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Genetic relationships among olive (*Olea europaea* L.) cultivars native to Croatia and Turkey

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Summary

The aim of the study is to determine genetic diversity and relationships among olive cultivars native to Croatia and Turkey. A total of twenty olive (*Olea europaea* L.) cultivars including fourteen from Croatia and six common cultivars from Turkey were analyzed for genetic diversity and relationships by using six microsatellite markers (DCA05, DCA09, DCA18, GAPU71B, GAPU101, UDO43). The number of polymorphic alleles ranged from 2 (UDO43) to 5 (DCA09), with an average of 3.6 fragments per marker. UPGMA cluster analysis based on simple matching similarity matrix grouped cultivars into three main clusters. Two pairs of cultivars from Croatia ("Buža muška" and "Levantinka"; "VLMD6" and "Drobnica") were thought to be different, although they produced identical SSR profiles. Cluster analysis points to some genetic relationships between Croatian and Turkish olive cultivars. The results also indicate efficiency of SSR markers to evaluate genetic diversity in olive and identify misnamed or synonym individuals.

Introduction

The olive (*Olea europaea*) is native to the coastal areas of the eastern Mediterranean Basin and it is estimated that the cultivation of olive trees began more than 7000 years ago (GREEN, 2002). In the last few years, olive cultivation is steadily expanding to more geographical zones, in response to increased demand for olive oil consumption owing to its nutritional value and recognized health benefits. Olives are now cultivated in many regions of the world with Mediterranean climates, such as South Africa, Chile, Peru, Australia, Argentina and USA (California) and in areas with temperate climates such as New Zealand. However these countries shared 15-16 percent of world's olive production (FAO, 2009).

The olive is of major agricultural importance in the Mediterranean region and olive oil is a basic constituent of the Mediterranean diet. Present production of olives (*Olea europaea*) is about 18.0 million tons green and black table olives and 3.3 million tons olive oil from 10.8 million ha (FAO, 2009). Of the total production, 86% is produced in the Mediterranean region with Spain, Italy, Greece and Turkey as the main producing countries. Mediterranean countries constituted a wide germplasm with a large number of cultivars. It is expected that there were more than 1200 olive cultivars in the world (BARTOLINI et al., 2005)

Olive has a great commercial importance in both Turkey and Croatia and both countries have long tradition in olive growing (POLJUHA et al., 2008; ERCISLI et al., 2011). It is used for local consumption mainly as olive oil in both countries. In Turkey, table olive consumption is also highly preferred. Olive growing in Turkey and Croatia are well established mainly around Aegean and Mediterranean regions of Turkey and Dalmatia region and Istria peninsula in Croatia (ERCISLI, 2004; POLJUHA et al., 2008). Turkey has continued olive production with its very old olive cultivars such as "Domat", "Uslu", "Ayvalik", "Gemlik" etc. for a long time. In contrast, new olive plantations has been established in Croatia predominantly with cultivars introduced from Italy including "Leccino", "Pendolino", "Frantoio", (PRIBETIC, 2006). However, there is increased interest to old autochthonous Croatian olive cultivars such as "Istarska bjelica" in new plantations in Croatia as well because of its adaptation to local conditions and high oil quality (POLJUHA et al., 2008).

Turkey (The Ottoman Empire) had an intense and long-lasting influence on the entire Balkan Peninsula until to the nearer past. Archeological and historical research in Anatolia, Turkey proves that this region was very important for the history of olive (DURGAC et al., 2010). Most contemporary olive cultivars are fairly old and of unknown genetic background. Olives have been propagated by vegetative means in Croatia for centuries and the introduction and spread of cultivars in Croatia may have come from countries that have in the past settled or conquered the area of the present Croatia. Until now, it has not been clarified all around the Mediterranean basin whether their olive cultivars have been introduced or are derived from local oleaster (HANNACHI et al., 2008).

To evaluate genetic relationships, trace phylogenetic origin and extent of ecologically differentiation in the same species, plant breeders need to have a definitive identification both of cultivars and selections of crop plants. Reliable and rapid methods of identification are also required for the establishment of plant variety rights (KJELDGAAD and MARSH, 1994).

