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Molecular characterization of ancient olive genotypes from Hatay Province in Turkey

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Abstract: Turkey's average share of world olive production is between 7% and 10% and the country is the fourth biggest table olive and olive oil producer in the world. More than fifty olive cultivars have been commercialized in Turkey and there are numerous olive genotypes in different olive-growing regions in Turkey that differ from each other in terms of leaf, flower, and fruit characteristics. The aim of the present study was to identify the 40 most widely grown olive genotypes in Hatay Province in Turkey using microsatellite or simple sequence repeat (SSR) markers. Ten SSR loci were selected and used to identify olive genotypes/cultivars. The number of alleles per locus was found to be between 4 (UDO4 and DCA13) and 16 (DCA9), indicating high polymorphism among olive germplasms. We did not determine identical cultivars in SSR analysis. Samandag2 and Dörtyol7 (0.85), Samandag7 and Saurani (0.75), Payas kalesi and Sarı ulak (0.75), and Yayladag4 and Samandag3 (0.70) genotype pairs showed higher similarity while Yayladag1 and Samandag8 (0.15), Reyhanlı1 and Yayladag6 (0.15), and Samandag8 and Hassa5 (0.15) were found to be the most genetically divergent genotypes.

Key words: Molecular characterization, olive, simple sequence repeat

1. Introduction

The olive tree has been cultivated for approximately 600 years in Mediterranean countries, where 95% of olive resources are located. Its habitat is determined by the Mediterranean climate, which is characterized by relatively mild winters and hot, dry summers. The areas belonging to this climate type lie between 30°N and 45°N. With the discovery of America, olive growing spread gradually on a limited scale to South and North America. The 19th century then saw its spread to Australia and today it is also grown in Peru, Argentina, India, Pakistan, Afghanistan, and other Asian, African, South American, and Middle Eastern countries (Galili et al., 1997). Outside of the Mediterranean, olive growing has developed basically through the introduction of varieties from other countries. This is the case in the United States, Argentina, and Australia (Bartolini and Petruccelli, 2002).

Some 850 million olive trees are grown in the world on approximately 8.7 × 10^6 ha of land (http://faostat.fao.org/). Around 10 × 10^6 t of olives is produced, 90% of which is channeled into oil production, and it is estimated that more than 2.5 × 10^6 t of olive oil is produced annually throughout the world (http://www.internationaloliveoil.org/noticias). The olive is important in the economy of many Mediterranean countries including Spain, Portugal, Italy, Greece, Turkey, and the countries of the Middle East through Morocco and Tunisia to Egypt (Boskou, 2009).

Turkey has a long history of olive tree cultivation and olive oil production. Currently, the country cultivates a number of trees that is triple its own population. According to the World Bank, the total population of Turkey is 77 million, while the number of olive trees is 250 million. That means that on average for every one person there are three olive trees. Most of these are grown along the Aegean and Mediterranean sea coasts in the west of the country. There are many different types of olives in southern Anatolia as well (Ercisli et al., 2011).

Traditionally, cultivar identification of horticultural plants including olive mainly relies on phenotypic characteristics, such as morphology and colors of leaf, flower, and fruit. However, phenotypic characteristics of plants are strongly affected by environment and also vary in different plant developmental stages (Barranco et al., 2000; Contento et al., 2002; Khakwani et al., 2005; Kaczmarska et al., 2015; Nemli et al., 2015).

The development of molecular techniques for genetic analysis has led to a great augmentation in our knowledge of crop genetics and our understanding of the structure and behavior of various crop genomes. These molecular techniques, in particular the applications of molecular markers, have been used to scrutinize DNA sequence variation(s) in and among the crop species and create

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new sources of genetic variation by introducing new and favorable traits from landraces and related crop species (Korir et al., 2013). Though restriction fragment length polymorphism (RFLP) markers have been the basis for most of the work in crop plants, valuable markers have been generated from random amplification polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Simple sequence repeats (SSRs) or microsatellite markers have been developed more recently for major crop plants including olive and this marker system is predicted to lead to even more rapid advances in both marker development and implementation in breeding programs (Muzzalupo et al., 2014; Abdessemed et al., 2015).

This study aims to detect the genetic diversity of the 40 olive genetic resources in Hatay Province in Turkey using SSR marker techniques.

2. Materials and methods

2.1. Plant material

In this study, we used 40 genotypes widely grown different parts of Hatay and we added 1 well-known foreign and 3 Turkish reference olive cultivars to make comparisons with them as well (Table 1). Leaf samples of all 40 olive genotypes and four cultivars used in this study were included in SSR analysis.

