 290	0.49	3
M14	JESPR- 14 R	(CTT)17	2	1	50	(0.29) 250	0.92	2
M15	JESPR- 15 R	(GAA)7	1	0	0	-------	0.00	1
M16	JESPR- 16R	(AT)12 TGA	6	1	16.67	(85.7) 360	0. 28	2
M17	JESPR- 17 R	(GAA)12	3	0	0	-------	0.00	1
M19	JESPR- 19R	(GTA)	6	5	83.33	(0.85) 60 , (0.21) 200 (0.07) 300 , (0.86) 400 (0.64) 420	0.91	4
M20	JESPR- 20R	(CTT) 9	6	5	83.33	(0.07) 190 , (0.07) 200 (0.07) 300 , (0.79) 500 (0.14) 600	0.35	4
M22	JESPR- 22R	(GAA) 12	5	1	20	(0.29) 250	0.15	2
M23	JESPR- 23R	(CTT)9	2	1	50	(0.14) 200	0.98	2
M24	JESPR- 24R	(CTT)1 (CTCTT)2 (CTT)2	3	1	33.33	(0.36) 150	0.87	2
M55	JESPR-55R	(CT)6 A(CA)11	6	4	66.67	(0.07) 200, (0.79) 120 (0.14) 200, (0.64) 220	0.06	3

Tables (1and2), show that there is no clear relationship between the number of repeat motifs and the number of alleles. Each SSR, EST-SSR and EST locus may have one (homozygous locus) or several (heterozygous locus) allelic forms. The locus that revealed different allelic forms varying in length among the cotton genotypes is considered as a polymorphic locus. Tables (1 and 2) showed a number of 2, 2 and 1 of the SSR,

Table 2: EST-SSR and EST primer pairs, total numbers of alleles, polymorphic alleles , percentage of polymorphism, observed allele size & (frequency) ,banding patterns and PIC value detected by different primer pairs for the 14 cotton varieties.

Code No.	Gene Bank Accession (s)	Repeat units	No. of alleles	Polymorphic alleles	% polymorphism	Observed polymorphic allele size & (Frequency)	PIC value	Banding pattern
EST-SSR C1	AW187078.1 AW187113.1	(CCA)7	1	0	0	-----	0.00	1
EST-SSR C2	A1731348.1 A1731677.1	(AG)12, (GA)3, (TTA)3	2	2	100	(0.35) 220 , (0.64) 225	0.25	2
EST-SSR C3	A1731348.1 A1731677.1	(AG)12, (TTA)3, (CGG)3	1	0	0	-----	0.00	1
EST-SSR C4	M16936.1	(GA)3	2	1	50	(0.85) 225	0.27	2
EST-SSR C6	A1727680.1 A1731036.1	(GAC)11, (ACA)3	4	4	100	(0.71) 220, (0.29) 225 (0.14) 230, (0.29) 240	0.32	3
EST-SSR C7	AW187936.1	(CT)10 , (TCT)4	3	2	75	(0.79)205 ,(0.85) 210	0.24	2
EST-SSR C8		(GA)14	4	4	100	(0.07) 250, (0.14) 255 (0.57) 290 ,(0.29) 295	0.57	4
EST-SSR C9	AW187121.1 AW187322.1 AW187585.1	(TCT)7 (AAG)3, (CTA)4	2	1	50	(0.29) 350	0.92	2
EST-SSR C10	AW187539.1 AW187811.1	(ATT)11	5	5	100	(0.57) 190 , (0.14) 200 (0.5) 290 , (0.57) 340 (0.71) 350	0.43	6
EST-SSR	AW186906.1 AW186907.1 AW187848.1	(CT)18	3	2	75	(0.86) 70, (0.14) 80	0.25	2
EST-E1			2	2	100	(0.29) 240 , (0.71) 250	0.42	3
EST-E3			2	2	100	(0.93) 400 , (0.07) 500	0.14	2
EST-E5			2	2	100	(0.29) 345 , (0.71) 350	0.42	2
EST-E7			1	0	0	------	0.00	1
EST-E8			2	1	50	(0.57) 230	0.67	2
EST-E9			2	1	50	(0.79) 550	0.38	2
EST-E10			3	3	100	(0.64) 500 , (0.79) 550 (0.93) 600	0.59	4

EST-SSR and ESTs loci revealing monomorphic patterns, respectively, however, monomorphic markers have no practical value in breeding. The data also reveal that there is no clear relationship between the number of repeated motifs and the level of polymorphism. This is in agreement with Hussein *et al.,*[12], who reported that primer pairs SH1 and SH5 comprised 17 and 18 repeats, respectively and exhibited only one allele across the 21 cotton genotypes.