Classifications and evaluations of the olive cultivars based on phenotypic expressions such as growth form, leaf morphology and fruit properties and information from these environmentally influenced morphological characteristics is not sufficient to identify olive cultivars. It is a predominantly allogamous species showing a high degree of outcrossing which leads to considerable levels of heterozygosity and DNA polymorphism among individuals. The wide genetic patrimony and the large number of synonyms and homonyms in olive require precise methods of discrimination for cultivar identification and classification. (ANGIOLILLO et al., 1999; RALLO et al., 2000).

Olive cultivars have been the subject of multiple studies given the large number of synonyms and homonyms generated during many centuries of vegetative multiplication and exchange. Nowadays several molecular markers (RFLPs, AFLPs, RAPDs, ISSRs, SSRs) are available and these markers provide an accurate and unambiguous tool for precise identification of cultivated olive germplasm (CARRIERO et al., 2002). Similarly to other crops, simple sequence repeat (SSR) markers have been preferred until now because of their high level of polymorphism, co-dominant nature and reliable repeatability (SZIKRISZT et al., 2011). SSR markers have been developed for olive by several groups (SEFC et al., 2000; CARRIERO et al., 2002; CIPRIANI et al., 2002), and this marker system was found to be the most reliable, effective and easy-to-use for identification of olive cultivars (SARRI et al., 2006). Simple Sequence Repeats (SSRs) are now widely used in olive (BRETON et al., 2008; POLJUHA et al., 2008; ROUBOS et al., 2010; ERCISLI et al., 2011).

In this study, six well-known cultivars from the Western part of Turkey and fourteen cultivars from Dalmatia and Istria regions of

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Croatia were analyzed by Simple Sequence Repeats (SSR) markers in order to determine the potential synonyms and genetic relatedness among them.

Materials and methods

Plant material

In the present study, a total of 20 olive cultivars including six well-known Turkish olive cultivars ("Domat", "Uslu", "Ayvalik", "Gemlik", "Tavsan Yuregi" and "Memecik"), which dominate commercial olive production in Turkey and 14 Croatian olive cultivars ("Istarska bjelica", "Buža muška", "Buža", "Piculja", "Mezanica", "Bjelica Dubrovnik", "Murgulja", "Uljarica", "Levantinka", "Oblica", "Grčka", "Drobnica", "Lastovka" and "VLMD6") were used for SSR analysis. Turkish and Croatian cultivars were selected according to dominancy in both countries' plantations. Six olive cultivars from Turkey share over 80% olive trees in Turkey. The cultivar "Buža " dominate old olive plantations in Croatia over 50 percents (Milotic and Setic, 2005) and the autochthonous cultivar "Oblica", makes up 75% of the total number of olive trees in Croatia (Strikic et al., 2007).

The Turkish cultivars were found together in the Atatürk Central Horticultural Research Institute, Yalova in Turkey. "Istarska bjelica", "Buža muška" and "Buža" were grown in Istria region of Croatia. "Levantinka" and "VLMD6" were sampled from middle Dalmatia and rest of the Croatian cultivars were sampled from South Dalmatia of Croatia.

DNA Isolation

Young leaves of olive trees were sampled for DNA extraction. Lyophilized leaf samples were ground to a fine powder using a mortar and pestle. DNA samples were extracted from 150 mg powdered leaf samples using a modified CTAB method described by Futterer et al. (1995). The concentrations of each DNA sample were measured using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and adjusted to 50 ng/mL for analysis.

Amplification, scoring and analysis of SSR's

Six previously developed and widely used SSR primers in molecular characterization of olive cultivars (DCA-05, DCA-09, DCA-18, GAPU-71B, GAPU-101, UDO-43) were used for amplification of SSR analysis in this study. Each 20-µL polymerase chain reaction (PCR) mixture for amplification of SSR markers consisted of 1.0 U DNA polymerase (Fermentas, Hanover, MD, USA) with the reaction buffer supplied at 1X concentration, 0.4 µM of each primer, dNTPs at 0.25 mM each, and 50 ng template DNA. Thermal cycling conditions were: 2 min at 94°C; 40 cycles of 40 s at 94°C, 45 s at annealing temperature of each primer pair, and 1 min and 30 s at 72°C, and a final extension step of 5 min at 72°C. An Applied Biosystems Thermal Cycler was used for these reactions. PCR products were separated on a 4% agarose SFR™ gel (Amresco Inc., Solon, OH, USA) in 1X TBE (89 mM Tris Base, 89 mM Boric Acid, 2 mM EDTA). Gels were stained with ethidium bromide (0.5 mg/mL; Sigma, St Louis, MO, USA) and photographed.

