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Original Article

Assessment of phylogenetic relationships of some worldwide-cultivated cotton genotypes (*Gossypium hirsutum* L.) by using ISSR and RAPD markers

Emre Sevindik
Faculty of Agriculture, Department of Agricultural Biotechnology, Adnan Menderes University, South Campus, Cakmar, Aydin, Turkey
ph.d-emre@hotmail.com (corresponding author)

Muhammed Ebrar Çayir
Faculty of Agriculture, Department of Agricultural Biotechnology, Adnan Menderes University, South Campus, Cakmar, Aydin, Turkey

Tülay Emrebaş
Aydın/ Nazilli Cotton Research Institute, Turkey

Ertuğrul Filiz
Duzce University, Department of Crop and Animal Production, Cilimli Vocational School, 81750, Cilimli, Duzce, Turkey

Abstract:

Cotton belongs to the genus of the Malvaceae family and presents an economically and biologically highly valuable multi-purpose crop grown as a source of fiber, food and feed worldwide. In this study, phylogenetic relationships of cotton genotypes of the thirteen countries (twelve *Gossypium hirsutum* and one *Gossypium barbadense* (GIZA 75 Egypt) as outgroup) taken from the Directorate of Nazilli Cotton Research Institute genetic stock were analyzed by using RAPD and ISSR markers. RAPD–PCR analysis obtained total of 53 DNA bands with the highest number of bands for OPA-02 and OPA-13 primers, no bands for OPE-08 primer and rate of polymorphism of 90.56%. ISSR –PCR analysis showed a total of 81 DNA bands, where the highest number of bands was obtained with UBC-811, UBC-834 and UBC-836 primers, no bands with UBC-853 primer and rate of polymorphism of 77.77%. Phylogenetic tree was obtained by using the UPGMA algorithm and the tree consisted of two large clades. Analysis of the results suggests that RAPD and ISSR markers are useful tools to demonstrate phylogenetic relationships between cotton genotypes.

Key words:
Gossypium hirsutum, RAPD, ISSR, molecular markers, phylogenetic

Apstrakt:

Utvrđivanje filogenetskih odnosa nekih genotipova pamuka (*Gossypium hirsutum* L.) kultivisanih širom sveta korišćenjem ISSR i RAPD markera

Pamuk pripada rodu *Gossypium* iz familije Malvaceae i predstavlja ekonomski i biološki veoma vredan višenamenski usev koji se gaji kao izvor vlakana i hrane širom sveta. U ovom istraživanju, analizirani su filogenetski odnosi genotipova pamuka iz trinaest različitih zemalja (dvanaest *Gossypium hirsutum* i jedna *Gossypium barbadense* (GIZA 75 Egypt) kao zasebna grupa) uzetih iz banke genotipova Nazili Instituta za istraživanje pamuka, a proučavani su korišćenjem RAPD i ISSR markera. RAPD–PCR analizom dobijeno je ukupno 53 DNK traka uz najveći broj traka sa OPA-02 i OPA-13 prajmerima, nije bilo trake za OPE-08 prajmer, a stopa polimorfizma iznosila je 90.56%. ISSR –PCR analiza pokazala je ukupno 81 DNK traka, gde je najveći broj traka dobijen sa UBC-811, UBC-834 i UBC-836 prajmerima, nije bilo traka za UBC-853 prajmer, a stopa polimorfizma iznosila je 77.77%. Filogenetsko drvo je dobijeno korišćenjem UPGMA algoritma i sadržalo je dve velike klade. Analiza rezultata sugeriše da RAPD i ISSR marker predstavljaju korisne alate za demonstriranje filogenetskih odnosa između genotipova pamuka.

