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MOLECULAR CHARACTERIZATION OF SOME SELECTED PERSIMMON GENOTYPES AND CULTIVARS BY SRAP AND SSR MARKERS

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In this study, SRAP and SSR markers were employed to determine genetic relationships among 42 persimmon genotypes (*Diospyros kaki* Thunb) obtained from Hatay province and 3 persimmon cultivars, 2 of which belong to *Diospyros kaki* Thunb and one belongs to *Diospyros oleifera* Cheng. Genetic relationships were determined by using a total of 29 molecular DNA primers (SRAP and SSR). Of these primers, 21 SRAP primer combinations produced a total of 107 bands and 77.6% of them were polymorphic; 8 SSR primers produced 26 polymorphic bands with an average polymorphism ratio of 84.6%. The SRAP and SSR markers produced 4.6 bands as average and the number of bands produced per marker was calculated as 3.6. The lowest similarity was observed between MK-113 (*Diospyros oleifera* Cheng) and the other genotypes all belongs to *Diospyros kaki* Thunb (with similarity ratios of 0.41-0.69 for SRAP primers, between 0.25-0.67 for SSR primers). The genotypes/cultivars belongs to *Diospyros kaki* had similarity ratio between 0.98-1.00 according to SRAP and SSR markers. This synonym or similarity could be results of clonal propagation rather than autogamy.

*Key words***:** Persimmon, selection, genetic diversity, molecular markers

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INTRODUCTION

Fruit trees are familiar to a wide cross-section of human society, both as a common food and for their spiritual importance. They have been using by people for food, either as edible products, or for culinary ingredients, for medicinal use for a long time. They are genetically very diverse group and play a major role in modern society end economy. Fruits are natural sources of vitamins, phytochemicals and minerals (SAHIN *et al*., 2002; BENJAK *et al*., 2005; ROP *et al*., 2014; CANAN *et al*., 2016; ZORENC *et al*., 2016). They are genetically very diverse group and play a major role in modern society end economy (KACZMARSKA *et al*., 2015; MISHRA *et al*., 2015; SARIDAS *et al*., 2016; YAZICI and SAHIN, 2016).

Persimmon was first cultivated in China between 5-6th centuries. There are more than 1050 cultivars in China (WANG *et al*., 1997; YONEMORI *et al*., 2000; ZHANG *et al*., 2009), about 500 cultivar in Japan (SATO and YAMADA, 2003) and 186 cultivars in South Korea (KANG and KO, 1997; YAMADA *et al*., 2002). There are other commercial cultivars and several local types in other major producer countries like Brazil, Australia, Italy, Israel and the United States of America (DUVAL, 1986; RYUGO *et al*., 1988; YONEMORI, 1997; BELLINI and GIORDANI, 2000; GIORDANI, 2002; NISSEN *et al*., 2003). Several local genotypes used in minor producer countries like Spain, South Africa, Taiwan and France (RABE, 2003; WEN, 2003). Majority of commercial persimmon cultivars have only female flowers, thus foreign pollination is quite high in this fruit and consequently there is a high genetic variation among the cultivars.

One of the main problems regarding diversity management of persimmon resources is the assignment of cultivar identity due to the existence of synonyms and homonyms among local varieties, misleading transliterations from Japanese, and incorrect labeling in the past. Traditionally, the method for cultivar identification was based on morphology of leaves, bud, flower, seed, and fruit characters (UPOV 2004). The main characteristics used as references for cultivar classification are the fruit astringency loss and the change in flesh color, resulting in the recognition of four groups of cultivars (YONEMORI *et al*., 2000; NAVAL *et al*., 2010).

Morphological and pomological characteristics have been used until quite recently for identification of fruit cultivar and species. However, assessments of morphological characteristics may vary from one researcher to another and may be influenced by abiotic (salinity, drought and etc.) and biotic stress conditions (YILDIRIM and KANDEMIR, 2001). The molecular marker technology with a rapid development during the last 20 years offered various new approaches in identification of genetic diversity among the cultivars, in chromosome mapping, characterization of gene sources. Today, molecular markers are efficiently used in plant systematics, breeding and assessment of gene sources (NAVAL *et al*., 2010).

