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Genetic characterization of some commercial cotton varieties using Td-DAMD-PCR markers

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Abstract Four species of the genus *Gossypium* L. is considered as the backbone of the plant based textile industry in the world. *Gossypium hirsutum* L. is the only cotton species commercially grown in Turkey for fiber and oil production. Cotton production in Turkey is still considered as organic cotton since there are no commercial genetically engineered cotton varieties growing. Almost 100 commercial cotton varieties are currently being grown in the four major cotton production areas of Turkey. In the present study we investigated genetic relationships among some commercial varieties and accessions developed in Turkey, Syria, Uzbekistan and the Unites States using a touchdown-directed amplification of minisatellite-region DNA polymerase chain reaction (Td-DAMD-PCR) technique. Analyses revealed that 10 minisatellite primers, which were selected from 22 primers, were suitable in genetic and molecular breeding studies of cotton. Commercial varieties and accessions of cottons were separated into 3 main clusters, each of which consisted of commercial varieties and accessions belonging to species of *G. hirsutum*, *G. barbadense* and *G. herbaceum*. Based on the analyses the following conclusions were drawn; i) commercial cotton varieties widely grown in Turkey have very narrow genetic base, ii) higher genetic similarities among the cultivars indicated that most of the cotton cultivars studied in the present study were developed from similar parental lines, iii) Td-DAMD-PCR technique may not be suitable for genetic diversity studies of commercial cotton cultivars with similar genetic background.

Keywords cotton, DNA markers, PCR, species diversity, touchdown-PCR, Turkish varieties

1. Introduction

Cotton is the world's most important plant based textile fiber and a significant oilseed crop. The word 'cotton' refers to four species in the genus *Gossypium* (Malvaceae) namely *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L. *Gossypium hirsutum*, also called New World or Upland cotton, and *G. barbadense*, also called Egyptian or Sea Island cotton or Pima cotton, supply most of the world's cotton fiber (more than 95%). *G. arboreum* and *G. herbaceum* produce relatively short fibers and they are grown in a few Asian countries. The worldwide economic impact of the cotton industry is estimated at approximately $500 billion per year with an annual utilization of about 30 million metric tons of cotton fiber. The main cotton producer countries are China (24%), the United States (19%), India (13%), Pakistan (8%), Brazil (6%), Uzbekistan (5%) and Turkey (4%) [1-4].

In 2014, 32.78 million hectares (mha) were planted cotton in the world. The largest land planted cotton was in India (11.7 mha) followed by China (4.8 mha), the United States (3.05 mha), Pakistan (3.0 mha), Uzbekistan (1.29 mha), Brazil (1.12 mha), Argentina (0.56 mha), Australia (0.44 mha) and Turkey (0.33 mha). Turkey is the fourth largest consumer of cotton in the world after China, India and Pakistan [2] and is the second largest cotton importer country, with only China importing more. Domestic cotton consumption was 1.32 million metric tons in 2012/13 in Turkey. The United States, Turkmenistan and Greece are the main cotton suppliers countries to Turkey. Turkey could remain one of the largest markets for the United States and other countries cotton exports due to a lack of local supplies [4].

Most cotton cultivars developed in Turkey are derived from a few germplasm resources brought from the United States, Turkmenistan, Uzbekistan, Syria and Egypt. Public and private sectors created a large number of cotton genetic germplasms, many of which are in the primer gene pool. These genetic resources contain fiber quality properties, yield and yield components, nematode resistance, *Verticillium* wilt resistance and *Fusarium* wilt resistance. It is difficult to improve most of these traits using conventional breeding methods. The use of DNA markers in plant breeding has been the choice of method worldwide because these markers are simple, quick, cheap and reliable in comparison to many other methods. DNA markers are being commonly used in identification of lines/varieties, seed certification, plant breeder rights implementation, genetic diversity, phylogenetic studies, and construction of genetic maps and gene cloning and gene identification in many plant species including cotton. However, literature review indicated that there were a few applications of DNA markers in commercial Turkish cotton varieties for genetic diversity studies [5-7]. Although microsatellite markers, also known as simple sequence repeat markers (SSRs), are very reproducible and easy to analyze, the levels of polymorphisms released by SSR technique are low in cotton [5, 8].

