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Genetic Similarity Among Tunisian Olive Cultivars and Two Unknown Feral Olive Trees Estimated Through SSR Markers

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Abstract We used eight informative microsatellite markers for fingerprinting and evaluation of genetic similarity among 15 Tunisian olive (*Olea europaea* L.) cultivars and two feral unknown trees named Soulela 1 and Soulela 2. Thirty-one alleles were revealed, and the number of alleles per SSR varied from 2 (UDO12) to 6 (GAPU71A). Cluster analysis grouped cultivars into three main clusters. The two unknown varieties could not be reliably classified into any of these cultivar groups. SSR analysis indicated the presence of three erroneous denominations of cultivars. We resolved two synonymy cases (Zalmati and Chemlali; Rkhami and Chetoui) and one case of homonymy (Chemlali Tataouine). Genetic analyses of DNA extracted from leaves, oils, and embryos of the two unknown cultivars and the two major Tunisian olive cultivars (Chemlali and Chetoui) were also studied. We conclude that the reliable identification of these two feral cultivars needs to be addressed by a larger set of markers.

Keywords *Olea europaea* L. · Microsatellite markers · Dendrogram · Similarity

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Introduction

Olive (*Olea europaea* L.) is one of the oldest cultivated plants and is an important oil-producing crop in the Mediterranean Basin. Trees are extremely long-lived (up to 1,000 years) and tolerant to drought, salinity, and almost total neglect and have been reliable producers of food and oil for thousands of years. There are two forms: cultivated olive (var. *europaea*) is clonally propagated by cuttings and grafting, and wild olive or oleaster (var. *sylvestris*) is reproduced from seeds (Khadari et al. 2008). Archeological data suggest that cultivated olive was derived from oleasters through vegetative multiplication of individuals presenting interesting traits such as fruit size and oil content (Khadari et al. 2008).

The olive tree is a species with a high degree of cross-pollination that leads to high levels of heterozygosity and genetic polymorphism (Angiolillo et al. 1999; Rallo et al. 2000). Over many centuries, most olive cultivars have been derived by random crosses or mutation. The majority of varieties are highly localized, but there are a few cultivars dispersed over widespread areas. The development of locally specific varietal populations was carried out by sexual reproduction, whereas other cultivars were established and maintained by vegetative means (Lumaret et al. 2004; Breton et al. 2006).

Olives have great commercial, economic, and social importance in Tunisia. In addition, Tunisia is a major producer and exporter of olive oil. The major varieties of olive oils in Tunisia are Chetoui and Chemlali.

Currently, there is much confusion in the identification of olive cultivars. Identification of cultivars is considered a major requirement because of the longevity of the crop and the need to improve efficiency in growing olives and extracting their oil. It is crucial that cultivars be identified using powerful techniques. In the past, olive cultivars were categorized by morphological traits, including tree, fruit, and leaf characteristics (Grati-Kamoun 1999), which are influenced by environmental factors. Reliance on phenotypic characters has possibly led to great confusion and uncertainty about the current classification of olive varieties in many countries. Recently, molecular techniques based on DNA markers have been shown to provide powerful tools for genetic analysis of olive cultivars (Belaj et al. 2001; Busconi et al. 2003; Pafundo et al. 2005). Among these markers, microsatellites (also known as SSR) have become the most popular in many species (Qin et al. 2012; Guo et al. 2012), and in olive cultivars they have a high potential for resolving issues of synonymies, homonymies, and misnamings (Taamalli et al. 2006; Rekik et al. 2008; Muzzalupo et al. 2009). Moreover, recent studies have shown that SSRs are reliable markers for tracing olive oil (Muzzalupo and Perri 2002; Testolin and Lain 2005; Ben Ayed et al. 2009, 2012).

In this work, we used eight SSR primers to assess the genetic diversity of Tunisian olive cultivars and to classify two unknown olive trees (Soulela 1 and Soulela 2) found in northeastern Tunisia by analyzing both extracted from leaves, oil, and embryos (stones).