So far, different DNA-based marker techniques such as Restriction Fragment Length Polymorphism (RFLP) (RFLP; KANZAKI *et al*., 2000a; MAKI *et al*., 2001), random amplification of polymorphic DNA (RAPD; LUO *et al*. 1995; BADENES *et al*. 2003; YAMAGISHI *et al*., 2005), and Amplified Fragment Length Polymorphism (AFLP; KANZAKI *et al*., 2000b; YONEMORI *et al*., 2008a; HU *et al*., 2008), chloroplast DNA (cpDNA; YONEMORI *et al*., 1998), in situ hybridization (CHOI *et al*., 2003), Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon-microsatellite Amplified Polymorphism (REMAP) (GUO *et al*., 2006; DU *et al*., 2012), Inverse Sequence-Tagged Repeat (ISTR) (DU *et al*., 2009) and Internal Transcribed Tpacer (ITS; YONEMORI *et al*., 2008b), have been applied to assess the genetic diversity and relationships between *Diospyros* species. Nevertheless, the relationships between *Diospyros* accessions of local and natural varieties are still not completely clarified in spite of all previous

efforts, probably because of the low resolution of the germplasm resource collection, conservation and exploitation.

SSR (Simple Sequence Repeat) is quite efficiently employed in investigating genetic diversity among the species with low polymorphism levels, identification of new cultivars and creating of genetic maps (STAUB *et al*., 1996). The method was also indicated as a reliable method in identification of genetic diversity among persimmons (GUO and LUO, 2006a; HU and LUO, 2006; SORIANO *et al*., 2006; NAVAL *et al*. 2010; GUO and LUO, 2011). On the other hand, SRAP has been shown to be more informative than other PCR-based techniques in detecting genetic diversity (BUDAK *et al*., 2004) and has been successfully used to study the genetic diversity of, and relationships among, several species (FENG *et al*., 2009; UZUN *et al*., 2009; CASTONGUAY *et al*., 2010; TALEBI *et al*., 2011; ABEDIAN *et al*., 2012, PINAR *et al*., 2013).

Elucidation of genetic structures of the material used in plant breeding and cultivar development works is a vital issue for the preservation, assessment and practical use of plant genetic resources. The present study was conducted to put forth the genetic similarities and differences among 42 persimmon genotypes selected from Turkey, 2 foreign cultivars (Fuyu and Vainiglia) and 1 local cultivar (Harbiye) by using SSR and SRAP molecular markers.

MATERIALS AND METHODS

Plant material

A total of 45 persimmon genotypes were used in this study. Of these genotypes, 42 were collected from different area of Hatay province (Table 1) and currently preserved at the experimental farm of Mustafa Kemal University in Hatay, Turkey.

Table 1. Locations of selected and preserved genotypes

Town	District-Village	Genotypes
Antakya	Harbiye	MK-1, MK-3, MK-13, MK-15, MK-16, MK-18, MK-37, MK-44, MK-60, MK-62, MK-67, MK-68, MK-80, MK-97
	Değirmenyolu	MK 71, MK 73
	Yeşilpınar	MK-35, MK-118
	Çardaklı	MK-69
	Bahçeköy	MK-39
Belen	Merkez	MK-24, MK-25
	Yapraklı	MK-27
	Benlidere	MK-42, MK-55
	Kömürçukuru	MK-94, MK-109, MK-111
	Çerçikaya	MK-30
	Müftüler	MK-120
Samandağ	Tavla	MK-103, MK-104
	Aknehir	MK-59
	Özbek	MK-102
	Vakıflı	MK-113 (*Diospyros oleifera* Cheng.)
Yayladağı	Yalaz (Lobas)	MK-119
	Gürışık	MK-85
	Çabala	MK-93
İskenderun	Merkez	MK-117
	Kurtbağı	MK-32
	Orhangazi	MK-43
	Karagöz	MK-45

One of these genotypes belongs to *Diospyros oleifera* Cheng (MK-113) and the rest of the genotypes belongs to *Diospyros kaki* Thunb. The cultivars Fuyu (Japan-originated), Vainiglia (Italy-originated) and Harbiye (local variety) were also used in this study.

Method

Total genomic DNA was extracted from the leaves following the method of DOYLE and DOYLE (1990). DNA amplification for SRAP analyses was performed in a PCR device (Apollo ATC 401, Nyx Technik Inc. San Diego, CA, USA) in accordance with GULSEN *et al*. (2005). For each 15 µl PCR reaction, 1.5 µl 10X PCR buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 1 U Taq DNA polymerase enzyme (Invitrogen), 2.00 mM from each primer pairs, 0.2 µg/µl BSA (Bovine serum albumin), 5.1 µl ddH_2O and 20 ng DNA were used.