SSR markers were scored as present (1) or absent (0) and simple matching similarity coefficients (Sneath and Sokal, 1973) were calculated for all pair-wise comparisons among 20 olive cultivars. Expected heterozygosity (*He*) and observed heterozygosity (*Ho*) were calculated according to the method of Nei (1973) using POPGEN32 software v.1.31 (Yeh et al., 1997). A dendrogram demonstrating the relative genetic relationship was generated using NTSYSpc version 2.11V (Exeter Software, Setauket, NY) (Rohlf, 2004) based on the unweighted pair-group method of arithmetic mean cluster analysis (UPGMA).

Tab. 1: Selected characteristics of olive cultivars from Croatia and Turkey used in this study.

Variety	Fruit mass (g)	Stone mass (g)	Fruit shape	Oil content (%)	Use of fruit	Origin
Domat	5.30	0.86	Oval	20.57	Table	Turkey
Uslu	3.53	0.52	Oval	21.50	Table	Turkey
Ayvalik	3.65	0.54	Cylindrical	24.72	Oil	Turkey
Gemlik	3.73	0.53	Oval	29.98	Table	Turkey
Tavsan Yuregi	6.08	0.83	Heart	20.20	Table	Turkey
Memecik	4.78	0.56	Oval	24.50	Table-Oil	Turkey
Istarska bjelica	3.09	0.40	Oval	23.80	Oil	Croatia
Buža muška	2.90	0.63	Ovoid	23.00	Oil	Croatia
Buža	4.38	0.53	Ovoid	20.05	Table-Oil	Croatia
Piculja	1.27	0.32	Ovoid	18.77	Oil	Croatia
Mezanica	3.00	0.59	Spherical	25,60	Table-Oil	Croatia
Bjelica Dubrovnik	2.97	0.50	Ovoid	24.57	Oil	Croatia
Murgulja	5.64	0.97	Spherical	18,02	Table	Croatia
Uljarica	2.73	0.65	Spherical	25.57	Oil	Croatia
Levantinka	4.50	0.52	Ovoid	20.00	Oil	Croatia
Oblica	5.05	0.8	Ovoid	22.10	Table-Oil	Croatia
Grčka	-	-	-	-	Oil	Croatia
Drobnica	2.18	0,49	Spherical	23.10	Oil	Croatia
Lastovka	2.80	0.48	Ovoid	24.00	Oil	Croatia
Unnamed (VLMD6)	-	-	-	-	Oil	Croatia

*Croatian cultivars were reported by Bakaric, P. (1995); Bakaric, P. (2002); Bencic, Đ. (2000); Bencic et al. (2009); Bencic et al. (2010) and Turkish cultivars were reported by Canozer (1991).

Results and discussion

Tab. 1 summarizes the data generated by the six SSR primer pairs in 20 olive cultivars from Croatia and Turkey. A total of 22 polymorphic alleles were identified. The number of polymorphic alleles ranged from 2 (UDO-43) to 5 (DCA-09), with an average of 3.6 fragments per primer. On the other hand, SSR locus DCA0-3 was excluded from our study because it was not polymorphic among the plant materials studied.

Expected heterozygosity (*He*) was the lowest as 0.14 at two loci (DCA-18) whereas it was the highest as 0.74 in GAPU-101 loci. Observed heterozygosity (*Ho*) was the highest in GAPU-101 (0.55). Except UDO-43, expected heterozygosity (*He*) was higher than the observed values (*Ho*) at all loci (Tab. 2).

According to SSR profiles of 20 autochthonous olive cultivars from Turkey and Croatia, two pairs of cultivars from Croatia ("Buža muška" and "Levantinka"; "VLMD6" and "Drobnica") were found to be synonyms. No synonyms were found among Turkish cultivars. In addition, synonyms were not observed between Turkish and Croatian cultivars.