2.2. 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, RNAse treatment was performed on the eluted DNA samples. Purity and concentration of the DNA were both checked on 1% (w/v) agarose gels and by NanoDrop ND-1000 spectrophotometer.

2.3. SSR analysis

Ten polymorphic SSR loci (DCA13, UDO4, UDO36, UDO26, UDO24, DCA9, UDO9, UDO39, DCA11, UDO11) were used in polymerase chain reaction (PCR) studies. PCR was conducted in a volume of 10 µL and contained 15 ng of genomic DNA, 5 pmol of each primer, 0.5 mM dNTP, 0.5 U of GoTaq DNA polymerase (Promega), 1.5 mM $MgCl_2$, and 2 µL of 5X buffer. The forward primers were labeled with WellRED fluorescent dyes D2 (black), D3 (green), and D4 (blue) (Proligo, Paris, France). Reactions without DNA were included as negative controls. PCR amplification was performed 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 h. 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 the CEQTM 8800 XL Capillary Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The analyses were repeated at least twice to ensure reproducibility of the results. Allele sizes were determined for each SSR locus using Beckman CEQTM Fragment Analysis software. In each run, foreign reference cultivars were included.

2.4. Genetic analysis

The genetic analysis program IDENTITY 1.0 [9] was used according to Paetkau et al. (1995) for the calculation of number of alleles, allele frequency, expected and observed heterozygosity, estimated frequency of null alleles, and probability of identity per locus. Genetic dissimilarity was determined with 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 UPGMA method (Sneath and Sokal, 1973) using the software NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, version 2.0) (Rohlf, 1988).

3. Results

In SSR analysis, 10 highly polymorphic SSR primer pairs were screened for amplification of 44 olive genotypes and cultivars. All SSR primers gave reproducible and scorable amplification products from 44 olive genotypes and cultivars. Table 2 shows that codes of SSR primers, the number of alleles for each primer, expected heterozygosity, and observed heterozygosity. A total of 85 polymorphic alleles were obtained across the 44 olive genotypes and cultivars. The number of amplified fragments (polymorphic alleles) ranged from 4 (DCA13 and UDO4) to 16 (DCA9), with an average of 8.5 fragments per primer. The results showed that all SSR primers gave polymorphic alleles (Table 2).

Expected heterozygosities (*He*) were variable across loci, reflecting the different number and frequencies of the alleles found. For 10 loci, UDO26 had the lowest expected heterozygosity (*He*) of 0.402 while the DCA11 loci gave the highest expected heterozygosity value of 0.857. It was especially visible for DCA9 loci, where a higher *Ho* was observed at 0.977. The observed heterozygosity was the lowest at 0.181 in UDO4 loci, indicating a dearth of heterozygotes at this locus. In general the expected heterozygosity (*He*) was higher than the observed values (*Ho*), except with DCA9 and UDO9 primers (Table 2). Allele size varied from 96 bp to 207 bp (Table 3).

Table 1. Utilization, origin, and growing areas of 40 olive genotypes and 4 cultivars.

Genotypes	Utilization	Origin and growing area
Reyhanlı1	Oil	Mediterranean
Reyhanlı2	Table and oil	Mediterranean
Reyhanlı3	Oil	Mediterranean
Reyhanlı4	Green, black-table	Mediterranean
Reyhanlı5	Green, black-table	Mediterranean
Reyhanlı6	Green, black-table	Mediterranean
Reyhanlı7	Table and oil	Mediterranean,
Yayladag1	Green-table	Mediterranean
Yayladag2	Table and oil	Mediterranean
Yayladag3	Table and oil	Mediterranean
Yayladag4	Green-table	Mediterranean
Yayladag5	Green-table	Mediterranean
Yayladag6	Oil	Mediterranean
Dörtyol1	Green-table	Mediterranean
Dörtyol2	Black-table	Mediterranean
Dörtyol3	Oil	Mediterranean
Dörtyol4	Oil	Mediterranean
Dörtyol5	Oil	Mediterranean
Dörtyol6	Table and oil	Mediterranean
Samandag1	Table and oil	Mediterranean
Samandag2	Table and oil	Mediterranean
Samandag3	Table and oil	Mediterranean
Samandag4	Oil	Mediterranean
Samandag5	Table and oil	Mediterranean
Samandag6	Oil	Mediterranean
Samandag7	Oil	Mediterranean
PayasKalesi	Green, black-table	Mediterranean
Samandag8	Green-table	Mediterranean
Hassa1	Table and oil	Mediterranean
Hassa2	Table and oil	Mediterranean
Hassa3	Oil	Mediterranean
Hassa4	Table and oil	Mediterranean
Hassa5	Oil	Mediterranean
Hassa6	Table and oil	Mediterranean
Hassa7	Green-table	Mediterranean
Kırıkhan1	Green, black-table	Mediterranean
Kırıkhan2	Oil	Mediterranean
Kırıkhan3	Oil	Mediterranean
Kırıkhan4	Oil	Mediterranean
Kırıkhan5	Green, black-table	Mediterranean
Sarı ulak	Green, black-table	Turkey, Mediterranean
Büyüktopak ulak	Green-table	Turkey, Mediterranean
Nizip yağlık	Oil	Turkey, Southern Anatolia
Saurani	Oil	Syria