Materials and Methods

Plant Material

For this study, we chose 17 Tunisian olive tree cultivars from different geographic regions of the country from north to south (Ben Ayed et al. 2012). Of those, 15 cultivars corresponded to the major and widely distributed cultivar groups and two others were of unknown filiations in the northeastern region of the country (Hawaria, Nabeul). They were arbitrarily named Soulela 1 and 2 by their discoverers.

DNA Extraction

Young leaves were frozen and powdered under liquid nitrogen using a mortar and pestle. Total DNA was extracted from leaves using the CTAB method, followed by two purification steps (Rekik et al. 2008; Ben Ayed et al. 2009).

DNA was extracted from oil using the QIAamp DNA stool kit (Qiagen) (Ben Ayed et al. 2009).

DNA was extracted from five embryos with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

Microsatellite Markers

Eight microsatellite markers were used in this study (Table 1). Three markers (DCA1, DCA3, DCA4) were from the primer set designed by Sefc et al. (2000), three (GAPU59, GAPU71A, GAPU71B) were from Carriero et al. (2002), and two (UDO12, UDO09) were from Cipriani et al. (2002). They were selected for their high polymorphism in many olive cultivars (Muzzalupo et al. 2009) and in Tunisian cultivars (Rekik et al. 2008; Ben Ayed et al. 2009, 2012).

PCR and Capillary Sequencer

The PCR was performed in a 15 µl volume consisting of 20 ng genomic DNA from young leaves, 2 mM $MgCl_2$, 0.05 mM each dNTP, 0.1 µM forward primer (labeled with FAM fluorescent dye), 0.4 µM reverse primer, 0.5 U Go Taq Flexi DNA polymerase (Promega), and 1× buffer Go Taq. The PCR amplifications were performed on a 96-well Veriti thermal cycler (Applied Biosystems). Conditions for the DCA1, DCA3, GAPU59, GAPU71B, and UDO12 primers were 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 45 s; then 72°C for 10 min. For the GAPU71A, UDO09, and DCA4 primers conditions were 95°C for 5 min; 35 cycles of 95°C for 30 s, 57°C for 45 s, and 72°C for 45 s; then 72°C for 10 min.

Five microliters of PCR products was mixed with 0.3 µl of marker (420 bp) and 14.7 µl deionized H_2O, centrifuged at 2,000 rpm for 1 min, denatured at 94°C for 3 min, cooled in ice and analyzed on a 3130XL Genetic Analyzer capillary sequencer (Applied Biosystems).

Table 1 Genetic diversity observed by typing 17 olive samples using 8 simple sequence repeat markers

Locus	Primer sequence (5′ → 3′)	Repeat motif	Tm (°C)	Reference	Size of amplicon (bp)	N_a	Heterozygosity		*PD*	*r*
							Observed	Expected		
DCA1	F: (FAM)CTCTGAAAATCTACACTCACATCC R: ATGAACAGAAAGAAGTGAACAATGC	$(GA)_{22}$	50	Sefc et al. (2000)	222–244	5.0	0.764	0.775	0.790	0.006
DCA3	F: (FAM)CCAAGCGGAGGTGTATATTGTTAC R: TGCTTTTGTCGTGTTTGAGATGTTG	$(GA)_{19}$	50	Sefc et al. (2000)	247–269	3.0	0.882	0.576	0.279	−0.194
DCA4	F: (FAM)TTAACTTTGTGCTTCTCCATATCC R: AGTGACAAAAGCAAAAGACTAAAGC	$(GA)_{16}$	57	Sefc et al. (2000)	208–222	4.0	0.214	0.434	0.595	0.153
GAPU59	F: (FAM)CCCTGCTTTGGTCTTGCTAA R: CAAAGGTGCACTTTCTCTCG	$(CT)_{10}$	50	Carriero et al. (2002)	210–228	5.0	0.882	0.712	0.798	−0.099
GAPU71A	F: (FAM)GATCATTTAAAATATTAGAGAGAGAGA R: TCCATCCATGCTGAACTT	$(AG)_6$	57	Carriero et al. (2002)	117–140	6.0	0.764	0.645	0.748	−0.072
GAPU71B	F: (FAM)GATCAAAGGAAGAAGGGGATAAA R: ACAACAAATCCGTACGCTTG	$(AG)_7(AAG)_8$	50	Carriero et al. (2002)	155–166	3.0	0.941	0.777	0.736	−0.091
UDO09	F: (FAM)TTGATTTCACATTGCTGACCA R: CATAGGGAAGAGCTGCAAGG	$(AG)_{16}$	57	Cipriani et al. (2002)	97–115	3.0	0.214	0.434	0.595	−0.197
UDO12	F: (FAM)TCACCATTCTTAACTTCACACCA R: TCAAGCAATTCCACGCTATG	$(GT)_{10}$	50	Cipriani et al. (2002)	149–179	2.0	0.941	0.653	0.696	−0.174
Total	–	–	–	–	–	31.0	–	–	–	–
Median	–	–	–	–	–	3.875	0.700	0.625	0.654	–