The dendrogram derived from an UPGMA cluster analysis of the total 22 SSR markers is shown in Fig. 1. Three main distinct groups were observed in the dendrogram. Group I consisted of "Ayvalik" from Turkey and "Uljarica" from Croatia with 77% similarity ratio and both cultivars have been utilized for olive oil production.

Tab. 2: Simple sequence repeats (SSRs), no. of detected alleles, observed heterozygosity (Ho) and expected heterozygosity (He) of 6 SSR markers on 20 olive cultivars investigated.

SSR Primers	Number of alleles	Expected heterozygosity (*He*)	Observed heterozygosity (*Ho*)
DCA-05*	3	0.18	0.10
DCA-09	5	0.63	0.40
DCA-18	4	0.14	0.10
GAPU-71B**	4	0.68	0.35
GAPU-101	4	0.74	0.55
UDO-43***	2	0.45	0.50
Total	22		
Average	3.6	0.47	0.33

* developed by SEFC et al. (2000)
**developed by CARRIERO et al. (2002)
***developed by CIPRIANI et al. (2002)

Group II included 3 Turkish and 6 Croatian autochthonous cultivars ("Murgulja", "Tavsan Yuregi", "Domat", "Grčka", "Uslu", "Lastovka", "Levantinka", "Buža muška" and "Oblica"). In this

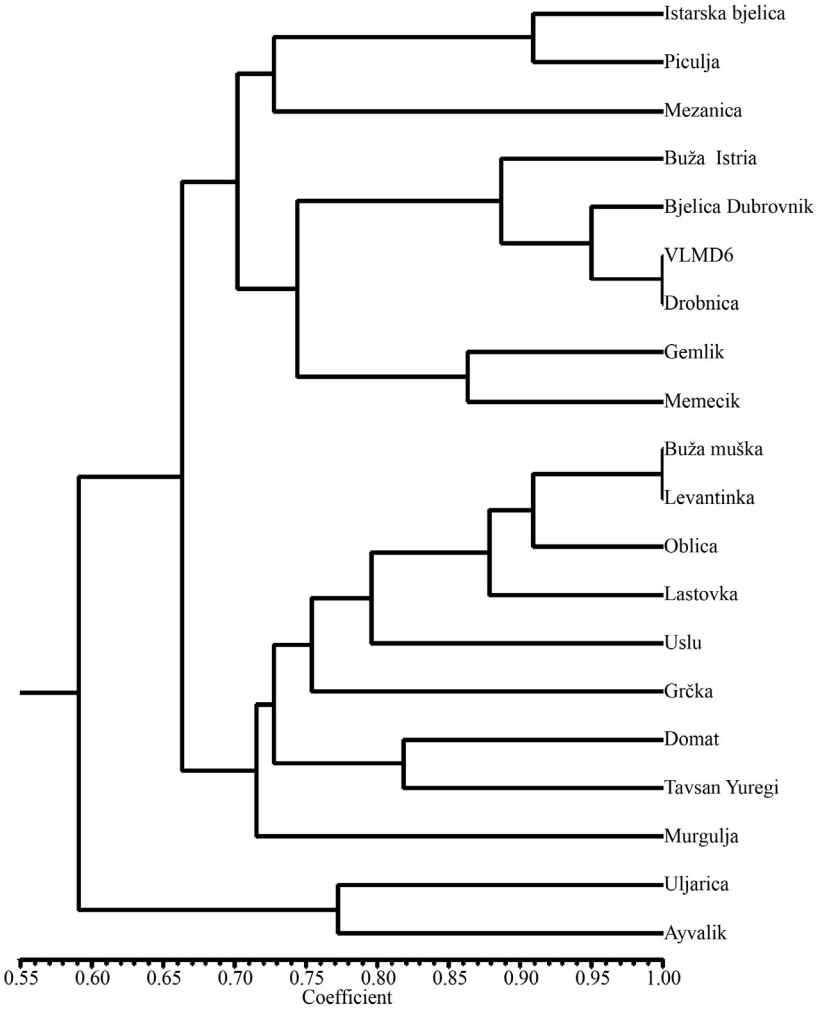

Fig. 1: The UPGMA dendrogram based on simple matching similarity matrix obtained using 22 SSR markers, illustrating the relative similarity among 20 olive cultivars from Croatia and Turkey.