The touchdown direct amplification of minisatellite-region DNA polymerase chain reaction (Td-DAMD-PCR) technique has not yet been applied in commercial Turkish cotton varieties. The DAMD technique specifically amplifies DNA region consisting of minisatellites or minisatellite regions, which are tandemly or almost tandemly repeated regions of eukaryotic genomes [9]. Amplified minisatellite regions show high levels of allelic length variations due to the differences in the number of repeat units [10-11]. Studies revealed that using touchdown polymerase reaction conditions enhance the reproducibility of the DAMD technique and its utilization rate [12-13].

This study was undertaken to determine suitable minisatellite primers to be used in the Td-DAMD-PCR method in cotton and to investigate genetic relationships among some commercial varieties and accessions developed in Turkey, Syria, Uzbekistan and the Unites States.

2. Materials and Methods

2.1. Cultivars and Varieties

Commercial varieties and accessions of cotton samples included "Aleppo-1", "Aydin-110", "DP-20", "DP-565", "Aleppo", "Lachata", "Maras-92", "McNair-235", "Nazilli-M503", "Nata", "Sahel-1", "Sealand-542", "Tashkent-3", "SDN-12", "Nazilli-84S", "Menderes-2005", "ADN-P01", "Beren", "Flash", "Theka", "Progen" and "Texas Marker 1" ("TM-1") belonging to *G. hirsutum* L., "Pima 3-79", "Giza-70" and "Pima-S7" belonging to *G. barbadense* L., and "Maydos Yerlisi" belonging to *G. herbaceum* L. Commercial varieties were randomly selected from a total of 100 varieties that are being maintained in Akdeniz University, Faculty of Agriculture, Field Crops Department, Antalya, Turkey. Accessions "Maydos Yerlisi", "TM-1", "Pima S7" and "Pima 3-79" were included in the analysis to trace the origins of the commercial Turkish cultivars.

2.2. Genomic DNA Extraction

Genomic DNA samples of commercial varieties and accessions mentioned above were extracted from bulked leaf samples using an established protocol [14]. Briefly, 1 g of leaf samples from samples were ground in a wide-sized mortar using liquid nitrogen. Plant samples were homogenized in the extraction buffer; following centrifugation, cells were lysed using lysis buffer. Genomic DNAs were purified from proteins, polysaccharides and pigments. DNA amount, purity, and integrity of the samples were determined using a spectrophotometer and agarose gel electrophoresis technique. Before polymerase chain reaction (PCR) studies, concentrations of DNA samples were readjusted so that each PCR contained 0.085 µg DNA in 8.5 µL volume for amplification. All chemicals used in this study were of molecular biology grade and purchased from Amresco Inc. (Solon, OH, USA) or Invitrogen Corp. (Carlsbad, CA, USA).

2.3. Touch-down DAMD-PCR

Target regions were amplified using touchdown polymerase chain reactions (Td-PCRs) based DAMD profile. Reactions were carried out in a 25 µL volume containing 0.06–0.08 µg of genomic DNA as a template, 2.4 µM of each primer listed in Table 1, 80 mM Tris-HCl (pH 8.8), 19 mM $(NH_4)_2SO_4$, 0.009% Tween-20 (w/v), 0.28 mM each dNTP, 3 mM $MgCl_2$, and 2 units of *Taq* DNA polymerase as reported in Ince and Karaca [15].

The Td-DAMD-PCRs were carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA,USA) with the following amplification profile: 3 min hold at 94°C, followed by 10 cycles of pre-PCR, consisting of 30 s at 94°C for denaturing, 30 s at 55°C for annealing, and 2 min at 72°C for primer extension reaction. Annealing temperature was reduced by 0.5°C per cycle during the first ten cycles. The PCR amplification was then continued for 30 more cycles at a constant 50°C annealing temperature, and the rest of the pre-PCR cycling parameters were kept unchanged. At the end of the PCRs, the samples were kept for 10 min at 72°C for the final extension reaction [15].

2.4. **Agarose Gel Electrophoresis of Td-DAMD-PCR Products**

Five microliters of DNA loading buffer consisting of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 40% (w/v) sucrose in sterile water was added to each 25 µL Td-DAMD-PCR amplified reaction, and 10 µL–12 µL of these mixtures were loaded in 3% (w/v) high resolution agarose gels (Serva, Heidelberg, Germany) containing 0.5 µg/mL of ethidium bromide and electrophoresed at 5 V/cm of constant voltage for 8–12 h in the presence of 1X Tris Borate EDTA buffer consisting of 89 mM Tris, 89 mM borate, and 2 mM EDTA (pH 8.3). Following electrophoresis, amplicons (bands) were visualized and photographed on a UV transilluminator for further analysis [15].