SSR analysis and PCR conditions were as follows: in 15 µl PCR mix, 1.5 µl 10X PCR buffer, 1.33 mM from each primer pairs, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase enzyme (Invitrogen), 4.3 µl ddH_2O and 20 ng DNA were used (GUO and LUO, 2011).

In PCR analyses, 20 SRAP primers (8 forward and 12 reverse), successfully used by GUO and LUO (2006a) in persimmons (Table 2) and 12 SSR primers, developed from Rojo Brillante persimmon by SORIANO *et al*. (2006) and successfully used by GUO and LUO (2011) in identification of persimmons (Table 3) were used. PCR was performed with 8 SSR primers with polymorphic bands and 21 SRAP combinations with the greatest number of bands.

Table 2. Primers and base sequences used in SRAP-PCR reactions

No	Forward Primer	Base Sequence (3'-5')	Reverse Primer	Base Sequence (5'-3')
1	Me1	TGA GTC CAA ACC GGA TA	Em1	GAC TGC GTA CGA ATT AAT
2	Me2	TGA GTC CAA ACC GGA GC	Em2	GAC TGC GTA CGA ATT TGC
3	Me3	TGA GTC CAA ACC GGA AT	Em3	GAC TGC GTA CGA ATT GAC
4	Me4	TGA GTC CAA ACC GGA CC	Em4	GAC TGC GTA CGA ATT TGA
5	Me5	TGA GTC CAA ACC GGA AG	Em5	GAC TGC GTA CGA ATT AAC
6	Me6	TGA GTC CAA ACC GGA CA	Em6	GAC TGC GTA CGA ATT GCA
7	Me7	TGA GTC CAA ACC GGA CG	Em8	GAC TGC GTA CGA ATT CAC
8	Me8	TGA GTC CAA ACC GGA CT	Em10	GAC TGC GTA CGA ATT CAT
9			Em11	GAC TGC GTA CGA ATT CTA
10			Em17	GAC TGC GTA CGA ATT GAG
11			Em18	GAC TGC GTA CGA ATT GCC
12			Em19	GAC TGC GTA CGA ATT TCA

Table 3. Primers and base sequences used in SSR-PCR reactions

No	Repeat	Primer	Base Sequence (5'-3')
1	$(GA)_{17}$	DQ097470	TTAATTTGGACACAAGTTCT -TCTCTTCAAGTCTTCTATCCT
2	$(GA)_{17}$	DQ097472	CATTTGAAAGCAGTCGTCCA - GCGCCAAATCATTGCTATCT
3	$(GA)_{13}$	DQ097481	GTAATTAGCTAAGACTTAAGGGG - TGCTACAACAACTGGAAGAC
4	$(GA)_{12}$	DQ097484	ACTACAACGGCGGTGAGAAC - GTCCTTCACTTCCCGCATT
5	$(GA)_{10}$	DQ097490	ATGAGAGAGAGAGAATGATTGATGC - CATTTTGCACGCAGTGAGAT
6	$(CT)_{15}$	DQ097493	GGGGTAATATGAATTGAATC - CTCAGAGAGGAGAAGAAATAG
7	$(GA)_{15}$	DQ097494	GGGAAATTAAGAGGGAAGAA - AGGAACTGGATCAGCATAAA
8	$(CCTTT)_8$	DQ097497	ATCATGAGATCAGAGCCGTC - CACGTTAACGTTACGGAACA
9	$(TG)_9$ $(AG)_{17}$	DQ097498	TGGTGATCGTGGTAGTGGTT - GGCCTAATCTCTGTCCATCC
10	$(CT)_{15}$	DQ097499	AGTTCTTGCGATGGGATTTG - GATGAGATGGGCTGATTGCT
11	$(GA)_{15}$	DQ097501	ACAGGGCACGAACAGATGAC - GCAAAATGGTCTGGACTGCT
12	$(GA)_{16}$	DQ097504	GGGAAGAACAAAGAGAACTG - ACGAAGTTGTAATCCTGAGC

PCR products were loaded to 2.0% agarose gel and electrophoresed under 115 V electric current for 3.5 hours. Following the electrophoresis process, gels were images in an imaging unit (Kodak Gel Logic 200, Eastman Kodak Company, USA) and gel images were assessed as (1) with a band, (0) without a band and (9) without an amplification. The resultant data were analyzed with NTSYS (Numerical Taxonomy Multivariate Analysis System, NTSYS-pc version 2.1, Exeter Software, Setauket, N.Y., USA; ROHLF, 1993) software to put forth the genetic relations with each other. Similarity indices were calculated with Dice (1945) method.