Ključne reči:
Gossypium hirsutum, RAPD, ISSR, molekularni markeri, filogenetski

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Introduction

The Malvaceae family consists of 243 genera with 4,225 known species, and contains various economically valuable species such as *Gossypium* (Cotton), *Corchorus* (Jute), *Hibiscus*, *Alcea, Theobroma cacao* (cacao), *Abelmoschus esculentus* (Okra) and *Malva* in tropical and subtropical regions (Pravin et al., 2018; Ben-Simchon et al., 2019; Wang et al., 2020). The main spreading center of these family members is South America. Besides, it spreads everywhere except for the very cold regions of the Earth (Uzunhisarcıklı & Vural, 2009). The members of the family Malvaceae include herbs,

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subshrubs, shrubs, trees and even woody climbers (Ţîţei & Teleuță, 2018). In addition, the Malvaceae family has mucilage, fixed oils and essential oils and is used for medicinal purposes. Plants of this family are used publicly for the treatment of respiratory and digestive system irritations and inflammations as expectorant, bronchodilator, diuretic and for the treatment of gastric ulcers (Erarslan & Koçyiğit, 2019). Cotton belongs to the *Gossypium* genus of the Malvaceae family and contains 51 species, among which 45 are diploid (2n = 26) and 6 are allotetraploid (2n = 52) (Elçi & Hançer, 2016). In addition, this genus includes only four cultivated cottons that are morphologically distinct and geographically widespread, namely: *G. arboreum* L. and *G. herbaceum* L. known as the Old World African-Asian cottons, *G. barbadense* L. (Sea Island cotton) and *G. hirsutum* L. and or others known as Upland cotton (Ibrahim et al. 2007). Eighty-seven percent of cotton growth area in the world occurs in developing countries (Nix et al., 2017). Cotton is an economically and biologically highly valuable multi-purpose crop grown as a source of fiber, food and feed worldwide. The cottons of *Gossypium hirsutum* L. and *Gossypium barbadense* L., are leading natural fiber crops as well as oilseed crops important to bioenergy, feed and food industries (Zhang et al. 2015; Noman et al., 2016; Bilval et al., 2017). The primary product of the cotton plant is mohair, which covers the seeds in the seed or cocoon. This mohair has been used for thousands of years to dress the people of ancient India, Asia, America and Africa (Abdellatif et al., 2012).

Molecular markers have developed a wide range of applications in the field of molecular biology, which may include phylogenetic study, genetic diversity, evolution, ecology, population genetics, and the study of complex genomic properties in both plants and animals (Atasagun et al., 2018; Raza et al., 2019). Genetic diversity can be estimated from pedigree analysis or from multivariate analysis carried out on a large number of plant attributes (Sharma et al., 2018). DNA markers offer a convenient way to study polymorphisms, taxonomy, physiology, embryology, genetic engineering and their use in product development (Kesawat & Kumar, 2009; Shaheen et al., 2013). PCR (polymerase chain reaction) based molecular markers such as ISSR, RAPD, SSR, and AFLP were found to be useful in various applications of genetic variation and plant breeding (Dongre et al., 2004; Cui et al., 2017). For the use in detecting genetic variation, the RAPD (Random Amplified Polymorphic DNA) marker has a number of advantages such as technical simplicity, speed of testing, minimum DNA requirements, and low costs. In addition, no prior knowledge about the sequence under investigation is required. RAPD markers have been used to analyze genetic relations and diversity in plant populations and cultivars (Ghasemi et al., 2014; Saha et al., 2019; Akinro et al., 2019). ISSR (Inter Simple Sequence Repeats) technique is a PCR based technique, reported by Ztetkiewicz et al. (1994), which can quickly show the differences of individuals with close relationships (Jafari et al., 2019). This marker is based on determining the random distribution of DNA nucleotides in plant genomes, independent of chromosome regions. ISSR markers are usually dominant markers, so it is difficult to determine whether individuals are heterozygous. This technique is much more sensitive and reproducible than RAPD technique (Gülşen & Roose, 2001; Vijayan, 2005; Güngör et al., 2020). In this study, genetic diversity of cotton genotypes of the thirteen countries (twelve *Gossypium hirsutum* and one *Gossypium barbadense* as outgroup) taken from the Directorate of Nazilli Cotton Research Institute genetic stock was analyzed by using RAPD and ISSR markers.