2.5. **Data Analysis**

Each Td-DAMD-PCR band was scored as present (1) or absent (0). Bands showing the same mobility were assumed to be the same and no attempts were made to code for band intensity in this study. Scored markers (bands) were used in the calculation of Nei and Li's genetic similarity indices. The Nei and Li's genetic similarity index values (GSI) were obtained using the following formula:

$$GSI_{XY}: 2a/(a + b) + (a + c)$$

where X and Y are the numbers of markers in varieties or cultivars X and Y, respectively, a is the number of markers shared between individuals X and Y, b is the number of markers present in individual X but absent in individual Y, and c is the number of markers absent in individual X but present in individual Y, using Multi Variety Statistical Package software (MVSP 3.13O, Kovach Computing Services, Pentraeth, UK). Also principal coordinate analysis (PCoA) was used to visualize the spatial relationships among commercial Turkish varieties and accessions based on Grower General Similarity Coefficient using MVSP software. In the PCoA axes each variety/accession was represented as a point in space.

3. Results and Discussion

In the present study, initially, 22 DAMD primers published in Ince et al. [16] were screened with four template DNAs, out of which 10 DAMD primers were selected since they produced clear and reproducible banding patterns (Table 1). In the present study not all the Td-DAMD-PCR bands were scored, we considered sharp and reproducible markers in the scoring studies. Bands greater than 3 kb and lower than 0.1 kb were not also used.

Table 1: Primers used in the Td-DAMD-PCR technique for genetic diversity studies of commercial Turkish cotton varieties and accessions

Primer[1]	5'→3' Nucleotide Sequences	Number of Amplified Bands	Number of Polymorphic Bands
URP1F	ATCCAAGGTCCGAGACAACC	12	8
URP2F	GTGTGCGATCAGTTGCTGGG	11	4
URP2R	CCCAGCAACTGATCGCACAC	13	5
URP4R	AGGACTCGATAACAGGCTCC	10	3
URP6R	GGCAAGCTGGTGGGAGGTAC	16	5
URP9F	ATGTGTGCGATCAGTTGCTG	10	3
URP13R	TACATCGCAAGTGACACAGG	8	2
URP17R	AATGTGGGCAAGCTGGTGGT	10	2
URP32F	TACACGTCTCGATCTACAGG	16	6
6.2H	CCCTCCTCCTCCTTC	14	4

1: Detailed information on the primers can be found in Ince et al. [16].

The size of the amplified Td-DAMD-PCR bands across the 26 cotton samples varied from 0.1 kb to 3 kb. The 10 selected DAMD primers amplified a total 120 DNA bands, 42 of which were polymorphic, with 35 % polymorphism across commercial Turkish cotton varieties and accessions studied. The highest numbers of amplified bands (16) were obtained with primers URP6R and URP32F, whereas the primer URP13R resulted in the lowest number (8) of amplified bands. The primer URP1F gave 66.67 % polymorphic bands (Fig. 1), whereas primer URP17R revealed 20% polymorphism among the cotton varieties and accessions (Table 1).

