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Original Article

Genetic Diversity and Relationships among Local Olive (*Olea europeaea* L.) Genotypes from Gaziantep Province and Notable Cultivars in Turkey, Based on SSR Markers

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Abstract

Olive and olive oil have a prominent place in the cultures of the countries within the Mediterranean basin including Turkey. The genetic relationships among 30 olive (*Olea europaea* L.) genotypes sampled from Gaziantep province in Turkey were examined using 10 simple sequence repeat (SSR) markers (DCA9, DCA11, DCA15, DCA18UDO4, UDO9, UDO11, UDO12, UDO22, UDO24). Also, three well known Turkish and one foreign olive cultivar were also included within the SSR analysis. The number of alleles per locus of the SSR markers ranged from 5 (DCA15, UDO9) to 14 (DCA9) (average 7.9), for a total of 79 alleles. Similarity coefficients were calculated on the basis of 79 amplified bands. A dendrogram was created according to the 10 SSR markers by the unweighted pair-group method. The banding patterns obtained from the SSR primers allowed all of the genotypes/cultivars to be distinguished. According to the dendrogram, the 33 olive genotypes and cultivars were clustered into five main clusters. The most closely related genotypes were 'Oguzeli 3' and 'Yavuzeli 1' with 0.80 similarity ratio. The most genetically divergent cultivars were 'Yavuzeli 6' and 'Kilis Yaglik' (0.30), 'Yavuzeli 6' and 'Saurani' (0.20), 'Nizip 7' and 'Yavuzeli 4' (0.15), 'Islahiye 5' and 'Nizip Yaglik' (0.10). In conclusion, SSR analysis can be an efficient method for olive genotypes and cultivar identification and can offer valuable informative data to identify olive genotypes and cultivars grown in Turkey.

Keywords: molecular markers, olive, Simple Sequence Repeat Marker

Introduction

The Mediterranean basin has very suitable environmental conditions for olive (*Olea europaea* L.) growing and the Mediterranean basin countries accounted for approximately 97% of the world's olive production currently accounting more than 800 million of olive trees (FAO, 2014). In main olive producer countries, more than 90% of olive fruits are used for oil production and the rest of the production is consumed as table olives; it is estimated that more than 2.500.000 tons of olive oil are produced annually throughout the world (IOOC, 2015).

The olive is one of the most widely cultivated and economically important fruit crop for several Mediterranean countries, mainly for Spain, Italy, Greece, Turkey and Portugal (Ercisli *et al*., 2011). These Mediterranean countries had cultivated (*Olea europaea* subsp. *europaea* var. *sativa*) and wild olive trees (*Olea europaea* subsp. *europaea* var. *sylvestris*) (Boskou, 2009). It is believed that cultivated varieties of *Olea europaea* supsp. *europaea* var. *sativa* were derived from the wild type *Olea europaea* subsp. *europaea* var. *sylvestris* in the Mediterranean region and then were spread throughout the world (Sesli and Yegenoglu, 2010). This crop is also having an increasing economic interest beyond Mediterranean basin countries, such as Argentina, Australia, Brazil, Chile, South Africa and USA. The olive tree has been naturalized in several regions of America, where it is used for the olive industry.

The number of olive oil consumers has been increasing, especially since recent evidence suggests health and nutritional benefits of virgin olive oil (Poljuha *et al*., 2008). Virgin olive oil (VOO) is a source of at least 30 antioxidant phenolic

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compounds and 100 aromatic compounds that contribute to its bitter taste and aroma; also, it is the only oil that can be eaten without refining. Olive oil is ranked sixth in level of world cooking oil production. (Navero *et al*., 2000; Besnard *et al*., 2007; Kole, 2011; Aparicio and Harwood, 2013).

Different techniques have been used to characterize olive diversity. Morphological criteria such as leaf, fruit, seed and growth behaviour have been used to evaluate olive diversity, as well as to determine the origin of olive trees. An evaluation of phenotypic diversity was used to discriminate olive cultivars with distinct morphological and pomological characters (Ipek *et al*., 2012). There are many systematic identification procedures that have been developed to help identify genetic diversity in olive trees. These include chemical (fatty acids and oil content) and phenological parameters (dates of first leaves, fruits and flowers) as reported by Lumaret *et al*. (2004) and Taamalli *et al*. (2006). Isozyme analysis has also been used to analyze the genetic diversity in cultivated and wild type olives because morphological traits have in general not been able to clearly differentiate between closely related cultivars (Kole, 2011). As well known, almost all morphological and biochemical characters are controlled by minor polygenic traits and are easily affected by environmental factors. Farming practices, age and developmental stages of plants affect morphological and biochemical characters as well (Khakwani *et al*., 2005; Kaczmarka *et al*., 2015; Nemli *et al*., 2015).