Fig. 1. Three-week appearance of thirteen different cotton varieties after planting

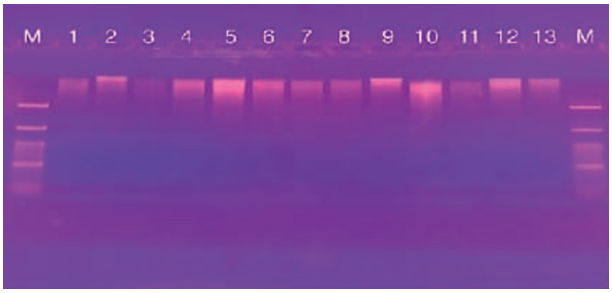

Fig. 2. Genotypes of gDNA isolation gel image

Materials and Methods

Plant materials and genomic DNA isolation

The cotton varieties of the thirteen countries

Table 1. Primers used in the RAPD-PCR reactions and their Tm degrees

Primers	DNA Sequences(5'-3')	TM (°C)	Amplifications	PCR protocol (35 Cycles)
OPA-02	5' - TGCCGAGCTG - 3'	34 °C	+	94 °C/2 min 94 °C/1 min 32-34 °C/1 min 72 °C/1 min 72 °C/10 min
OPA-05	5' - AGGGGTCTTG - 3'	32 °C	+	
OPA-13	5' - CAGCACCCAC - 3'	34 °C	+	
OPA-15	5' - TTCCGAACCC - 3'	32 °C	+	
OPA-16	5' - AGCCAGCGAA - 3'	32 °C	+	
OPA-18	5'- AGGTGACCGT - 3'	32 °C	+	
OPA-20	5' - GTTGCGATCC - 3'	32 °C	+	
OPE-08	5' - TCACCACGGT - 3'	32 °C	-	
OPA-07	5'-GAAACGGGTG-3'	32 °C	+	
OPA-03	5'-AGTCAGCCAC-3'	32 °C	+	

were obtained from Nazilli Directorate of Cotton Research Institute and this study was carried out in the Biotechnology Laboratory of the Directorate. In addition, the 13 cotton varieties were planted in two repeats and three seeds (**Fig. 1**). The seedlings were grown for about three weeks until they had four leaves. Those who did not germinate to yield four leaves were waited or replanted until they grown four leaves. The cottons, which were sown in three seeds and two repeats, were diluted into the best growing single plant after they had four leaves. Two or three-days old leaves were collected by observing the selected plants every day. After being washed with pure water and dried, the leaf samples were stored at -86 ºC for genomic DNA isolation. Power Plant DNA Isolation Kit (QIAGEN) was used for genomic DNA isolation from the specimens. The obtained genomic DNA samples were made ready for use and stored at -20 ºC. For preparation of the gDNA (genomic DNA) gel image, the mixture was prepared by using 1 µL gDNA, 8 µL dH_20 and 1 µL loading dye. Then 1% agarose gel electrophoresis was prepared and the samples were loaded. gDNA gel images of the 13 cotton varieties are shown in **Fig. 2.** The purity of the genomic DNA was assessed with a nanodrop spectrophotometer (IMPLEN Nanophotometer

Table 2. Primers used in the ISSR-PCR reactions and their Tm degrees

ISSR Primers	DNA Sequences(5'-3')	TM (°C)	Amplifications	PCR protocol (35 Cycles)
UBC-831	5'-CTCTCTCTCTCTCTCTT-3'	50 °C	+	94 °C/1 min 94 °C/1 min 48-53 °C/1 min 72 °C/1 min 72 °C/10 min
UBC-830	5'-TGTGTGTGTGTGTGTGG-3'	52 °C	+	
UBC-807	5'-AGAGAGAGAGAGAGAGT-3'	50 °C	+	
UBC-819	5' - GTGTGTGTGTGTGTGTA -3	50 °C	+	
UBC-808	5'-AGAGAGAGAGAGAGAGC-3'	52 °C	+	
UBC-836	5'-AGAGAGAGAGAGAGAGYA-3'	52 °C	+	
UBC-856	5'–ACACACACACACACACYA-3'	52 °C	+	
UBC-853	5' - TCTCTCTCTCTCTCTCRT -3'	52 °C	-	
UBC-855	5'-ACACACACACACACACYT-3'	52 °C	+	
UBC-810	5' -GAGAGAGAGAGAGAGAT-3'	50 °C	+	
UBC-826	5'-ACACACACACACACACC-3'	52 °C	+	
UBC-811	5'-GAGAGAGAGAGAGAGAC-3'	53 °C	+	
UBC-834	5'-AGAGAGAGAGAGAGAYT-3'	52 °C	+	
UBC-873	5'-GACAGACAGACAGACA-3'	48 °C	+	

