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Characterization and Identification of Tunisian Olive Tree Varieties by Microsatellite Markers

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Abstract. **In the Mediterranean basin, a large number of olive varieties are present. This poses a series of problems concerning germplasm characterization and management. In addition, there is a problem arising from the existence of homonyms and synonyms. This makes cultivar identification very difficult and complex. Microsatellites or simple sequence repeat (SSR) are locus-specific codominant markers showing a high degree of polymorphism and multiple alleles per locus. Their high informativeness makes them the markers of choice in genetic diversity studies. This work presents the results of molecular characterization and identification of 20 Tunisian olive varieties using 10 SSR markers. All the SSR amplification products were sequenced to determine the number of repeats and the range of allele size. The number of alleles per SSR varied from three to six and the average heterozygosity rate ranged from 30% to 95%. Hierarchical classification of varieties base on similarity measures and clustering was globally consistent with the grouping of varieties by end use and phenotypic characteristics. The result is that varieties having the same name were found to have a clonal relationship. Paternity analysis showed also clone relationships between varieties not known to be related.**

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In Tunisia, olive (*Olea europaea* L.) is cultivated throughout the country. Olive farms cover more than one-third of arable land producing 6.0% of the world's olive production and contributing 45.0% of food export receipts, 4.5% of total exports, and 10.0% of the total agricultural production value. The Tunisian olive grove lands, although dominated by two major varieties, 'Chemlali' in the South and the Center and 'Chetoui' in the North, is rich in cultivars. Many of these cultivars have been characterized by pomological and chemical traits (Grati Kamoun et al., 2000; Grati Kamoun and Khlif, 2001; Mehri et al., 1997) with isozyme markers (Grati Kamoun et al., 1992, 1999, 2002) and more recently with amplified fragment length polymorphism markers (Grati Kamoun et al., 2006; Taamalli et al., 2006) and simple sequence repeat (SSR) markers (Taamalli et al., 2006, 2007). However, there is still a need for better genetic diversity assessment and varietal identification using high throughput marker technologies such as SSR markers.

Morphological and biometrical characters have been widely used to describe olive germplasm (Grati-Kamoun et al., 1999, 2000; Lombardo et al., 2003, 2004; Ruby, 1918). Recently, biochemical and molecular markers have been used to get better insight into the diversity of olive genetic resources. The first technique to be used was isoenzymes (Grati Kamoun et al., 1992, 2002; Lumaret et al., 2004; Perri et al., 1995; Trujillo and Rallo, 1995), which were shown to be very useful for varietal identification of olive. However, the lack of specificity of isoenzymes and their sensitivity to environmental and developmental factors have limited the widespread use of this technique for routine genotypic identification (Zhang et al., 1999). With the advent of molecular techniques, several types of DNA markers have been used in genetic diversity assessment of olive cultivars. The first technique to be used was randomly amplified polymorphic DNA because of its simplicity and low cost (Besnard et al., 2001; Belaj et al., 2002; Bogani et al., 1994; Bronzini de Caraffa et al., 2002; Fabbri et al., 1995; Muzzalupo et al., 2007a; Perri et al., 2002; Wiesman et al., 1998). The second type of DNA markers to be used was amplified fragment length polymorphism (AFLP) (Angiolillo et al., 1999; Bandelj et al., 2004; Belaj et al., 2003; Grati Kamoun et al., 2006; Montemurro et al., 2005; Sensi et al., 2003). More recently, several microsatellites have been isolated from the olive tree. Currently, ≈106 SSR markers are available and have been characterized on many olive cultivars worldwide (Carriero et al., 2002; Diaz et al., 2006; Rallo et al., 2000; Sarri et al., 2006; Sefc et al., 2000). This technique has gained interest and has become the standard approach to study genetic diversity of olive germplasm and is also used for the development of linkage maps (Wu et al., 2004).

SSR markers are easily amplified by polymerase chain reaction (PCR) and are highly reproducible among different laboratories. Almost all reports of cultivar identification using SSRs assessed differences in lengths of amplified alleles (Bandelj et al., 2002, 2004; Belaj et al., 2004; Breton et al., 2006; De La Rosa et al., 2004; Hess et al., 2000; Khadari et al., 2003; Muzzalupo et al., 2006a, 2008a, 2008b; Sajjad et al., 2006; Taamalli et al., 2006, 2007). More recently, it was reported that DNA sequence analysis of microsatellites enhances the

efficiency of classification and identification of Italian olive cultivars (Muzzalupo et al., 2006b). In fact, alleles with similar or even identical lengths may have different sequences. The presence of sequence variants within microsatellites, including single nucleotide polymorphisms, could be particularly useful in the development of molecular markers for characterization of germplasm (Reale et al., 2006).