The identification of olive cultivars and their area of origin are very important in order to expand cultivation of those commercial varieties with superior yields, that are best adapted to specific local environmental conditions (Sarri *et al*., 2006; Poljuha *et al*., 2008). The presence of synonymous clones and mislabelling has been reported in olive orchards. Researchers have failed to accurately evaluate these two forms by using morphological studies due to the similarities in phenotypes (Belaj *et al*., 2003) and environmental influence (phenotypic plasticity) over specific traits.

DNA-based markers are more reliable for cultivar and subspecies identification than phenotypic traits, since they are not influenced by environmental conditions (Sesli and Yegenoglu, 2010). Molecular markers have been developed for olives in order to facilitate accurate cultivar identification (Belaj *et al*., 2003). This enables clear identification of genetic polymorphism within and among olive cultivars. Previous research clearly indicated that the SSR technique was more appropriate than AFLPs and RAPDs for polymorphic detection, which more clearly distinguishes among closely related cultivars (Belaj *et al*., 2003; Montemurro *et al*., 2008; Muzzalupo *et al*., 2014; Abdessemed *et al*., 2015). In olive cultivar identification, microsatellites have a lot of advantages compared to the other PCR (Polymerase Chain Reaction) based molecular markers techniques due to co-dominant and easily reproducible characteristics, frequent and random distribution, thus allowing a wide coverage of the genome. Microsatellites detect a high level of variation and reduce the number of markers required to distinguish between genotypes in olive (Kole, 2011).

In light of the general lack of detailed information about genetic diversity and relationships in native olive cultivars from Turkey, the present study evaluated genetic diversity and relationships among the most important olive genetic resources in Turkey.

Materials and Methods

Plant material

In the study there were used thirty genotypes widely grown within different parts of Gaziantep; one well-known foreign and three Turkish reference olive cultivars were added to the study in order to have valuable comparison data (Table 1). For this reason, leaf samples of all thirty olive genotypes and the four cultivars used in the hereby study were collected in Turkey and finally a total of thirty-four olive genotypes were included in SSR analysis.

DNA extraction

Genomic DNA was extracted from young leaf tissue using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the instructions provided by the manufacturer. Subsequently, a RNAse treatment was performed on the eluted DNA samples. Purity and concentration of the DNA were checked both on 1% (w/v) agarose gels and by NanoDrop® ND-1000 spectrophotometer.

SSR analysis

Ten widely used SSR loci (DCA15, DCA18, UDO12, UDO24, UDO4, UDO9, DCA11, DCA9, UDO22, UDO 11) were used in Polymerase Chain Reaction (PCR) studies. PCR was conducted in a volume of 10 μL and contained 15 ng genomic DNA, 5 pmol of each primer, 0.5 mM dNTP, 0.5 unit GoTaq DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 2 μL 5X buffer. The forward primers were labelled with Well RED fluorescent dyes D2 (black), D3 (green) and D4 (blue) (Proligo, France). Reactions without DNA were included as negative controls. PCR amplification was performed by using the Biometra® PCR System. The amplification conditions consisted of an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 52-56 °C and 2 min at 72 °C, with a final extension at 72 °C for 10 min. The PCR products were first separated on a 3% (w/v) agarose gel run at 80 V for 2 hrs. The gel was then stained with ethidium bromide at a concentration of 10 mg/mL. A DNA ladder (100 bp) (Promega) was used for the approximate quantification of the bands. The amplification products were visualized under UV light and their sizes were estimated relative to the DNA ladder. For further determination of polymorphisms, the PCR products were run on CEQTM 8800 XL Capillary Genetic Analysis System (Beckman Coulter, Fullerton, CA). The analyses were repeated at least twice to ensure reproducibility of the results. Allele sizes were determined for each SSR locus using the Beckman CEQTM Fragment Analysis software. In each run, foreign reference cultivars were included.