Tm annealing temperature for PCR amplification, N_a number of alleles, *PD* power of discrimination, *r* probability of null alleles

Data Analysis

The alleles detected for each microsatellite were recorded in a data matrix as present (1) or absent (0), with each allele representing a band. Allele frequencies and heterozygosities (both observed and expected under Hardy–Weinberg equilibrium) were calculated using the GDA program (Weir 1996). The power of discrimination (*PD*) was calculated for each SSR locus according to Brenner and Morris (1990):

$$PD = 1 - \sum_{i=1}^{g} p_i^2$$

where p_i is the frequency of the ith genotype for the locus and the sum is over all genotypes.

The combined power of discrimination over all loci was then calculated as:

$$1 - \prod_{l=1}^{L} (1 - PD_l)$$

where index l is relative to the loci and the product is taken for all loci. The probability of null alleles was estimated according to the formula of Brookfield (1996):

$$r = (H_e - H_o) / (1 + H_e).$$

The data matrix was converted into a matrix of similarity (*S*) values using Jaccard's coefficient (Jaccard 1908). For a pair of two cultivars, i and j, this coefficient is calculated as:

$$S_{ij} = \frac{n_{ij}}{n_{ij} + n_i + n_j}$$

where n_i is the number of bands present in cultivar i and absent in cultivar j, n_j is the number of bands present in j and absent in i, and n_{ij} is the number of bands shared by the two cultivars.

A tree is then inferred using the unweighted pair group method with an arithmetic average clustering algorithm. All analyses were done using NTsysPC version 2.1 (Rohlf 1999).

Results and Discussion

SSR Characterization and Discrimination Capacity

We observed 31 alleles across the eight markers, with the number of alleles per locus ranging from 2 (UDO12) to 6 (GAPU71A) (Table 1). The lowest allelic frequency (0.029) was observed for 238 bp of locus DCA1 found in cultivar Zarrazi Zarzis (Table 2). The most frequent (0.821) and therefore less polymorphic allele is reported to be 149 bp of locus DCA4. In effect, several SSR markers are currently available for genetic analysis in olive species (Sefc et al. 2000; Carriero et al. 2002;

Table 2 Allele size and frequency for eight SSR loci in 17 olive genotypes

Locus	Number of alleles	**Allele length (bp)** Frequency					
DCA1	5	**222**	**230**	**232**	**234**	**238**	
		0.294	0.323	0.058	0.294	0.029	
DCA3	3	**247**	**255**	**259**			
		0.441	0.470	0.088			
GAPU59	4	**208**	**212**	**214**	**222**		
		0.264	0.500	0.147	0.088		
GAPU71A	5	**210**	**212**	**214**	**226**	**228**	
		0.117	0.264	0.382	0.117	0.117	
GAPU71B	6	**117**	**120**	**122**	**123**	**126**	**140**
		0.117	0.294	0.176	0.058	0.058	0.294
UDO12	3	**155**	**157**	**166**			
		0.352	0.470	0.176			
UDO09	3	**97**	**101**	**115**			
		0.558	0.294	0.147			
DCA4	2	**149**	**179**				
		0.821	0.178				