In a recent study, Taamalli et al. (2006) investigated the diversity of 25 Tunisian olive cultivars using 10 SSR markers among those proposed by Sefc et al. (2000). Another paper (Taamalli et al., 2007) used 11 SSR markers to study diversity within the two major cultivars (or groups of synonymous cultivars): Chemlali and Chetoui. However, they did not address in deep details the problem of homonymy (cultivars having the same name but that are genetically different) nor synonymy (cultivars having different names but that are genetically the same).

In this article, we used 10 microsatellite markers to study 20 Tunisian olive cultivars of major commercial interest. The objective of the present study was the first to assess the potential of SSR markers to differentiate a number of Tunisian olive cultivars and second to address the problem of synonymy and homonymy in major cultivars.

Materials and Methods

Plant materials. Twenty Tunisian olive tree cultivars were selected from different geographical regions of the country from north to south (Table 1; Fig. 1). Six cultivars were selected from Gafsa region, five from Zarzis, four from Sfax, three from Tunis, one from Kairouan, and one from Siliana region. This includes 12 olive oil varieties having generally small fruit size (1.2 to 2.8 g), three table olive varieties (6.5 to 10.0 g), and five varieties with both end uses (3.0 to 4.0 g; Table 1).

Total DNA was extracted from young leaves of 20 *Olea europaea* cultivars (Table 1). The DNA extraction protocol described by Fabbri et al. (1995) was modified as follows: 0.5 g plant material were ground in liquid nitrogen and incubated in 2 mL CTAB buffer (100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.07 g PVP, 7 μL β-mercatoethanol) for 1 h at 65 °C. After centrifugation, the supernatant was extracted twice with chloroform-isoamyl alcohol (24:1; by volume) and DNA was precipitated from the recovered aqueous phase 0.6 volumes isopropanol. The pellet was resuspended in 1 mL ddH_2O and proteins were precipitated with 0.5 volumes 7.5 M ammonium acetate. After ethanol precipitation of the DNA, the pellet was resuspended in 200 μL ddH_2O, an RNase A digest with 50 $\mu g \cdot mL^{-1}$ and a second protein precipitation. The supernatant was extracted with phenol–chloroform–isoamyl alcohol (25:24:1; by volume). DNA was precipitated from the aqueous phase with ethanol, washed, and resuspended in 200 μL ddH_2O.

Table 1. List of the olive accessions analyzed from the Tunisian olive germplasm collection.

Cultivar name	Abbreviation	Growing region[z]	End use	Avg fruit wt (g)
Beldi	Beldi	Gafsa	Both	3.90
Besbessi	Besbessi	Tunis	Table olive	10.00
Chemcheli	Chemcheli-S	Sfax (Taous[y])	Both	3.00
Chemcheli	Chemcheli-G	Gafsa	Both	3.00
Chemlali ontha	Chem-ontha	Zarzis	Oil	1.00
Chemlali	Chemdok	Sfax (Dokhane[y])	Oil	1.07
Chemlali	Chem-Sfax	Sfax (Sfax)	Oil	1.07
Chemlalizarzis	Chem-Zarzis	Zarzis	Oil	0.80
Chetoui	Chetoui-Sfax	Sfax	Oil	2.80
Chetoui	Chetoui-BA	Tunis	Oil	2.80
Chetoui	Chet-ML	Siliana	Oil	2.80
Fougi	Fougi	Gafsa	Both	4.00
Horr	Horr	Kairouan	Oil	1.20
Jemribouchouka	Jemri-Bou	Gafsa	Oil	1.80
Lguim	Lguim	Gafsa	Oil	1.60
Meski	Meski	Tunis	Table olive	6.50
Toffehi	Toffehi	Zarzis	Oil	2.00
Tounsi	Tounsi	Gafsa	Table olive	9.00
Zalmati	Zalmati	Zarzis	Oil	1.30
Zarrazi	Zarrazi	Zarzis	Both	3.00

[z]Department from which the cultivar tree was sampled.
[y]Dokhane is located 20 km north of Sfax town and Taous is located ≈20 km east of Sfax town.