RESULTS AND DISCUSSION

PCR and electrophoresis were performed over DNA samples obtained from different persimmon genotypes and cultivars with 8 SSR primers and 21 SRAP combinations and bands were assessed following gel imaging. While 77.6% of 107 bands obtained from 21 SRAP primer combinations yielding a PCR product were polymorphic, 84.6% of 26 bands obtained from SSR primers were polymorphic (Table 4). Respectively 5.1 and 3.3 bands were obtained for each SRAP and SSR primers and number of polymorphic bands per primer was respectively calculated as 4.0 and 2.8. When the primers were compared, it was observed that Me3-Em4 SRAP primer combination had the greatest number of bands (12 bands) and it was followed by Me6-Em2 and Me2-Em3 SRAP primer combinations with 6 bands. Polymorphism ratios varied between 50.0-100.0% with the lowest values in Me3-Em1 SRAP primer combination and DQ097504 SSR primer.

Table 4. Number of bands and polymorphism ratios of different persimmon genotypes obtained with SRAP and SSR primers

No		Primers	Total number of bands	Number of polymorphic bands	Polymorphism ratio (%)
1		Me1-Em1	6	4	66.7
2		Me3-Em1	6	3	50.0
3		Me4-Em2	6	4	66.7
4		Me6-Em2	9	6	66.7
5		Me7-Em2	7	5	71.4
6		Me2-Em3	9	5	55.6
7		Me3-Em3	5	4	80.0
8		Me4-Em3	4	3	75.0
9		Me5-Em3	5	4	80.0
10		Me8-Em3	5	4	80.0
11	SRAP	Me2-Em4	3	3	100.0
12		Me3-Em4	12	12	100.0
13		Me4-Em4	4	4	100.0
14		Me5-Em4	4	3	75.0
15		Me6-Em4	3	2	66.7
16		Me7-Em4	3	3	100.0
17		Me1-Em10	2	2	100.0
18		Me2-Em10	3	2	66.7
19		Me8-Em10	4	3	75.0
20		Me6-Em19	3	3	100.0
21		Me6-Em19	4	4	100.0
Average			**5.1**	**4.0**	**77.6**
1		DQ097470	4	4	100.0
2		DQ097484	2	2	100.0
3		DQ097490	6	4	66.7
4	SSR	DQ097497	4	3	75.0
5		DQ097498	2	2	100.0
6		DQ097499	2	2	100.0
7		DQ097501	4	4	100.0
8		DQ097504	2	1	50.0
Average			**3.3**	**2.8**	**84.6**

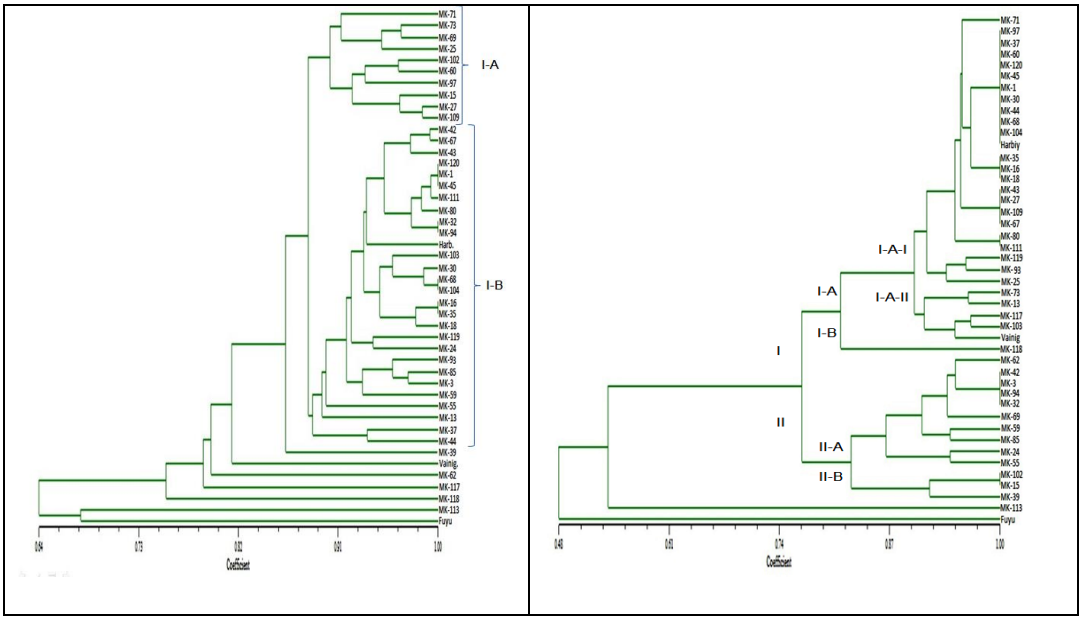
MK-113 belongs to *D. oleifera* was reported to be oily persimmon (YILDIZ *et al*., 2012) and it had quite different genetic structure from the other genotypes belongs to *Diospyros kaki*. According to SRAP technique, while MK-118 had the greatest genetic distance (0.41) to MK-113, genetic similarity of the remaining 41 genotypes in *Diospyros kaki* species varied between 0.54-1.00. The similarity ratios of these 41 genotypes with Harbiye cultivar varied between 0.74-0.95, with Vainiglia cultivar varied between 0.68-0.86 and with Fuyu cultivar varied between 0.58-0.71. According to SSR techniques, the similarity ratios of MK-113 genotype with 41 genotypes in *Diospyros kaki* species and 3 cultivars varied between 0.25-0.67. The similarity ratio among 41 genotypes in *Diospyros kaki* varied between 0.50-1.00. With SSR, similarity ratio of higher number of genotypes was identified as 1.00 than SRAP. Similarity ratios of these genotypes with Harbiye, Vainiglia and Fuyu cultivars respectively varied between 0.61-1.00, 0.61-0.97 and 0.27-0.63. Closer genetic similarities with Vainiglia than with Fuyu were probably because European cultivars were brought to Hatay region and produced throughout the initial persimmon culture of Turkey.