Table 2. Simple sequence repeats (SSRs), no. of detected alleles, observed heterozygosity (*Ho*), and expected heterozygosity (*He*) of 10 SSR markers on 40 genotypes and 4 olive cultivars investigated.

SSR primers	Number of alleles	Expected heterozygosity (*He*)	Observed heterozygosity (*Ho*)
DCA13	4	0.621	0.250
UDO4	4	0.568	0.181
UDO36	14	0.757	0.363
UDO26	5	0.402	0.204
UDO24	7	0.729	0.545
DCA9	16	0.830	0.977
UDO9	8	0.408	0.431
UDO39	7	0.739	0.659
DCA11	13	0.857	0.318
UDO11	7	0.740	0.704
Total	85	6.651	4.632
Average	8.5	0.665	0.463

The dendrogram resulting from UPGMA cluster analysis showed that the studied genotypes and cultivars could be divided into four main clusters. The first cluster contained only Payas kalesi that originated from Turkey. In cluster there were 2 subclusters; subcluster I included Reyhanlı3 and Hassa5 and subcluster II included Samandag8 and Hassa4. The majority of cultivars were placed in cluster 3. The closest genotypes Dörtyol17 and Samandag2 (0.85 similarity ratio) were also in cluster 3. Cluster 3 included genotypes and cultivars from Turkey and abroad without geographical isolation. Cluster 4 included 4 genotypes and was also further divided into 2 subgroups. We could not observe any genotypes or cultivars genetically identical (Figure).

In the present study there were no identical cultivars in SSR analysis. Samandag2 and Dörtyol7 (0.85), Samandag7 and Saurani (0.75), Payas kalesi and Sarı ulak (0.75), and Yayladag4 and Samandag3 (0.70) genotype pairs showed higher similarity while Yayladag1 and Samandag8 (0.15), Reyhanlı1 and Yayladag6 (0.15), and Samandag8 and Hassa5 (0.15) were found to be the most genetically divergent genotypes (Figure).

4. Discussion

Every SSR marker primer pair successfully amplified the target DNA in 44 olive genotypes and cultivars. This study has demonstrated the utility of 10 universal SSR markers among olive germplasm in Turkey. Previously those 10 SSR markers showed high polymorphism in Slovenia (Poljuha et al., 2008), Italy (Cipriani et al., 2002; Alba et al., 2009; Muzzalupo et al., 2014), and Algeria (Abdessemed et al., 2015). The number of average polymorphic alleles per primer (6.6) was higher than that obtained by Cipriani et al. (2002); comparable to those of Belaj et al. (2003), Poljuha et al. (2008), Alba et al. (2009), and Roubos et al. (2010); and lower than that of Abdessemed et al. (2015). The values found in this study average 6.6 alleles/locus and are therefore consistent with the literature, whereas a smaller number of genotypes (44) were evaluated. The allele size ranges found in this study are similar to those of Poljuha et al. (2008). Variations reported in the number of alleles in olive cultivars by different scientists may be related to variations in the loci studied as well as the number of genotypes and their localities (Lopes et al., 2004).

In all the studied genotypes/cultivars, the observed heterozygosity (mean $H_0 = 0.665$) was lower than expected (mean $He = 0.463$), except for the DCA9 and UDO 9 primers. Muzzalupo et al. (2014) found similar results in olive germplasm by using SSR markers.

The primers DCA9, UDO28, DCA18, and DCA3 were found to be more polymorphic. Alba et al. (2009), Noormohammadi et al. (2009), and Muzzalupo et al. (2014) also found high polymorphism with the DCA9 primer. Poljuha et al. (2008) found that the DCA3, DCA10, and DCA16 primers had high discrimination capacity among Istrian olive cultivars in Slovenia and Croatia.

As expected, the most closely related cultivars were within local genotypes from Hatay in Turkey. A partial clustering was observed among cultivars from two gene pools, suggesting that Turkish and foreign olive cultivars

Table 3. Allele sizes of olive genotypes and cultivars.