Polymorphism Information Content (PIC): The SSR s revealed high level of polymorphism. Data obtained by the 21 SSR primer pairs that were assayed on 14 cotton genotypes are illustrated in Table (1). These were 78 alleles, with an average of 3.7 alleles per locus. Ten EST-SSR produced 27 alleles with an average 2.7 alleles per locus and seven ESTs produced 14 alleles with an average of 2 alleles per locus (Table 2). PIC values detected by SSR primer pairs ranged from 0.06 in M55 to 0.98 in M23, while it

Table 3: The cotton genotypes characterized by unique positive and/or negative EST, EST-SSR and SSR markers, and total number of markers identifying each genotype.

Primer code	Unique Negative Markers			Unique Positive Markers							
	G.77	G.80	G.86	G.45	G.75	G.76	G.80	G.85	G.86	G.87	G.90
C7			230								
C8				220	230						
C8				250							
C10										300	
M12											290
M13									200		
M20						300	200				
M22	250										
M55								100			
E3		200					210				
E10	550										
Total	2	1	1	2	1	1	2	1	2	1	1

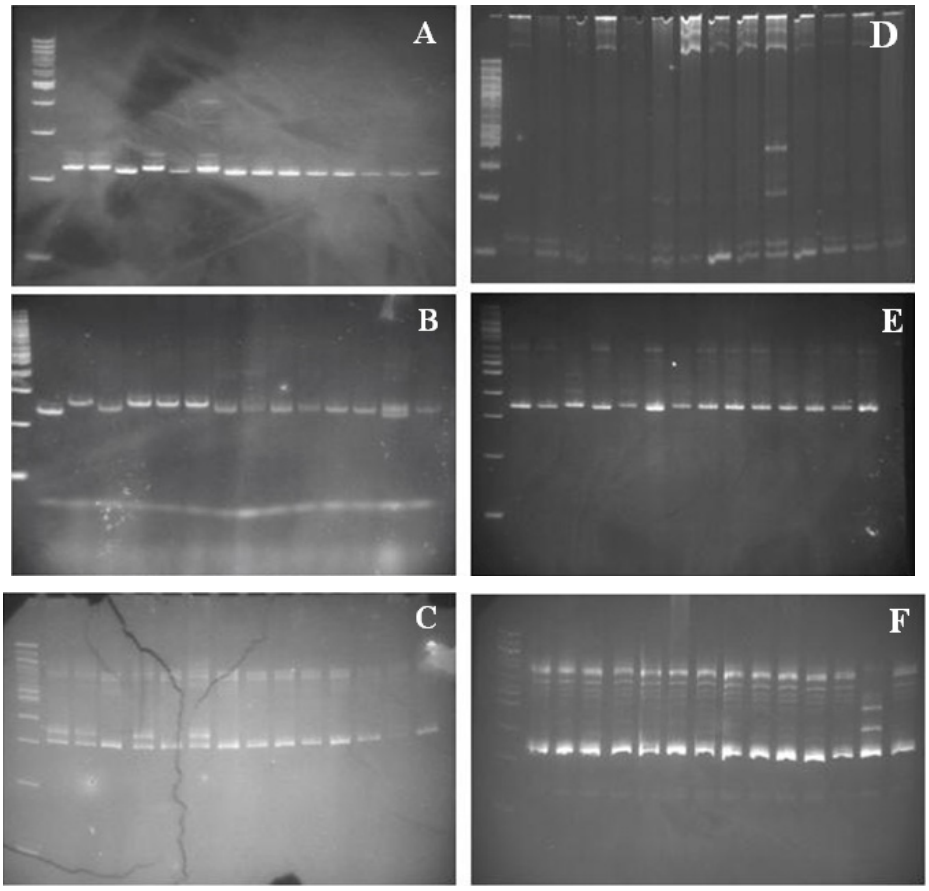

Fig. 1: EST-SSR and EST profiles of the 14 cotton genotypes as revealed by primer pairs C6 (A), C8 (B), C9 (C), E8 (D), E5 (E) and E1 (F) on polyacrylamide gels stained with ethidium bromide.