P330). Blanking was applied with the PD7 Solution we used in the last stage of the DNA isolation kit. The amount of DNA was determined by taking 1 µL of each sample and reading of the OD_{260} and OD_{280} nm in a nanodrop spectrophotometer. The purity amount of DNA was determined by OD_{260}/OD_{280} ratio. According to nanodrop spectrophotometer measurements after isolation, the lowest DNA amount was obtained from Brown Egyptian and the highest DNA amount was obtained from UJCHI 2 UZBEK. According to OD_{260}/OD_{280} ratios which are the indications of purity, the highest value was obtained from HARIDOST and the lowest value was obtained from BULGARIAN 73.

PCR amplifications

RAPD and ISSR primers chosen for PCR amplifications are shown in **Tab. 1** and **Tab. 2**. Ready mixes were used as an alternative to manual PCR loadings. Amplification was carried out by adding 2 µL genomic DNA, 1 µL primers and 5 µL of master mix (Cat. No: RP02-II-400, RP02-II-2000 0.75U of Taq DNA polymerase, reaction buffer, 2mM $MgCl_2$, 250µM dNTPs and enzyme stabilizer) and 17 µL dH_2O into PCR tube. Amplification products were analyzed by electrophoresis on 1% agarose gel buffered with 1.0X TAE (Tris-Acetate-EDTA), stained with ethidium bromide and photographed under ultraviolet light. As a results of RAPD-PCR, some of the gel images were shown in **Fig. 3A**, **Fig. 3B**, **Fig. 3C** and **Fig. 3D**.

Data and Phylogenetic analysis

Following the PCR analysis, DNA bands were scored as "1" in the presence of DNA, "0" in the absence of DNA and a ''?'' for missing data. Genetic relationship of the some *Gossypium* genotypes used in the study was analyzed using PAUP 4.0b10 (Swofford, 2001), and the genetic distance matrix between the populations was calculated by drawing the UPGMA (unweighted pair group method) phylogenetic tree in the same program in accordance with the arithmetic mean of the pedigree trees. The phylogenetic trees were evaluated with bootstrap test on 10,000 resamplings (Felsenstein, 1985) and these values were added to the UPGMA tree.