The dendrogram created with the data obtained from SRAP markers revealed that genotypes were gathered under 2 main groups with a similarity ratio of about 88%. The first group included 10 genotypes and the second group included 27 genotypes (Fig 1a). The common Harbiye genotype of the region was placed in the first group. In the second group, MK-39 numbered genotype was placed in the nearest position with a similarity ratio of 86%. While the MK-62, MK-113, MK-117 and MK-118 numbered genotypes were separated from the other genotypes with a maximum similarity ratio of 79%, Vainiglia cultivar exhibited a closer similarity ration (81%) than these genotypes. Japan-originated Fuyu cultivar and MK-113 numbered genotype were placed at the furthest position. The dendrogram created with SSR technique revealed that all genotypes except for MK-113 numbered genotype and Fuyu cultivar were placed in two main groups with a similarity ratio of about 76% (Fig 1b). The first main group was divided into two sub-groups (I-A and I-B). The I-A group included only MK-118 numbered genotype and I-B group divided into two sub-groups including Harbiye cultivar and 23 genotypes in one group (I-A-I group) and Vainiglia cultivar and 4 genotypes in the other group (I-A-II group). The second main was also divided into two sub-groups with 10 genotypes in II-A and 3 genotypes in II-B group.

YONEMORI *et al*. (2008a) investigated the relationships among 61 persimmon (*Diospyros kaki* Thunb.) cultivars (including 17 Italian, 11 Spanish, 13 Japanese, 6 Korean, 5 Chinese, 1 Israeli, and 8 of unknown origin) through AFLP analysis and reported that similarities among groups were generally less than 0.60 and cultivars were separated as European and Asian cultivars. Diversity within groups was greater than diversity between groups. Most cultivars were quite polymorphic (only 0.60–0.80 similarity between cultivars). NAVAL *et al.* (2010) reported polymorphism ratio of 71 persimmon cultivars from Japan, Italy, and Spain as 74% by SSR markers. JING *et al*. (2013) reported that 11 primer combinations produced high number of polymorphic bands (303) and 27.5 polymorphic bands per primer combination were detected for 48 persimmon accessions.

GUO and LUO (2011) analyzed genetic relationships among 30 *Diospyros* spp. samples including 14 Japanese and 10 Chinese genotypes of Japanese persimmon (*Diospyros kaki* Thunb.) and 6 related species and detected 262 with 18 SSR primers. All SSR primers developed from *Diospyros kaki* were successfully employed to reveal the polymorphism in other species of

Diospyros. Most of the primers were highly polymorphic, with a degree of polymorphism equal to or higher than 0.66.



(a) (b)

Figure 1. Dendrogram of 45-genotypes generated based on a: SRAP, b: SSR.