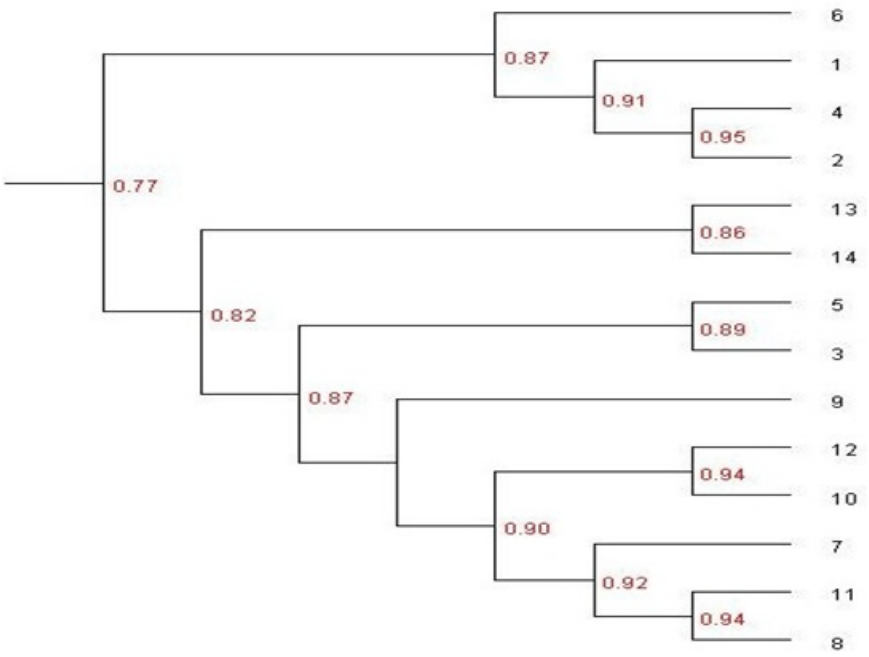

Fig. 2: Dendrogram of the 14 cotton genotypes as revealed by combined data (SSR s, EST-SSR s and ESTs).

ranged from 0.24 in C7 to 0.92 in C9 in EST-SSR. Finally in EST, the PIC value ranged from 0.14 in EST-E3 to 0.67 in EST-E8. In this regard, Senior *et al.*[31] found that the PIC value for the SSR loci ranged from 0.17 to 0.92 in 70 maize SSR primers. In barley, Pillen *et al.* [22] reported lower PIC values 0.73 to 0.15 in EST-SSR and 0.50 to 0.21 in EST. Cho e*t al.*,[7] compared genomic library derived with Gen Bank–derived microsatellites in rice and reported lower PIC values for the latter. In general, Matus and Hayes [19] found higher PIC values for SSRs with higher numbers of alleles, as did Pillen *et al.*,[22] also Matuy and Hayes [19] did find high PIC values for some SSR s, even in the narrow genetic base of the BARI germplasm, which indicates that some SSRs are useful for differentiating between closely related accessions. PIC scores represent the gene diversity for a specific locus. Martinez *et al.,*[18] found that the expected heterozygosis in *Vitis vinifera* varieties varied between 0.71 and 0.89, and the PIC ranged from 0.70 to 0.88 indicating that the SSRs were highly informative.