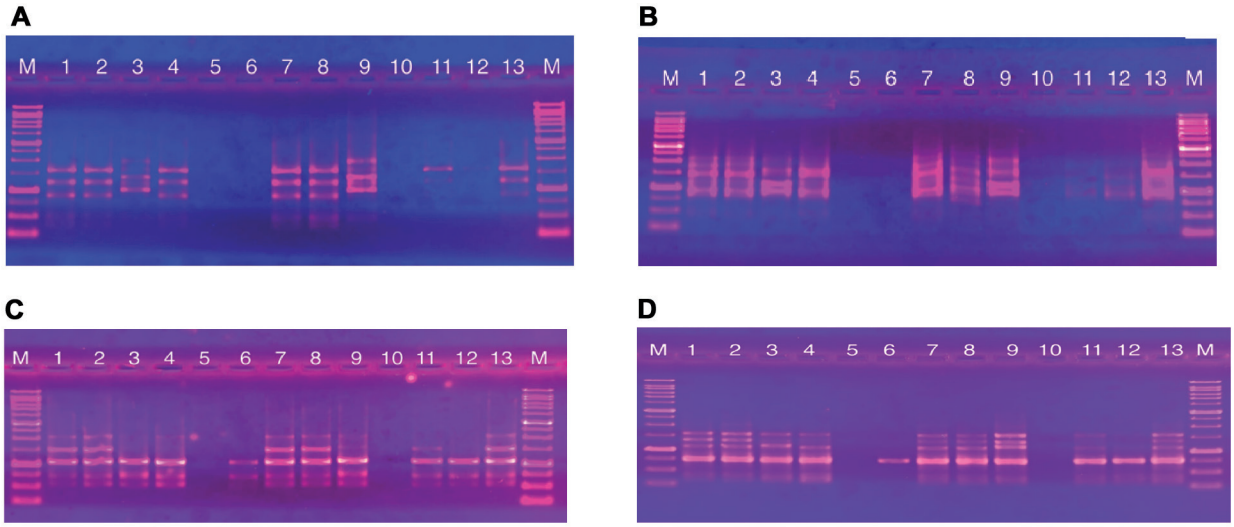

Fig. 3. Gel image of RAPD-PCR bands amplified with OPA-02 (**A**) and OPA-13 (**B**). Gel image of ISSR-PCR bands amplified with UBC-807 (**C**) and UBC-836 (**D**)

Results and discussion

Molecular markers developed over the last 30 years have an important role in breeding programs in agriculture (Kalkışım et al., 2016). DNA markers based on PCR are versatile tools in different aspects of genomic studies. It is possible to analyze closely related genera to evaluate phylogenic and genetic relationships using these markers (Zarei et al., 2017). As a result of RAPD-PCR analysis, a total of 53 bands were obtained. Of these, 48 were polymorphic and the rate of polymorphism was about 90.56%. Most of the bands were obtained from OPA-02 and OPA-13 primers and no band was obtained from OPE-08 primer. Phylogenetic tree and genetic distances between genotypes were calculated using the PAUP 4.0b10 analysis program. According to the PAUP analysis, the closest genetic distance was 0.06 (TAM C66 - 16 ELS and SC-2079), while 1.0 was the farthest genetic distance (ARCOTA-129 and ZIROATKAR-81,

Table 3. Pairwise genetic distance matrix obtained from RAPD primers

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13
TAM C66 - 16 ELS	-	0.14	0.37	0.21	0.40	0.40	0.14	0.25	0.22	0.58	0.58	0.42	0.06
TROPICAL 225	7	-	0.35	0.10	0.60	0.60	0.17	0.14	0.22	0.64	0.55	0.47	0.21
BROWN EGYPTIAN	15	14	-	0.32	0.20	0.20	0.37	0.42	0.20	0.58	0.55	0.47	0.40
AzGR-7711	10	5	13	-	0.36	0.27	0.10	0.08	0.34	0.64	0.52	0.33	0.27
ARCOTA-129	2	3	1	4	-	0.09	0.40	0.60	0.54	0.60	1.00	0.54	0.60
BULGAR 73	2	3	1	3	1	-	0.40	0.60	0.63	0.60	1.00	0.45	0.60
EVA	7	8	15	5	2	2	-	0.10	0.32	0.64	0.52	0.47	0.17
FIBERMAX 832	12	7	17	4	3	3	5	-	0.32	0.64	0.52	0.47	0.23
GIZA 75	9	10	8	16	6	7	13	13	-	0.47	0.58	0.40	0.20
UJCHI 2 UZBEK	10	11	10	11	3	3	11	11	8	-	0.30	0.23	0.58
ZIROATKAR-81	21	20	20	19	5	5	19	19	21	4	-	0.19	0.55
HARIDOST	9	10	10	9	6	5	10	10	11	3	4	-	0.38
SC-2079	3	10	16	13	3	3	8	11	8	10	20	8	-