Tab. 3: Similarity matrix among 14 Croatian and 6 Turkish olive cultivars based on "Simple Matching" coefficient.

	Istarska bjelica	Buža muška	Buža Istria	Meza-nica	Mur-gulja	Uljarica Piculja	Bjelica Dubrovnik	Oblica	Levan-tinka	Grčka	VLMD6	Drob-nica	Las-tovka	Ayvalik	Domat	Gemlik	Meme-cik	Tavsan Yuregi	Istarska bjelica
Buža muška	0.77																		
Buža Istria	0.64	0.68																	
Mezanica	0.68	0.64	0.68																
Murgulja	0.50	0.67	0.67	0.39															
Uljarica	0.59	0.45	0.68	0.55	0.39														
Piculja	0.91	0.68	0.64	0.77	0.44	0.68													
Bjelica Dubrovnik	0.77	0.73	0.86	0.64	0.61	0.73	0.77												
Oblica	0.77	0.91	0.68	0.55	0.78	0.45	0.68	0.73											
Levantinka	0.77	1.00	0.68	0.64	0.67	0.45	0.68	0.73	0.91										
Grčka	0.59	0.73	0.68	0.64	0.72	0.45	0.59	0.73	0.82	0.73									
VLMD6	0.83	0.78	0.89	0.61	0.67	0.72	0.78	0.94	0.78	0.78	0.72								
Drobnica	0.73	0.68	0.91	0.59	0.67	0.77	0.73	0.95	0.68	0.68	0.68	1.00							
Lastovka	0.68	0.91	0.77	0.64	0.72	0.55	0.68	0.73	0.82	0.91	0.73	0.83	0.77						
Ayvalik	0.64	0.59	0.64	0.50	0.50	0.77	0.64	0.68	0.59	0.59	0.59	0.83	0.73	0.68					
Domat	0.82	0.77	0.64	0.59	0.72	0.50	0.73	0.59	0.86	0.77	0.68	0.72	0.64	0.77	0.55				
Gemlik	0.73	0.77	0.73	0.68	0.61	0.59	0.64	0.77	0.68	0.77	0.68	0.94	0.82	0.77	0.73	0.64			
Memecik	0.77	0.64	0.59	0.73	0.44	0.55	0.68	0.64	0.55	0.64	0.55	0.78	0.68	0.64	0.59	0.68	0.86		
TavsanYuregi	0.64	0.68	0.73	0.50	0.72	0.59	0.55	0.68	0.77	0.68	0.68	0.72	0.73	0.68	0.45	0.82	0.64	0.59	
Uslu	0.55	0.77	0.64	0.59	0.72	0.41	0.55	0.59	0.77	0.77	0.77	0.72	0.64	0.86	0.64	0.73	0.73	0.68	0.64

group, "Murgulja" formed a subgroup and the rest of cultivars formed another subgroup within Group II. Croatian and Turkish cultivars distributed within Group II without any geographical isolation. Croatian cultivars, "Levantinka" and "Buža muška" showed 100% identical SSR banding profiles. Group III included "Gemlik", "Memecik", "Istarska bjelica", "Buža", "Piculja", "Mezanica", "Bjelica Dubrovnik", "Drobnica" and "VLMD6" cultivars. There were 3 subgroups within this cluster and Turkish cultivars "Gemlik" and "Memecik" clustered together but related to Croatian cultivars within Group III. There were 100% similarities between the last two Croatian cultivars within Group III.

Similarity matrix indicated that the "Ayvalik" and "Tavsan Yuregi" were the most distant cultivars among Turkish samples with 0.45 similarity ratio. The highest similarity (0.86) was observed between "Gemlik" and "Memecik" olive cultivars among Turkish samples. Considering Croatian samples, cultivars "Murgulja" and "Mezanica" was found the most distant from each other with a similarity ratio of 0.39. "Levantinka" - "Buža muška" and "Drobnica" - "VLMD6" had identical allelic SSR profile (Tab. 3).