	DCA13	UDO4	UDO36	UDO26	UDO24	DCA9	UDO9	UDO39	DCA11	UDO11
Reyhanlı1	121–121	141–141	145–145	96–112	166–182	161–197	96–102	117–117	176–176	112–124
Reyhanlı2	119–119	147–147	167–167	96–96	166–166	193–205	96–116	111–115	170–170	112–124
Reyhanlı3	121–121	143–143	141–141	96–96	182–182	187–203	96–128	111–123	152–166	116–124
Reyhanlı4	119–119	143–143	159–183	96–96	182–186	193–203	96–96	115–115	172–172	114–118
Reyhanlı5	117–117	143–143	183–183	96–96	166–166	175–193	96–96	115–123	168–168	112–116
Reyhanlı6	119–137	143–143	145–145	96–112	192–192	187–193	96–96	111–115	176–176	112–124
Reyhanlı7	119–137	143–143	141–141	96–96	166–186	175–193	96–96	111–123	174–180	116–116
Yayladag1	119–137	141–141	139–145	96–96	166–184	193–205	96–96	111–115	174–174	124–124
Yayladag2	117–117	147–147	141–141	96–96	182–186	171–193	96–96	143–143	176–176	120–120
Yayladag3	119–119	147–147	137–145	96–96	182–182	193–203	96–96	123–123	168–168	116–124
Yayladag4	119–119	141–147	141–141	106–106	166–166	193–203	96–96	111–123	174–174	112–116
Yayladag5	119–119	147–147	141–173	116–116	182–182	187–193	96–96	123–123	168–168	112–124
Yayladag6	119–137	141–151	145–145	102–102	166–166	171–177	96–96	115–123	174–174	112–116
Dörtyol1	117–117	147–147	141–151	102–102	166–182	187–193	96–132	111–117	172–172	112–124
Dörtyol2	119–137	143–147	145–161	96–96	182–186	197–205	96–96	111–111	174–174	124–124
Dörtyol3	117–117	143–143	157–157	96–96	166–186	161–193	112–112	111–115	192–192	116–124
Dörtyol4	117–121	143–143	145–145	96–96	184–186	193–197	96–114	117–117	168–168	112–116
Dörtyol5	117–117	147–147	141–141	96–112	184–184	175–193	96–96	123–123	170–170	112–112
Dörtyol6	117–117	147–147	145–153	96–96	182–186	171–193	96–116	111–123	172–186	116–122
Samandag1	119–119	147–147	145–145	96–96	166–182	187–193	96–96	111–123	174–174	124–124
Samandag2	119–119	147–147	143–143	96–112	182–196	187–193	96–102	115–123	172–172	112–124
Samandag3	119–119	147–147	141–141	96–96	166–182	187–193	96–96	111–123	174–174	112–124
Samandag4	119–119	143–143	145–159	102–102	166–182	187–193	96–114	117–123	166–176	112–124
Samandag5	119–119	147–147	141–145	96–96	166–182	175–187	96–128	123–123	174–174	112–124
Samandag6	119–137	147–147	145–145	96–96	186–186	185–185	96–96	111–123	178–186	114–124
Samandag7	119–119	147–147	145–145	96–96	166–166	171–193	96–96	117–123	176–176	124–124
PayasKalesi	119–137	147–147	143–147	96–96	166–166	171–193	96–114	111–123	196–196	112–124
Samandag8	119–119	147–151	147–147	102–102	182–186	175–203	96–96	123–123	166–180	116–122
Hassa1	117–121	143–147	143–143	96–116	182–186	171–193	96–102	119–123	170–176	114–114
Hassa2	119–119	143–147	141–145	96–112	166–166	171–175	96–96	115–123	174–174	124–124
Hassa3	119–119	147–147	145–145	96–96	186–186	171–193	96–114	111–123	166–176	112–116
Hassa4	117–117	151–151	145–145	96–96	166–182	187–191	96–96	115–115	168–174	112–118
Hassa5	117–117	143–143	143–153	96–112	182–182	193–199	96–122	115–123	170–182	112–124
Hassa6	117–121	147–151	147–159	96–112	182–182	193–203	96–114	111–123	174–174	116–116
Hassa7	119–119	147–147	141–141	96–112	166–192	161–175	96–102	123–123	174–174	114–122
Kırıkhan1	119–119	143–147	137–145	96–96	166–186	171–193	96–114	111–117	170–170	116–124
Kırıkhan2	119–119	147–147	141–141	96–96	166–166	171–207	96–96	117–123	174–174	114–116
Kırıkhan3	119–119	143–143	137–145	96–96	168–184	185–203	96–102	115–115	172–172	112–124
Kırıkhan4	119–119	147–147	145–145	96–96	182–182	161–203	96–96	123–123	168–192	112–124
Kırıkhan5	121–121	147–147	145–145	96–96	166–166	187–187	96–114	111–115	172–172	114–114
Turkish and foreign cultivars										
Saurani	119–119	143–143	145–145	96–96	166–166	171–193	96–96	117–117	178–178	124–124
Sarı ulak	119–137	147–147	145–145	96–96	166–166	171–193	96–112	111–123	168–176	112–124
Büyüktopak ulak	117–117	147–147	141–141	96–96	166–184	185–193	96–96	113–119	172–186	116–124
Nizip yağlık	121–121	147–147	145–145	96–96	184–184	181–207	96–96	113–123	186–186	124–124