BULGAR 73 and ZIROATKAR-81) (**Tab. 3**). The UPGMA tree constructed using the PAUP 4.0b10 phylogenetic analysis program consists of the two large clades (**Fig. 4**). Clade 1 was subdivided into multiple subclades. Subclade A1, consisted of TAM C66-16 ELS, SC-2079, TROPICAL 225, AzGR-7711, FIBERMAX 832, EVA and GIZA-75 (*Gossypium barbadense*) genotypes. Subclade A2, consisted of BROWN EGYPTIAN, ARCOTA-129 and BULGAR 73 genotypes and this subclade has a 53% bootstrap value. Clade 2 consisted of UJCHI 2 UZBEK, ZIROATKAR-81 and HARIDOST genotypes genotypes with bootstrap value of 83%. As a result of ISSR-PCR analysis, a total of 81 bands were obtained. Of these, 63 were polymorphic and the rate of polymorphism was 77.77%. Most of the bands were obtained from UBC-811, UBC-834 and UBC-836 primers and no band was obtained from UBC-853 primer. According to the PAUP analysis, the closest genetic distances were 0.00 (UJCHI 2 UZBEK and ZIROATKAR-81, HARIDOST and ZIROATKAR-81), while the farthest genetic distances were 0.77 (ARCOTA-129 and FIBERMAX 832, ARCOTA-129 and SC-

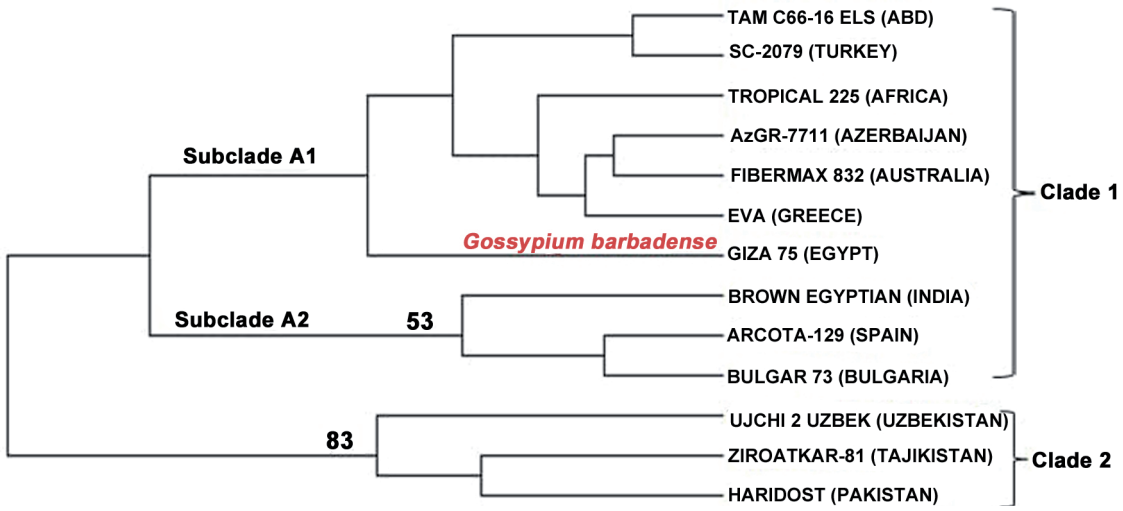

Fig. 4. The UPGMA tree generated using RAPD data

Table 4. Pairwise genetic distance matrix obtained from ISSR primers

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13
TAM C66 - 16 ELS	-	0.07	0.23	0.11	0.66	0.55	0.04	0.08	0.28	0.57	0.24	0.31	0.06
TROPICAL 225	6	-	0.28	0.13	0.66	0.60	0.07	0.13	0.28	0.71	0.27	0.36	0.08
BROWN EGYPTIAN	18	22	-	0.20	0.44	0.53	0.24	0.22	0.20	0.28	0.32	0.31	0.24
AzGR-7711	9	11	16	-	0.77	0.53	0.11	0.07	0.33	0.14	0.19	0.25	0.07
ARCOTA-129	6	6	4	7	-	0.33	0.66	0.77	0.44	0.00	0.44	0.44	0.77
BULGAR 73	36	39	35	35	3	-	0.53	0.52	0.60	0.00	0.38	0.33	0.56
EVA	4	6	19	9	6	35	-	0.08	0.32	0.57	0.25	0.32	0.06
FIBERMAX 832	7	11	17	6	7	34	7	-	0.32	0.42	0.20	0.27	0.07
GIZA 75	22	22	16	26	4	39	25	25	-	0.51	0.40	0.41	0.32
UJCHI 2 UZBEK	4	5	2	1	0	0	4	3	4	-	0.00	0.00	0.57
ZIROATKAR-81	19	21	25	15	4	25	20	16	31	0	-	0.09	0.23
HARIDOST	24	28	4	20	4	22	25	21	32	0	7	-	0.32
SC-2079	5	7	19	6	7	37	5	6	25	4	18	25	-