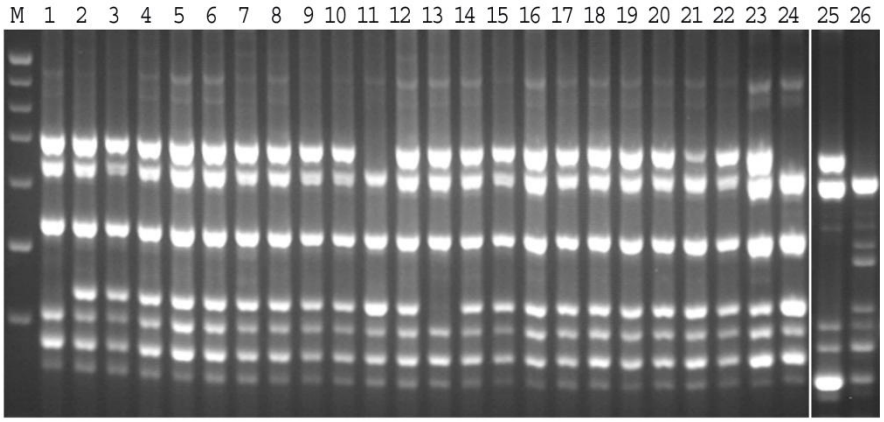

Figure 1: The Td-DAMD-PCR profile of some commercial Turkish cotton varieties and accessions amplified with URP1F primer. M: DNA size markers ranging from 400 bp to 1000 bp. Number 1 to 26 are "Aleppo-1", "Aydin-110", "DP-20", "DP-565", "Aleppo", "Lachata", "Maras-92", "McNair-235", "Nazilli-M503", "Nata", "Pima S7", "Sahel-1", "Sealand-542", "Tashkent-3", "SDN-12", "Nazilli-84S", "Menderes-2005", "ADN-P01", "Beren", "Flash", "Theka", "Progen", "TM-1", "Pima 3-79", "Giza-70" and "Maydos Yerlisi", respectively. Td-DAMD-PCR markers are separated in 3% high resolution Serva agarose gels

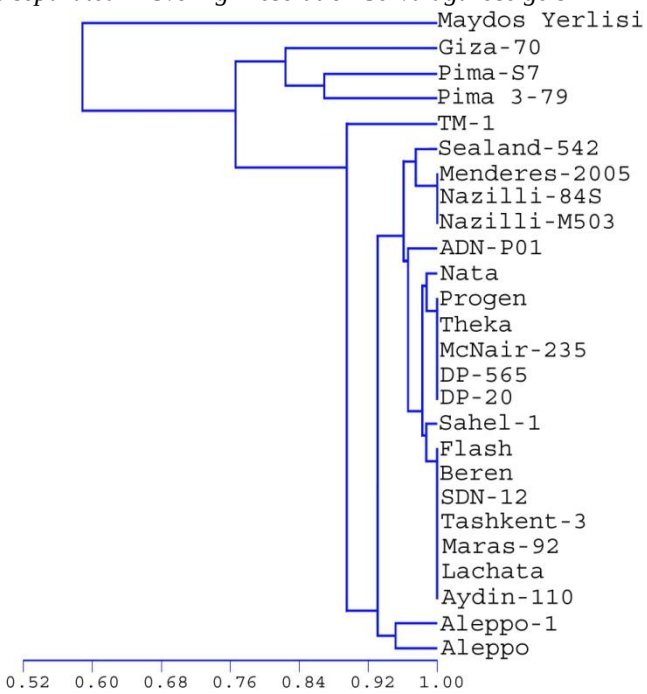

Figure 2: UPGMA cluster of some commercial Turkish cotton varieties and accessions. The bar below the tree shows Ne-Li's genetic similarity indices

Table 2. Similarity matrix for some commercial varieties of Turkish cotton and accessions determined on the basis of Td-DAMD-PCR markers using Nei & Li's coefficients