Genetic analysis

The genetic analysis program "IDENTITY" 1.0 [9] was used according to Paetkauet *et al.* (1995) for the calculation of number of alleles, allele frequency, expected and observed heterozygosity, estimated frequency of null alleles, and probability to identity per locus. Genetic dissimilarity was determined by the program "MICROSAT" (version 1.5) (Minch *et al*., 1995) using proportion of shared alleles, which was calculated by using "ps (option 1- (ps))", as described by Bowcock *et al.* (1994). The results were then converted to a similarity matrix, and a dendrogram was constructed with the

Table 1. Utilization, origin and growing areas of thirty olive genotypes and four cultivars

Genotype	Utilization	Origin and growing area
'Islahiye 1'	Oil	Mediterranean
'Islahiye 2'	Oil	Mediterranean
'Islahiye 3	Oil	Mediterranean
'Islahiye 4'	Table and Oil	Mediterranean
'Islahiye 5'	Table and Oil	Mediterranean
'Oğuzeli 1'	Table and Oil	Mediterranean
'Oğuzeli 2'	Oil	Mediterranean,
'Oğuzeli 3'	Oil	Mediterranean
'Oğuzeli 4'	Oil	Mediterranean
'Oğuzeli 5'	Oil	Mediterranean
'Karkamış 1'	Table and Oil	Mediterranean
'Karkamış 2'	Table and Oil	Mediterranean
'Karkamış 3'	Oil	Mediterranean
'Karkamış 4'	Oil	Mediterranean
'Karkamış 5'	Oil	Mediterranean
'Nizip 1'	Oil	Mediterranean
'Nizip 2'	Oil	Mediterranean
'Nizip 3'	Oil	Mediterranean
'Nizip 4'	Table and Oil	Mediterranean
'Nizip 5'	Table and Oil	Mediterranean
'Nizip 6'	Table and Oil	Mediterranean
'Nizip 7'	Table and Oil	Mediterranean
'Yavuzeli 1'	Oil	Mediterranean
'Yavuzeli 2'	Table and Oil	Mediterranean
'Yavuzeli 3'	Oil	Mediterranean
Yavuzeli 4'	Oil	Mediterranean
'Yavuzeli 5'	Oil	Mediterranean
'Yavuzeli 6'	Green-Table	Mediterranean
'Yavuzeli 7'	Table and Oil	Mediterranean
'Yavuzeli 8'	Table and Oil	Mediterranean
cv. 'Sarı ulak'	Green, Black-Table	TR, Mediterranean
cv. 'Nizip Yağlık'	Oil	TR, Southern Anatolia
cv. 'Kilis Yağlık'	Oil	TR, Southern Anatolia
cv. 'Saurani'	Oil	Syria

TR: Turkey

UPGMA method (Sneath and Sokal, 1973) using the software NTSYS-pc (Numerical Taxonomy and Multiware Analysis System, version 2.0) (Rohlf, 1988).

Results and Discussion

A matrix of 10 SSR primers for the 34 olive individuals (Table 1) was used to evaluate the genetic relationships among local genotypes, national and new introduced cultivars. Using DNA samples isolated from the 34 olive genotypes and cultivars as templates, polymorphic DNA fragments were amplified from all 10 highly polymorphic simple sequence repeat (SSR) primer pairs and the results of molecular analysis of the 34 genotypes and cultivars generated by SSR primer pairs are summarized in Table 2. Ten SSR loci were also used aiming to identify if duplicate olive cultivar samples were present in the dataset.

A total of 79 polymorphic alleles with the average alleles per locus of 7.9 were detected within the 10 SSR loci. The maximum alleles were observed at the loci of DCA9 as 14, while the lowest number of alleles was observed at the loci of DCA15 and UDO9 as 5, respectively.

Previous studies conducted on olive cultivars in different olive growing countries also revealed high polymorphism by using SSR markers and DCA9 primer was found more discriminative for olive, that support the current findings as well (Bandelj *et al*., 2002; Poljuha *et al*., 2008; Alba *et al*., 2009; Abdessemed *et al*., 2015). Noormohammadi *et al*. (2009) and Muzzalupo *et al*. (2014) also found high polymorphism in olive cultivars with DCA9 marker.