2079, AzGR-7711 and ARCOTA-129) (**Tab. 4**). The UPGMA tree constructed using the PAUP 4.0b10 phylogenetic analysis program consists of two large clades (**Fig. 5**). Clade one was subdivided into multiple subclades. Subclade A1 consisted of TAM C66 - 16 ELS, EVA, SC-2079, TROPICAL 225, AzGR-7711 and FIBERMAX 832 genotypes. Subclade A2 is composed of BROWN EGYPTIAN and GIZA-75 (*Gossypium barbadense*) genotypes. This subclade has 81% bootstrap value. Clade two was subdivided into multiple subclades and this subclade has a 73% bootstrap value. Subclade B1 is composed of ARCOTA-129, UJCHI 2 UZBEK and BULGAR 73 genotypes.Subclade B2 is composed of ZIROATKAR-81 and HARIDOST genotypes.

In our study, both RAPD and ISSR results showed that TAM C66 - 16 ELS, EVA, SC-2079, TROPICAL 225, AzGR-7711 and FIBERMAX 832 genotypes belong to the same group. UJCHI 2 UZBEK, ZIROATKAR-81 and HARIDOST genotypes appeared in the same group. In addition, these three genotypes have been geographically supported. In RAPD results, BULGAR 73, ARCOTA-129 and BROWN EGYPTIAN appeared