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. Aleppo-1	1.00																									
2. Aydin-110	0.95	1.00																								
3. DP-20	0.94	0.99	1.00																							
4. DP-565	0.94	0.99	1.00	1.00																						
5. Aleppo	0.95	0.93	0.91	0.91	1.00																					
6. Lachata	0.95	1.00	0.99	0.99	0.93	1.00																				
7. Maras-92	0.95	1.00	0.99	0.99	0.93	1.00	1.00																			
8. McNair-235	0.94	0.99	1.00	1.00	0.91	0.99	0.99	1.00																		
9. Nazilli-M503	0.92	0.97	0.96	0.96	0.95	0.97	0.97	0.96	1.00																	
10. Nata	0.92	0.97	0.99	0.99	0.90	0.97	0.97	0.99	0.95	1.00																
11. Pima-S7	0.75	0.80	0.79	0.79	0.74	0.80	0.80	0.79	0.78	0.78	1.00															
12. Sahel-1	0.94	0.99	0.97	0.97	0.94	0.99	0.99	0.97	0.96	0.96	0.79	1.00														
13. Sealand-542	0.92	0.95	0.96	0.96	0.93	0.95	0.95	0.96	0.97	0.95	0.75	0.94	1.00													
14. Tashkent-3	0.95	1.00	0.99	0.99	0.93	1.00	1.00	0.99	0.97	0.97	0.80	0.99	0.95	1.00												
15. SDN-12	0.95	1.00	0.99	0.99	0.93	1.00	1.00	0.99	0.97	0.97	0.80	0.99	0.95	1.00	1.00											
16. Nazilli-84S	0.92	0.97	0.96	0.96	0.95	0.97	0.97	0.96	1.00	0.95	0.78	0.96	0.97	0.97	0.97	1.00										
17. Menderes-2005	0.92	0.97	0.96	0.96	0.95	0.97	0.97	0.96	1.00	0.95	0.78	0.96	0.97	0.97	0.97	1.00	1.00									
18. ADN-P01	0.92	0.97	0.96	0.96	0.90	0.97	0.97	0.96	0.95	0.95	0.77	0.96	0.92	0.97	0.97	0.95	0.95	1.00								
19. Beren	0.95	1.00	0.99	0.99	0.93	1.00	1.00	0.99	0.97	0.97	0.80	0.99	0.95	1.00	1.00	0.97	0.97	0.97	1.00							
20. Flash	0.95	1.00	0.99	0.99	0.93	1.00	1.00	0.99	0.97	0.97	0.80	0.99	0.95	1.00	1.00	0.97	0.97	0.97	1.00	1.00						
21. Theka	0.94	0.99	1.00	1.00	0.91	0.99	0.99	1.00	0.96	0.99	0.79	0.97	0.96	0.99	0.99	0.96	0.96	0.96	0.99	0.99	1.00					
22. Progen	0.94	0.99	1.00	1.00	0.91	0.99	0.99	1.00	0.96	0.99	0.79	0.97	0.96	0.99	0.99	0.96	0.96	0.96	0.99	0.99	1.00	1.00				
23. TM-1	0.85	0.90	0.91	0.91	0.86	0.90	0.90	0.91	0.90	0.90	0.73	0.89	0.90	0.90	0.90	0.90	0.90	0.87	0.90	0.90	0.91	0.91	1.00			
24. Pima 3-79	0.73	0.77	0.76	0.76	0.72	0.77	0.77	0.76	0.75	0.75	0.87	0.76	0.73	0.77	0.77	0.75	0.75	0.75	0.77	0.77	0.76	0.76	0.73	1.00		
25. Giza-70	0.73	0.78	0.77	0.77	0.71	0.78	0.78	0.77	0.75	0.75	0.82	0.77	0.73	0.78	0.78	0.75	0.75	0.75	0.78	0.78	0.77	0.77	0.73	0.82	1.00	
26. Maydos Yerlisi	0.55	0.61	0.60	0.60	0.55	0.61	0.61	0.60	0.59	0.59	0.56	0.60	0.55	0.61	0.61	0.59	0.59	0.61	0.61	0.61	0.60	0.60	0.57	0.56	0.57	1.00

The Ne-Li's genetic similarity data (Table 2) were used to generate the UPGMA dendrogram depicted in Figure 2 (Fig. 2) and PCoA analysis depicted in Fig 3. UPGMA dendrogram showed that the Upland and Pima cotton varieties were distinctly separated from "Maydos Yerlisi", which belongs to *Gossypium herbaceum* L. Within the varieties studied, the Egyptian cotton variety; "Giza 70" and Pima accessions "Pima 3-79" and "Pima S7" which belong to *G. barbadense* L. were distinctly separated from commercial *G. hirsutum* varieties (Fig. 2).
Among the Turkish cotton varieties "Menderes-2005", "Nazilli-84" and "Nazilli-M503" were found to related to "Sealand-542", which carries some alleles from *G. barbadense*. Among the Turkish varieties these varieties formed a cluster (Fig. 2). These varieties showed closer genetic similarity indices as they can be seen in Table 2.