We obtained a high level of polymorphism among olive cultivars in the present study. A high level of polymorphism in olive cultivars by using SSR markers was also reported previously (Carriero et al., 2002; Sarri et al., 2006; Muzzalupo et al., 2006; Gomes et al., 2009; Alba et al., 2009). In our study, the number of average polymorphic alleles per primers (3.6) was higher than obtained by Cipriani et al. (2002) and comparable to Carriero et al. (2002), Sarri et al. (2006) and Muzzalupo et al. (2006) and lower than those reported by Belaj et al. (2003), Poljuha et al. (2008), Alba et al. (2009) and Roubos et al. (2010). In this study, we used relatively few SSR loci; nevertheless, the most were highly polymorphic and therefore allowed unequivocal identification of all the plant material. Previously Gomes et al. (2009) and Roubos et al. (2010) also discriminated olive cultivars by using only six SSR loci. The SSR locus DCA-03 was excluded from our study because it was not polymorphic among the cultivars used. The marker UDO-43 showed the lowest and DCA09 showed the highest polymorphism in this study. In contrast, Belaj et al. (2003) and Alba et al. (2009) reported a high discrimination capacity of the UDO-43 marker. However, Alba et al. (2009) and Noormohammadi et al. (2009) also found high polymorphism with DCA-09 marker. Poljuha et al. (2008) found that the markers DCA-03, DCA-10 and DCA-16 were of high discrimination capacity among Istrian olive trees. Variations reported in the number of alleles in olive cultivars by different scientists may be related to variation in the loci studied as well as the number of genotypes and their localities (Lopes et al., 2004).

As indicated before some of Croatian cultivars had identical allelic SSR profile. Previously Poljuha et al. (2008) reported three synonym cultivars "Crna", "Karbonera" and "Karbuna" from Croatia by SSR analysis. Ercisli et al. (2011) reported synonymous names of "Ziraat" and "Gemlik"; "Isrange" and "Tuz" and "Patos" and "Yag" olive cultivars from the Black Sea region in Turkey. These results also indicat that synonym olive cultivars from Croatia might be renamed or misnamed in a new locality where those were cultivated.

Our results also highlight the genetic diversity of olive cultivars grown in Turkey and Croatia. It seems that the six SSR markers used in this study had high discriminating capacity for 20 olive cultivars. SSR markers have been previously used in genetic diversity and relationships studies in olive cultivars and most scientists conclude that SSR markers are a powerful tool for cultivar identification and analysis of genetic structure (Khadari et al., 2003; Belaj et al., 2003; Gomes et al., 2009).

As expected, the most closely related cultivars were within each gene pool ("Levantinka" and "Buža muška", "Drobnica" and "VLMD6" from Croatia with 100% identical SSR banding profiles and "Gemlik" and "Memecik" from Turkey with 85% similarity ratio (Fig. 1). The use of synonym or mislabeling is one of the most important problems in olive germplasm from different Mediterranean countries. Discrimination of synonymous cases in olive germplasm has also been reported by using SSR and other molecular markers (Muzzalupo et al., 2006; Gomes et al., 2009). No particular clustering was observed among cultivars from two countries and Turkish olive cultivars distributed in all three main clusters and were clustered with Croatian olive cultivars suggesting that Turkish and Croatian olive cultivars continue to be related. This result may be due to the germplasm exchange and selection for similar climatic

environments (e.g. selecting for similarly important adaptation genes). However, the divergence among Croatian cultivars was most likely caused by breeding for adaptation and cultivar improvement after the introduction of olive cultivars from Turkey and other related ancestral cultivars from Mediterranean countries. For Croatian olive cultivar improvement, it is possible to use those Turkish olive cultivars that are related to Croatian olive cultivars as parents to possibly add new alleles without introducing too much new genetic diversity, or the genetic diversity that is most closely related to previous/historical parents.

These results also indicate 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 based on their geographical origins. The result was similar to 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.

In conclusion, the SSR analysis was useful for the detection of genetic differences among the olive accessions from Turkey and Croatia. The outcome of this study could be useful for varietal survey and the construction of a database of olive cultivars in both Croatia and Turkey. Our results also suggest that older Turkish olive cultivars have not diverged greatly from the Croatian olive cultivars based upon the UPGMA dendrogram (Fig. 1).

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