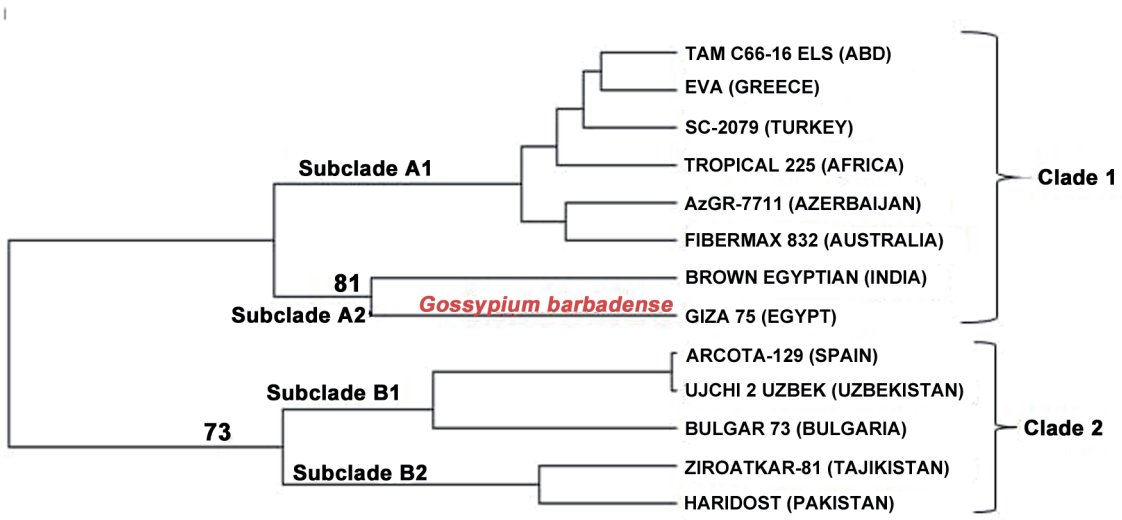

Fig. 5. The UPGMA tree generated using ISSR data

together, while ISSR results showed only BROWN EGYPTIAN genotype in a separate group. In our study, both RAPD and ISSR results were consistent with each other. In previous studies, Hossain et al. (2017) in Bangladesh, determined the genetic diversity of the 4 varieties of the *Gossypium hirsutum* through the RAPD technique. Five RAPD primers were used in the study; they yielded 53 bands in total and determined the polymorphism rate to be 90.56%. Maleia et al. (2010) determined the genetic difference between African and American varieties and inbred cotton lines (*Gossypium hirsutum* L. race *latifolium* H.) through RAPD molecular markers. 24 RAPD primers were used in the study; 166 bands were obtained in total, and the rate of polymorphism was determined to be 90.96%. Consequently, the use of RAPD molecular markers has been effective for genetic differentiation in cotton varieties and genotypes. Surgun et al. (2012) determined the identification and genetic diversity of some Turkish cotton (*Gossypium hirsutum* L.) genotypes through RAPD-PCR analysis. In the study, genetic difference of nine cotton genotypes was determined through RAPD-PCR technique. Among the genotypes, a total of 42 RAPD primers were used to detect polymorphism; assessable results were obtained from 34 of these primers, and a total of 319 RAPD-PCR bands were obtained. The rate of polymorphism among the studied genotypes was determined as 18.1% and genetic similarity between any 2 genotypes ranged from 90.2% to 96.5%. The results revealed that genotypes can be separated at the molecular level with the help of RAPD markers. In the study conducted by Khan et al. (2000), 45 RAPD primers were used in 35 *Gossypium* species in total containing 31 species, 3 subspecies and 1 interspecific hybrid. In the study, a total of 579 DNA bands were obtained and the rate of polymorphism was 99.8%. The results obtained in the study provide good correspondence to the taxonomic sections recognized within the genus *Gossypium*. These results show that the RAPD technique produces reliable results to create the phylogenetic history of the *Gossypium* genus. In a study carried out in Turkey, Şahin et al. (2020) determined the genetic relationship between 30 cultivated cotton varieties (*Gossypium hirsutum* L.) through ISSR-PCR method. 24 ISSR primers were used to determine polymorphism in 30 cotton varieties; while creating a total of 41 bands from the primers, it was determined that an average of 22.3 of these bands were polymorphic. The polymorphic information content values for ISSR primers ranged from 0.19 to 0.68 and it was found as 0.49 in average. As a result of the study, they concluded that ISSR molecular marker technique can be successfully applied in the studies of determining genetic diversity in cotton; in addition, due to its high polymorphism and reproducibility, it can be successfully applied in many laboratories with limited laboratory facilities. Bilval et al. (2017) analyzed genetic variation among nine cotton (*Gossypium*) genotypes by using RAPD, ISSR and SSR markers. In the study, 25 RAPD, 22 ISSR and 24 SSR primers were used. The number of polymorphic amplicons was found to be 156 (RAPD), 142 (ISSR) and 24 (SSR) accounting for polymorphism of 91.22% (RAPD), 67.94% (ISSR) and 72.72% (SSR), respectively. As a result of their work, they explained the suitability and reliability of the molecular markers (RAPD, ISSR and SSR) to predict genetic diversity and genetic relationships between cotton genotypes. Bardak and Bolek (2012) used 5 ISSR and 39 SSR primers to determine the genetic relationship between diploid and tetraploid 25 cotton genotypes (*Gossypium* spp.) cultivated in different regions of the world. They found that 155 (89.60%) out of 173 alleles obtained as a result of the study were polymorphic. The average number of alleles per SSR and ISSR markers was 3.93, ranging from 1 to 8 alleles. Genetic diversity ranged from 0.04 to 0.58 among all the genotypes inspected. This ratio was 0.04-0.23 within *G. hirsutum* and 0.07-0.26 within *G. barbadense* species. As a result of the study, the use of wild cotton species has been suggested to increase the diversity in the gene pool and it has been stated that it will be useful in the selection of the desired properties. In conclusion, RAPD and ISSR markers were used in this study to investigate the genetic diversity of some worldwide cultivated cotton genotypes. The results of the present research have confirmed that these molecular markers can detect polymorphism among *Gossypium hirsutum* genotypes, predict if they are related, and identify genotype-specific RAPD and ISSR markers. According to the results obtained with RAPD markers, polymorphism was found to be high and this rate is 90.56 and 77.77% respectively. In addition, the findings obtained from this study are thought to contribute to breeding programs, development of germplasm sources and future marker studies.

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