Bold values represent allele length

Cipriani et al. 2002) and have been shown to be valuable tools for genetic identification. In our study, high polymorphism was detected, and the average number of alleles per locus was over 3.875 polymorphic alleles per SSR primer, which is lower than that obtained by Carriero et al. (2002), averaging 5.7 alleles over 10 loci in 20 olive varieties, and Rallo et al. (2000), averaging 5.2 alleles over 10 loci in 46 olive cultivars. However, our level of polymorphism is comparable to that reported by Cipriani et al. (2002). The high degree of polymorphism in the alleles of SSR markers corroborates the high genetic variation of Tunisian olive cultivars.

Overall, observed heterozygosity values per marker ranged from 0.214 to 0.941, with an average value of 0.7. The values of observed heterozygosities are higher than those of expected heterozygosity for all loci except DCA4 and DCA1 (Table 1). Locus DCA4 has a high probability of null alleles ($r = 0.153$), which might indicate a genotyping problem. The average heterozygosity detected by SSRs in this paper was 0.7 observed (0.62 expected). In general, we found high values of observed and expected heterozygosity for most of the SSR markers, and the observed heterozygosity scored higher than expected in five of the eight loci. A high level of heterozygous varieties and higher observed values were previously described for other SSR markers (Diaz et al. 2006).

The *PD* ranged from 0.279 (DCA3) to 0.798 (GAPU59), averaging 0.65 (Table 1) and yielded a combined value of 0.99997, which means that the probability of finding two cultivars with the same genotype combination for the eight SSR markers is about 1 in 3,000, indicating the high discrimination of the

marker system used. The *PD* was slightly lower than that found by Rekik et al. (2008) in 20 Tunisian cultivars (0.71) but significantly higher than those reported by Cipriani et al. (2002) in 12 Italian cultivars (0.44) and by Muzzalupo et al. (2006) in 39 Italian cultivars (0.38). The four best loci were DCA01, GAPU59, GAPU71A, and GAPU71B. The *PD* values of the GAPU markers were consistent with that found by Rekik et al. (2008), but this is higher than that found by Muzzalupo et al. (2010).

Patterns of Genetic Diversity

The cluster analysis showed three distinct groups, identified by cutting the dendrogram (Fig. 1) at a genetic similarity (*GS*) reference value of 0.72. The first cluster, containing nine cultivars at the top of the dendrogram, can be identified as the Chemlali cluster. It includes all of the Chemlali cultivars growing in different regions of the country and the Zalmati cultivar, all of which have identical genotypes, as well as Soulela 1, from the Hawaria region. The second cluster contains three morphological fruit cultivars (Oueslati, Zarrazi, and Chemlali Tataouine). The third group includes Chetoui cultivars from three regions and the Rkhami cultivar, all of which have the same genotype. The second unknown variety (Soulela 2) is included in the third cluster. This last group is more heterogeneous and contains three cultivars of similar fruit size.

The unknown cultivar Soulela 1 showed the highest similarity with the Chemlali cluster ($GS = 0.54$); the other, Soulela 2, was most similar to the Chetoui cluster