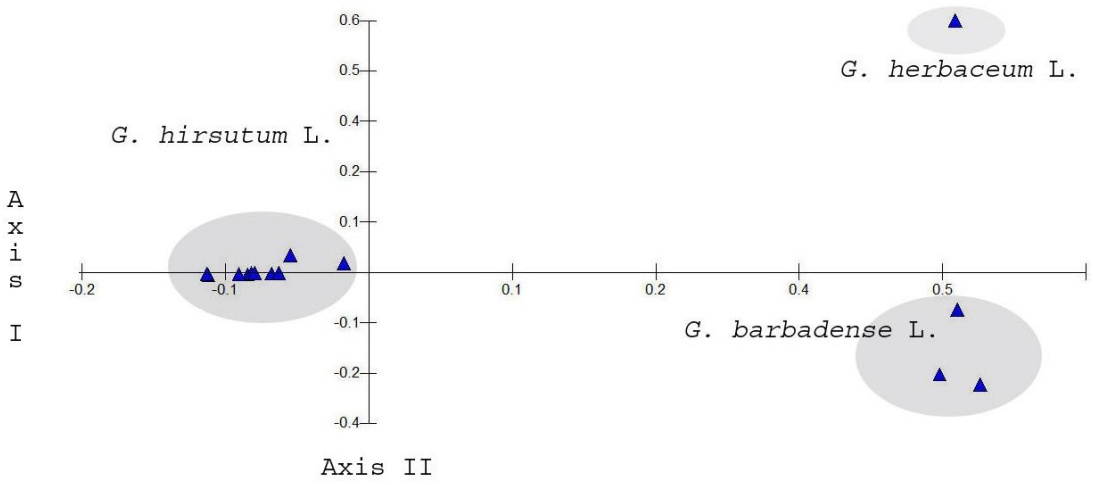

Figure 3: Principal coordinate analysis (PCoA) of commercial Turkish cotton varieties and accessions. All Turkish varieties clustered in G. hirsutum L. The 1st and 2nd axis explained 39.11% and 16.89% of the variance, respectively

The second cluster consisted of those commercial varieties other than "Aleppo-1" and "Aleppo", which formed the third cluster (Fig. 2). Varieties of "Alleppo-1" and "Aleppo" are from Syrian germplasm. Analysis indicated that some of the Turkish varieties originated from Syrian germplasms while other originated from the US and Uzbekistan germplasms. However most of the cotton varieties have very narrow genetic base and show very high level of genetic similarity indices (Table 2). Low level of genetic similarity for the Turkish cotton varieties was also reported in the previous works [5-6, 8]. Also there are many reports revealing the low level of genetic diversity in cultivated cotton varieties in the world [1, 17].
Some of the cotton varieties clustered in one of the three main groups could not be separated from the other members of the main group. For instance in group 1 (cluster 1) "Menderes-2005", "Nazilli-84S" and "Nazilli-M503", in group 2, "Progen", "Theka", "McNair-235", "DP-565" and "DP-20" and again in group 2, "Flash", "Beren", "SDN-12", "Tashkent-3", "Maras-92", "Lachata" and "Aydin-110" could not be separated from each other.
PCoA, which is an ordination method similar to principal component analysis, uses the distance matrix (rather than the values) to plot the axes. In the present study PCoA was used to determine and reveal spatial relationships among the commercial varieties.. In the PCoA analysis axes 1 and 2 represented 38.11% and 16.89% of the variation among the commercial Turkish varieties and accessions, respectively. PCoA analysis also clearly showed that all the commercial cotton varieties belonged to *G. hirsutum* L. (Fig 3). PCoA analysis also clearly showed that commercial Turkish cultivar has a narrow genetic base. However some of the cultivars could not be separated from other using Td-DAMD-PCR analyses. Indeed some of these varieties could be easy differentiated morphologically. For instance, some of these cultivars were separated on the basis of 4 fiber

quality traits including fiber length, strength, fineness, and uniformity [7]. However they could not be separated using Td-DAMD-PCR technique indicating that Td-DAMD-PCR technique is not suitable for diversity studies of Turkish cotton varieties.

According to cotton textile industry desirable cotton traits are micronaire between 3.8 and 4.4, minimum 27.4 mm for 2.5% span length, 28 g/tex for strength, and 6% for elongation, and maximum of 5% short fiber content and 15 per gram of seed coat fragments, and at least 83 % for length uniformity ratio. However desirable cotton traits for producers are increased yield, early maturity, disease and pest resistance such as *Verticillium*/*Fusarium* fungi, nematodes, and viruses while producing longer and stronger fibers. However with the narrow genetic diversity it is very difficult to accomplish these desirable cotton traits. One solution to the narrow genetic diversity of cotton is to collect, evaluate, and utilize a broader range of cotton germplasm.

Conclusion

Based on the analysis the following conclusions were drawn: Td-DAMD-PCR technique could be very useful in secondary and tertiary gene pools rather than primary gene pool of cotton genomic resources. Td-DAMD-PCR technique may not be suitable for genetic diversity studies of commercial cotton varieties with similar genetic background. Commercial cotton varieties widely grown in Turkey have very low genetic base so new varieties are required to increase the yield potential of Turkish cotton production. We also concluded that the most of the varieties studied in the present study were developed from similar parental lines.

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