Different results can be obtained by using different DNA molecular marker techniques in the same plant species. For example, genetic similarity of 59 persimmon accessions representing 34 landraces from 4 different geographic regions in Jiangxi province was evaluated for genetic diversity with 28 morphological traits and IRAP molecular markers ranged from 0.44 to 0.95. (DU *et al*., 2013). The analysis of genetic relationships among 27 genotypes including Japanese persimmon (*Diospyros kaki* Thunb.) genotypes from Japan and China and the related species revealed that the Japanese, Chinese genotypes and related species were distinctly separated using Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon–Microsatellite Amplified Polymorphism (REMAP) markers were used to evaluated genetic relationships. It was stated that related species were clearly clustered in a separated group, suggesting that they are very closely related (GUO *et al*., 2006). YAMAGISHI *et al*. (2005) conducted a study using RAPD markers and 25 persimmon genotypes clarified the phenotic relationship between six species and subspecies of *Diospyros*: *D. lotus*, *D. lotus* subsp. *glabbra* and *D. taitoensis* nested at one group.

For SSR technique to define persimmons, GUO and LUO (2006b) developed 9 primers with 5-20 alleles in each locus and SORIANO *et al*. (2006) developed 22 primers with 1-6 allelles in each locus.

Sequence-related amplified polymorphism (SRAP) markers were used to evaluate the genetic diversity of seven persimmon species and to exploit valuable wild resources for breeding new cultivars. A total of 303 bands amplified with 11 SRAP primer combinations revealed high polymorphism. The 48 persimmon genotypes were divided into five groups. It was stated that SRAP was a better molecular marker that could provide more polymorphisms information and the capacity of average discriminating was relatively more informative than SSR, ISSR, RAPD and AFLP (BUDAK *et al*., 2004; DONG *et al*., 2010). GUO and LUO (2006a) reported the polymorphic percentage of 27 genotypes as 80.88% based on SRAP markers. GUO and LUO (2006a) used SRAP markers in assessing genetic diversity among persimmons and reported the average number of bands per primer as 5.5 and polymorphism ratio as 80.1%. UZUN *et al*. (2010) in another study used SRAP markers in local and foreign apricot species and observed polymorphism in 63 of 87 bands (73%). Previous researchers indicated that SRAP marker technique could successfully be used in genetic diversity assessments, marker-assisted selection (MAS), core collection formation and genetic mapping works (CRAVERO *et al*., 2007; LI *et al*., 2008; WANG *et al*., 2008).

Present findings revealed one more time that SRAP and SSR markers could successfully be used in phylogenetic researches, genetic characterization, genetic diversity and relationship assessments and cultivar identification in persimmons. Considering the entire findings, it was observed that the genotypes with a similarity ratio of 0.98-1.00 in both market techniques (MK-1, MK-45 and MK-120; MK-43 and MK-67; MK-80 and MK-111; MK-27 and MK-109; MK-32 and MK-94; MK-30, MK-68 and MK-104; MK-16, MK-18 and MK-35 numbered genotypes) had the same genetic structure. Such synonyms in persimmon genotypes were mainly because of clonal propagation of the species rather than autogamy.

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MOLEKULARNA KARAKTERIZACIJA SELEKCIJA KAKI GENOTIPOVA I KULTIVARA SA SRAP I SSR MARKERIMA

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Izvod

U ovom radu, SRAP i SSR marker su korišćeni za određivanje genetičkih međuodnosa između 42 kkai genotipova (*Diospyros kaki* Thunb) iz Hatay provincije i 3 kaki kultivara, od koji 2 pripadaju *Diospyros kaki* Thunb i jedan *Diospyros oleifera* Cheng. Genetički međuodnosi su određeni korišćenjem ukupno 29 molekularnih DNK prajmera (SRAP I SSR). Od ovih prajmera, 21 SRAP primer kombinacije daju ukupno 107 traka i 77.6% su polimorfne; 8 SSR primera daju 26 polimorfnih traka sa prosečnim polimorfizmom 84.6%. SRAP i SSR markeri daju 4.6 traka u proseku i broj traka po marker je 3.6. Najmanja sličnost je dobijena između MK-113 (*Diospyros oleifera* Cheng) i drugih genotipova koji pripadaju *Diospyros kaki* Thunb (sličnost 0.41-0.69 za SRAP primere, 0.25-0.67 za SSR primere). Genotip/kultivari pripadaju *Diospyros kaki* imaju sličnost 0.98-1.00 prema SRAP i SSR markerima. Ova sličnost može da bude rezultat klonalne propagacije pre nego autogamije.

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