Genetic Relationships among 14 Cotton Genotypes: In this study, the genetic similarity (GS) ranged from 74.3 % to 94.9% in SSR, from 52% to 98.1% in ESTs and EST-SSR while, it was from 68.9% to 95.5% in combined data (SSR, EST and EST-SSR). The highest (GS) revealed by SSRs analysis was between G.89 and G.87, while the lowest (GS) was between G.45 and G.86. The highest (GS) in ESTs and EST-SSR was between G.83 and G.85 and the lowest (GS) was between Hindi and *G*.76. The combined data revealed (GS) ranging from 68.9% between (Hindi and *G*.45) to 95.5% between (*G*.76 and *G*.70). Three dendrograms were developed based on Dice similarity index obtained by the SSR, EST- SSR and ESTs and the combined data. All the three dendrograms grouped all cotton genotypes into two clusters. The first SSR dendrogram included genotypes *G*.45, *G*.70, *G*.76 and *G*.90 in one cluster and the rest allocated in the second cluster. The second EST and EST-SSR dendrogram grouped genotypes *G*.45, *G*.70, *G*.76 and *G*.80 in one cluster and the rest in the second cluster. The third dendrogram based on combined data assigned cotton genotypes as in EST-SSR dendrogram (Fig. 2). In this respect, Powell *et al.*[24] reported that several factors might affect the estimates of genetic relationships between individuals i.e., number of markers used, distribution of markers in the genome (genome coverage) and the nature of evolutionary mechanisms underlying the variation measured. Joobeur *et al.,*[13], Hussein *et al.*,[12] and Adawy *et al.*,[2,3] reported that combining data was more powerful in detecting the maximum amount of genetic variation. Multani and Lyon[20] found in Australian cotton that the genetic similarity ranged from 92.1 to 98.9% among nine cultivars of *G. hirsutum* L and also found that *G. barbadense* L. v. Pima S-7 showed about 57% similarity with the *G. hirsutum* L. varieties which is in agreement with our

results. Rana and Bhat [28] clustering 59 cotton cultivars using UPGMA cluster analysis, showed that all *G. barbadense* cultivars grouped in one cluster and having maximum similarity, another cluster contained all *G. hirsutum* except cultivars CPD 423, CP 15/2, Laxmi and G. Cot12 which did not cluster with the rest of the *G. hirsutum* cultivars. Genomic SSRs derived from *G. hirsutum* were used for mapping in other species, such as *G. barbadense, G. nelsonii fryx.*, and *G. australa* f. Muell [16, 17, 26]. These results indicate that there is a high degree of sequence conservation between the different diploid and tetraploid species of *Gossypium*. The ability to amplify these markers by PCR, conservation of sequence across different species of cotton, a high polymorphic rate, and the potential for sequence based comparative mapping make the EST-SSR markers ideal for comparative mapping in cotton. EST-SSR sequences compared with sequences of other cotton species, as well as the sequences of other crops and model organisms using BLAST revealed that about 74% of the EST-SSR loci had sequence similarity with a gene expressed in fiber tissues of *G.hirsutum*. Forty eight percent of the non-redundant EST-SSR sequences matched with ESTs from *G. arboretum* derived from fiber tissues.

Cultivar Identification by Unique Markers: In the present investigation 11 SSR and EST primer pairs detected unique specific markers identifying 9 out of 14 cotton genotypes. Each of these primer combinations revealed unique markers characterizing one or more genotypes. As shown in Table (3) the number of unique positive markers (UPM) ranged from 1 to 2 in the different cotton genotypes (G.45, G.75, G.76, G.80, G.85, G.86, G.87 and G.90). Four unique negative markers (UNM) characterized genotypes (G.77, G.80 and G.86) and ranged from 1 to 2 in each genotype. In this context, Hussein *et al.*,[9] identified genotype specific DNA markers when applying SSR on 13 cotton genotypes. They stated that only one unique marker characterized G.75 and G.87 while Hindi was characterized with 3 unique markers. Moreover, Hussein *et al*.,[12] identified specific DNA marker when applying RAPD, ISSR and SSR markers on 21 cotton genotypes. Adawy *et al*.,[3] detected unique specific markers identifying 8 out of the 21 cotton genotypes using AFLP markers. The total number of unique bands across the 21 cotton genotypes was 47 including 14 (UPM) and 33 (UNM).The number of UPM ranged from 1 to 4 and the number of UNM ranged from 1 to 22 in the different cotton genotypes. The highest number of UPM and UNM was 4 and 22, respectively, characterized the genotype (Delcero)**.** In addition, their results revealed that 14 out of 16 AFLP primer combinations distinguished *G.hirsutum* from *G.barbadense* genotypes.

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