Table 2. Simple sequence repeats (SSRs), number of detected alleles, observed heterozygosity (*Ho*) and expected heterozygosity (*He*) of 10 SSR markers on thirty olive genotypes and four cultivars

SSR Primers	Number of alleles	Expected heterozygosity (*He*)	Observed heterozygosity (*Ho*)
DCA15	5	0.474	0.529
DCA18	10	0.844	0.676
UDO12	6	0.650	0.500
UDO24	7	0.767	0.523
UDO4	6	0.547	0.494
UDO9	5	0.333	0.323
DCA11	12	0.841	0.529
DCA9	14	0.874	0.764
UDO22	8	0.526	0.676
UDO11	6	0.780	0.535
Total	79	6.636	5.549
Average	7.9	0.663	0.554

The number of average polymorphic alleles per primers was 7.9 thus comparable with the results of Cipriani *et al*. (2002), Poljuha *et al*. (2008), Alba *et al*. (2009) and Roubos *et al*. (2010), but lower than data published by Lopes *et al*. (2004) and Abdessemed *et al*. (2015). The contrary results of the

Table 3. Allele size of the olive genotypes and cultivars under study

Genotype	DCA15	DCA18	UDO12	UDO24	UDO4	UDO9	DCA11	DCA9	UDO22	UDO11
'Islahiye 1'	244-244	174-180	155-165	186-186	147-147	96-96	182-182	193-203	196-200	114-114
'Islahiye 2'	244-244	170-186	155-163	182-182	143-147	96-102	140-146	185-203	200-200	124-124
'Islahiye 3'	244-264	170-174	155-163	186-186	147-147	96-114	140-140	185-193	196-200	124-124
'Islahiye 4'	244-264	174-174	163-163	184-184	143-143	96-96	140-140	161-175	196-200	124-124
'Islahiye 5'	244-264	174-174	155-165	186-186	143-143	104-104	146-160	187-187	196-204	124-124
'Oğuzeli 1'	244-244	168-174	155-155	186-186	143-147	96-96	140-146	175-193	196-200	124-124
'Oğuzeli 2'	244-264	170-178	155-155	166-186	143-147	96-96	160-182	175-187	196-200	134-134
'Oğuzeli 3'	244-264	170-178	155-155	182-182	143-143	96-96	180-180	175-187	196-200	112-122
'Oğuzeli 4'	264-264	172-174	165-165	166-166	147-147	96-114	140-180	193-193	196-200	118-118
'Oğuzeli 5'	244-244	180-192	165-165	184-184	143-147	96-96	146-146	175-187	196-200	122-122
'Karkamış 1'	244-244	168-176	155-165	166-184	147-147	96-96	178-178	193-203	196-200	114-114
'Karkamış 2'	244-244	174-174	155-155	186-186	147-147	96-114	184-184	185-203	200-200	112-112
'Karkamış 3'	244-264	174-174	155-163	166-166	147-147	96-96	140-146	185-193	196-200	116-122
'Karkamış 4'	256-264	168-168	165-165	180-186	147-147	96-96	140-140	187-187	196-200	112-112
'Karkamış 5'	244-254	168-168	155-165	166-186	147-147	96-96	140-140	175-193	196-200	112-124
'Nizip 1'	244-264	172-180	139-151	184-184	147-147	96-96	146-160	175-187	196-200	124-124
'Nizip 2'	244-268	172-180	155-163	166-184	149-149	96-96	140-146	175-187	196-200	122-122
'Nizip 3'	244-244	172-182	155-155	186-186	145-145	96-96	160-182	193-193	196-200	124-124
'Nizip 4'	244-264	168-192	155-157	166-166	137-143	96-96	180-180	171-175	196-204	112-112
'Nizip 5'	244-244	170-170	165-165	180-186	147-147	96-96	140-180	175-187	196-196	112-122
'Nizip 6'	244-244	170-170	155-163	166-186	147-151	96-96	142-152	197-197	200-200	122-122
'Nizip 7'	244-264	176-176	155-155	166-188	147-151	96-114	142-142	173-177	200-200	116-116
'Yavuzeli 1'	244-264	170-176	155-155	166-188	143-143	96-96	140-180	173-187	196-200	112-122
'Yavuzeli 2'	244-244	168-168	155-155	168-182	143-147	96-96	140-182	197-203	196-200	112-112
'Yavuzeli 3'	244-264	168-174	155-163	182-182	143-147	96-114	146-182	171-171	196-196	116-116
'Yavuzeli 4'	244-264	170-178	163-163	166-180	143-143	96-96	140-140	171-203	196-196	112-112
'Yavuzeli 5'	264-264	170-178	165-165	184-184	147-147	96-102	140-182	175-203	196-200	112-112
'Yavuzeli 6'	244-244	172-174	155-165	182-182	143-147	96-102	182-182	175-203	196-200	114-114
'Yavuzeli 7'	244-244	180-192	139-151	186-186	147-147	96-102	146-178	197-197	200-200	114-114
'Yavuzeli 8'	244-244	168-176	155-155	166-186	147-147	96-96	140-178	183-203	200-200	116-116
Turkish and Foreign cultivars										
'Sarı Ulak'	244-264	168-168	155-163	184-184	147-147	96-96	168-176	181-207	196-200	124-124
'Nizip Yağlık'	244-264	170-174	155-155	166-166	143-143	96-96	186-186	171-193	196-200	124-124
'Kilis Yağlık'	244-244	168-176	155-155	166-166	147-147	96-102	180-180	163-163	196-196	112-124
'Saurani'	244-264	168-176	155-165	166-166	147-147	96-112	178-178	171-193	200-200	112-124