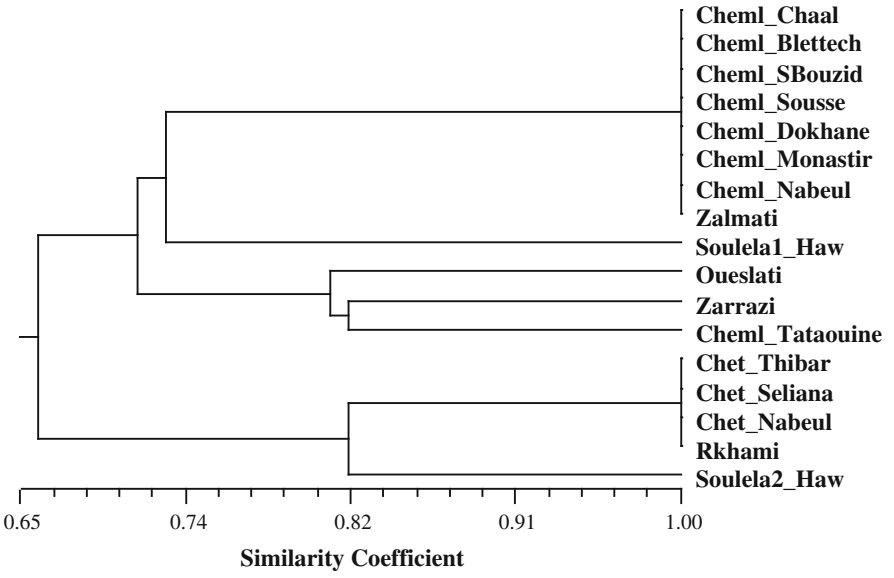

Fig. 1 Localization of two unknown olive cultivars, Soulela 1 and 2. Dendrogram based on SSR data using Jaccard's similarity coefficient and UPGMA clustering method

($GS = 0.67$). According to the dendrogram (Fig. 1), three clusters were detected, and the grouping was globally consistent with the phenotypic characteristics of the cultivars but not correlated to the geographic origin. This finding was also reported by Besnard et al. (2001) and Grati-Kamoun et al. (2006). Conversely, Rao et al. (2009), who used AFLPs to discriminate between several olive cultivars located in Campania (south of Italy), assessing suspected cases of synonyms and homonyms and evaluating their relationships with morphological markers, concluded that the morphological and molecular data yielded different hierarchical patterns.

Using eight microsatellite loci, we were able to identify a case of homonymy between the Chemlali (all seven identical cultivars) and Chemlali Tataouine cultivars, which differed at seven SSR alleles. This result was supported by differences observed between these two cultivars at the morphological and chemical levels. The same result was produced by Grati-Kamoun et al. (2006) with AFLP markers. We think that Chemlali Tataouine was so-named because of the morphological similarity of the fruit and leaves to the Chemlali.

The maximum genetic similarity between nonsynonymous varieties ($GS = 1$) was found between the Chemlali cluster cultivars and the Zalmati cultivar. The high similarity between Zalmati and Chemlali is well established in both morphological and chemical characteristics and is even apparent in our previous molecular data (Grati-Kamoun et al. 2006; Rekik et al. 2008). However, this is the first time that we show, using high-resolution genotyping, that Zalmati is genetically identical to Chemlali and that the differences found in our previous work are due to genotyping errors.

Another case of original synonymy revealed by our study is that between the Rkhami and Chetoui cultivars. These cultivars, growing in northern Tunisia, have very similar morphological and chemical characteristics. By analyzing oil composition and characteristics, we found that Rkhami has a profile similar to Chetoui, within the range of environmental variation of Chetoui oil (unpublished data).

Comparison of Embryo, Oil, and Leaf Profiles from the Two Unknown Cultivars

When we compared the profiles of genomic DNA extracted from Chemlali with those of Soulela 1 and the Chetoui cultivars with those of Soulela 2 (Table 3), we found that at least one allele was identical between Soulela 2 and Chetoui (the same genotype for marker UDO12) and between Soulela 1 and Chemlali (the same genotype for markers UDO09 and DCA3). When comparing the profiles of DNA obtained from embryos of the two unknown cultivars (Soulela 1 and Soulela 2) and the profiles of genomic DNA extracted from leaves from two major olive cultivars in Tunisia (Chemlali and Chetoui), we showed that for some markers (GAPU71A and UDO12) embryo DNA from Soulela 1 has one identical allele with genomic DNA from the Chetoui cultivar. It is also worth noting that alleles from the Chemlali cultivar were detected in embryo samples from Soulela 2 for markers GAPU71A, GAPU71B, and UDO09 (Table 3). The two unknown cultivars, discovered in an isolated area in the northeast, could not be reliably attached to any group based on our data. Although there is a clear genetic similarity between Soulela 1 and Chemlali cultivars on one hand and between Soulela 2 and Chetoui