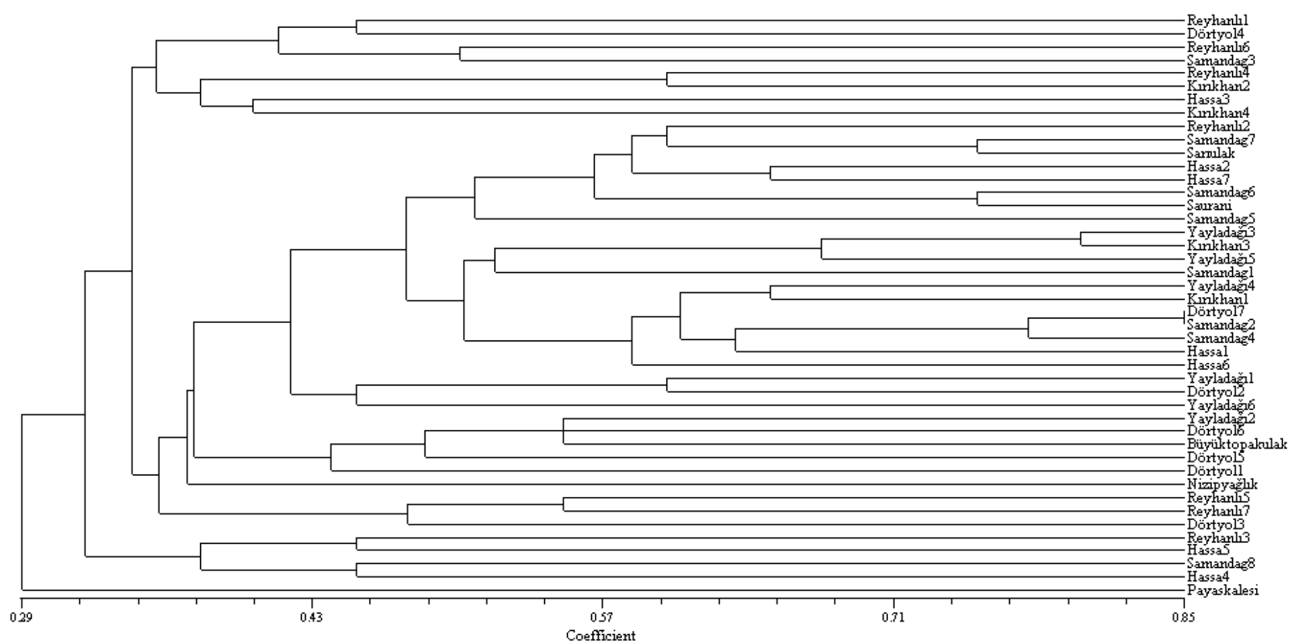

Figure. The UPGMA dendrogram based on simple matching similarity matrix obtained using 10 SSR markers, illustrating the relative similarity among 40 olive genotypes and 4 cultivars from Turkey and other countries.

continue to be related. These results also indicate that grouping genotypes based on 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 based on 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.

This study showed that molecular marker technologies are the most advanced and possibly the most effective means for understanding the basis of genetic diversity in olive. They are efficient and accurate tools with which genetic variation can ultimately be identified and assessed in a rapid and thorough manner. By applying molecular technologies to approach the biological questions underlying the understanding of genetic diversity, we can make significant progress in the speed and depth at which we attain adequate and appropriate conservation and thus genetic resources made available for its use in crop improvement.

Associated with the high reproducibility of the SSR markers, the results obtained in this study support the use of these markers as an important tool in the molecular characterization of olive varieties in germplasm banks, in the identification of duplicates, in the correct identification of cultivars, and of genetically divergent potential parents to be used in breeding programs.

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