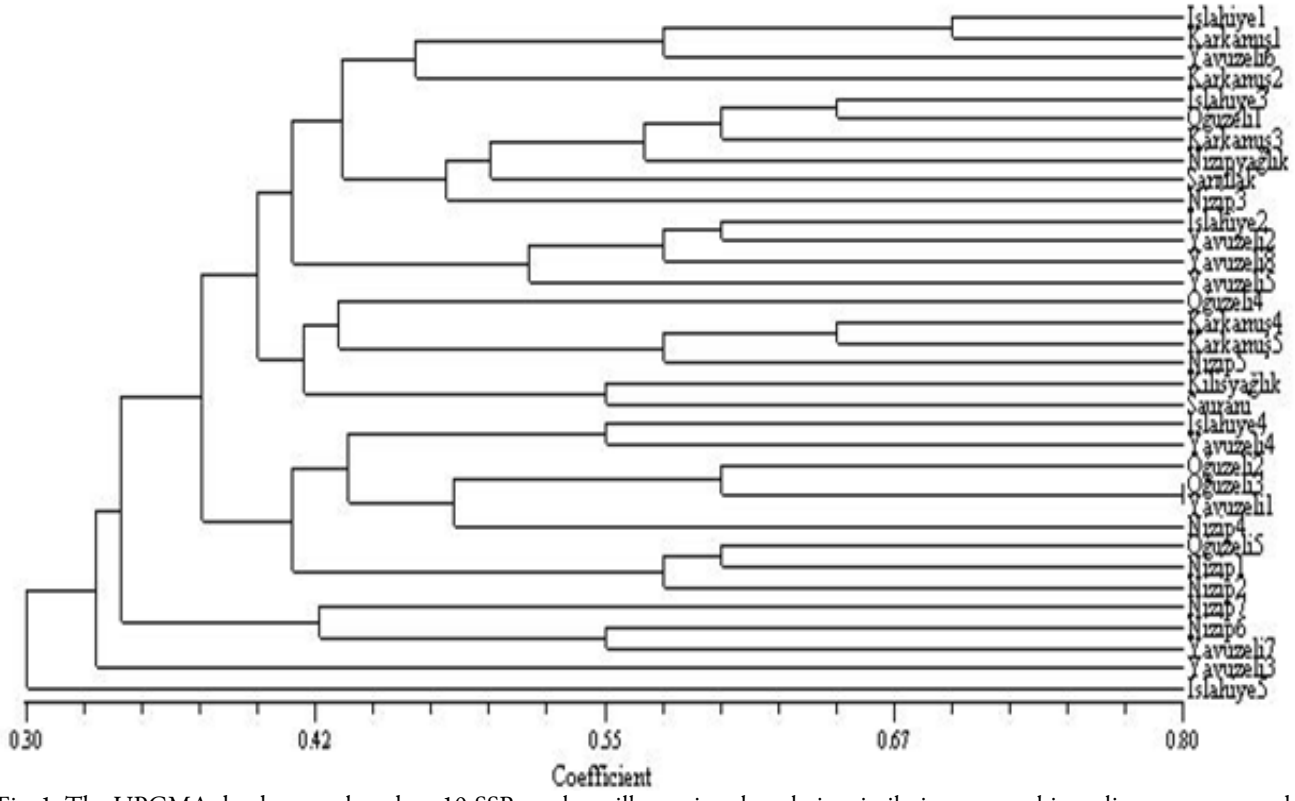

Fig. 1. The UPGMA dendrogram based on 10 SSR markers, illustrating the relative similarity among thirty olive genotypes and four cultivars from Turkey and Syria

molecular marker researches on olive might result from the different genetic bases of the accessions tested, primer numbers, types of molecular marker, higher number of analyzed samples, as well as due to the more diverse genotypes analyzed.