Table 3 SSR genotypes obtained from oil, leaves, and embryos of four olive varieties

Variety_DNA source	Locus							
	DCA1	DCA3	GAPU59	GAPU71A	GAPU71B	UDO12	UDO09	DCA4
Chemlali_leaves (Sfax)	230/234	247/255	208/212	212/214	120/140	155/157	97/101	149/149
Chemlali_embryos (Sfax)	230/234 + *222* + *242*	247/255 + **259** + *269* + *261*	208/212 + *214* + **222**	212/214	120/140	155/157	97/101 + *115*	149/149
Chemlali_oil (Sfax)	230/234	247/255 + **259**	208/212 + **218** + **220** + **222**	212/214	120/140 + **126** + **123** + **117**	155/157 + **166**	97/101	149/149
Chetoui_leaves (Siliana)	222/222	247/255	212/214	210/228	117/122	157/166	97/***101***	149/179
Chetoui_embryos (Siliana)	222/222 + *230*	247/255 + *269*	212/214 + *208* + **218**	210/228 + *214*	117/122 + *126*	*155*/166 + *163*	97/115	147/149
Chetoui_oil (Siliana)	222/222	247/255 + **261** + **263**	212/214 + **218** + **222**	210/228	117/122 + **140**	157/166	97/115	149/179 + **147**
Soulela1_leaves	232/234	247/255	222/222	226/226	120/123	157/166	97/101	–
Soulela1_embryos	232/234	247/255	222/222	*210/210*	120/123	157/166 + *163*	97/101	*157/183*
Soulela1_oil	232/234	247/255	222/222	–	120/123	157/166	97/101	–
Soulela2_leaves	222/232	255/255	212/222	226/226	122/122	157/166	97/115	149/149
Soulela2_embryos	222/232	247/255	212/222	*210/214*	120/122	157/166 + *163*	97/115 + **101**	149/149 + *157/183*
Soulela2_oil	222/232	255/255	212/222	–	122/122	157/166	97/115 + **105** + **101**	–
Allele interval (bp)	222–242	247–263	208–222	210–228	117–140	155–166	97–115	147–183

Italic allele present in the embryos and not in the leaves and oil

Bold allele present in the oil and not in the leaves

Bold italic allele present in the leaves and not in the embryos and oil

cultivars on the other, the level of similarity is quite low (0.54 and 0.67, respectively). Features shared by these two cultivar pairs, including similar fruit size and chemical characteristics (unpublished data), suggest that it is very likely that Soulela 1 and Soulela 2 have a common ancestry with Chemlali and Chetoui, respectively. Taking into account all the obtained results, two hypotheses can be suggested: either these trees are hybrid genotypes resulting from cross pollination between cultivars currently in use, or they are unique specimens of ancestral trees brought by conquerors (Phoenicians, Romans). These hypotheses can be supported by the age of the trees, estimated at about 2,000 years.

In conclusion, our work shows that SSR markers can be successfully used to describe genetic diversity in olive cultivars. Particularly, we resolved two synonymy cases (same genotype having two different denominations) and one case of homonymy (two different denominations applied to the same genotype). We recommend the synonymy of Zalmati and Chemlali and that of Rkhami and Chetoui. We also suggest that Chemlali Tataouine is in fact not a Chemlali cultivar. Moreover, the use of SSR markers may allow identification of unknown varieties, although we were unable here to resolve definitely the case of two feral trees. In addition, we primarily solved the identification of the two unknown cultivars Soulela 1 and Soulela 2, using DNA extracted from leaves, oil, and embryos.

We also found the same phenomenon that we reported previously (Ben Ayed et al. 2009, 2012), in that DNA extracted from oil samples could indicate the variety of origin of unknown olive oil samples (from monovarietal oil). We will use high-throughput genotyping techniques with SSR or SNP markers to confirm our findings and provide automated tools for identifying olive cultivars and oil authenticity.

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