The value of observed heterozygosity (*Ho*) was less than the value of expected heterozygosity (*He*) for all SSR loci, except DCA15 and UDO22. UDO9 loci revealed the lowest expected heterozygosity (*He*) as 0. 333, while the loci DCA9 gave the highest expected heterozygosity value as 0.874. Observed heterozygosity (*Ho*) was the highest in DCA9 loci as 0.764, while the lowest, as 0.323, was noted in UDO9 loci (Table 2). The results indicate that the studied population may exhibit a high level of inbreeding within isolated and closely related individuals. Muzzalupo *et al*. (2014) reported higher expected heterozygosity among 489 olive cultivars by using 11 nuclear SSR markers.

Based on SSR profiles of the thirty Gaziantep genotypes as well the data for the one foreign and the three Turkish cultivars, nu synonyms were observed neither among Turkish cultivars or foreign ones and neither synonym were determined between the two group of genotypes.

Allele size varied from 96 bp to 268 bp and the most frequent alleles were 96 bp (16.17%), followed by 244 bp (13.23%), 147 bp (12.05%), 155 bp (10.58%), 200 bp (10.29%) and 196 bp (9.11%), respectively (Table 3).

The most closely related genotypes were 'Oğuzeli 3' and 'Yavuzeli 1' with 0.80 similarity ratio. The most genetically divergent cultivars were 'Yavuzeli 6' and 'Kilis Yağlık' (0.30), 'Yavuzeli 6' and 'Saurani' (0.20), 'Nizip 7' and 'Yavuzeli 4' (0.15), 'Islahiye 5' and 'Nizip Yağlık' (0.10), indicating a fairly big range, thus signifying that the olive genotypes and cultivars tested showed some diverse relationships.

UPGMA cluster analysis of the 34 olive genotypes and cultivars using the 10 polymorphic SSR markers resulted in the dendrogram shown in Fig. 1, which displayed a good fit to the genetic similarity matrix. Five groups could be distinguished by truncating the dendrogram and local genotypes 'Islahiye 5' and 'Yavuzeli 3' was clustered in a solitary group (Group I and Group II). Group III included 3 local genotypes, namely 'Yavuzeli 7', 'Nizip 7' and 'Nizip 6'. The Group IV also include 9 local genotypes and was further divided 2 sub-groups. The first subgroups included local 'Nizip 2', 'Nizip 1' and 'Oguzeli 5' genotypes, while subgroup 2 included 'Nizip 4', 'Yavuzeli 1', 'Oguzeli 3', 'Oguzeli 2', 'Islahiye 4' and 'Yavuzeli 4' (Fig. 1). 'Yavuzeli 1' and 'Oguzeli 3' were the closest genotypes, with a similarity coefficient of 0.80 (Fig. 1). Group V contained the rest of 20 genotypes and cultivars. The standard Turkish cultivars 'Nizip Yaglik' and 'Kilis Yaglik' and also the Syrian cultivar 'Saurani' were placed together within Group V. 'Saurani' and 'Kilis Yaglik' showed 0.55 similarity coefficient. Group V was further clustered into 4 major subgroups containing 6, 4, 6 and 4 genotypes/cultivars.

As expected, the most closely related cultivars were within each gene pool. A partly clustering was observed among cultivars from two gene pools, suggesting that Turkish and foreign olive cultivars continue to be related. These results also indicated that grouping genotypes based on the geographic origin is not useful in olive. Besnard *et al*. (2001) found that olive genotypes from different countries clustered together within a group and they did not find any grouping pattern based on their geographical origins. The result was similar to that of Poljuha *et al*. (2008) who studied genetic diversity among Slovenian and Croatian olive cultivars and found that Croatian olive cultivars clustered with olive cultivars from Slovenia. Previous studies indicated that olive genotypes have been freely exchanged among collectors in different countries for centuries.

Conclusions

In conclusion, the SSR analysis was found to be useful for the detection of genetic differences among the olive accessions. The genetic relationships among olive cultivars may facilitate the selection of genitors in various breeding programs with the hypothesis that the more genetically diverse the parents, the more likely they are to possess unique alleles for traits of interest for their descendants. This study will help to restructure the Turkish olive cultivar database, to widen the genetic base and give the premises to introduce new varieties.

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