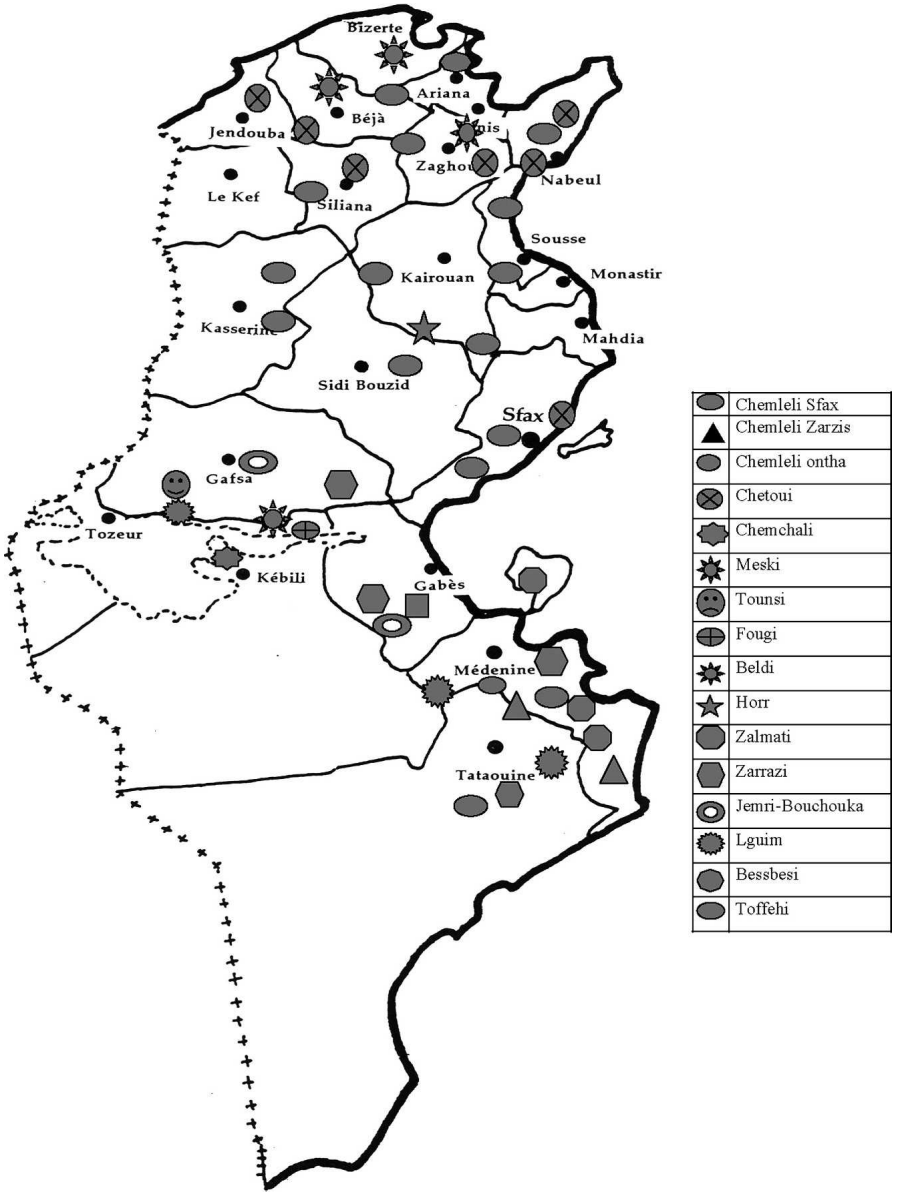

Fig. 1. Map of geographic origin of cultivars used in this study.

DNA was quantified by H33258 dye incorporation detected by a Hoefer DyNA Quant®200 fluorometer (Amersham Pharmacia Biotech, Milan, Italy). Genomic DNA was stored undiluted in TE 1 × pH 8.0 (10 mM Tris, 1 mM EDTA) at –20 °C.

Microsatellite markers. Ten published microsatellite markers were used in this study. Four markers (GAPU59, GAPU71A, GAPU71B, GAPU103A) from the primer set designed by Carriero et al. (2002), four markers (UDO03, UDO12, UDO28, UDO39) from Cipriani et al. (2002) and two markers (DCA9, DCA18) from Sefc et al. (2000) were selected. These markers were in fact highly polymorphic and very informative in Italian olive cultivars (Muzzalupo et al., 2006a, 2006b, 2008a, 2008b; Sajjad et al., 2006). The majority of the markers were of the dinucleotide repeat type (Table 2).

Polymerase chain reaction amplification and genotyping. SSR amplification was carried out as described by Muzzalupo et al. (2006a) using primers given in the original publications.

PCR products were analyzed with a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) by using DNA 500 LabChip® kits (Burns et al., 2003; Muzzalupo et al., 2007b) providing an estimation of the length of any amplified product.

Sequencing of polymerase chain reaction products. The PCR product were run in 4% agarose gels (FMC BioProducts, Vallensbaek Stand, Denmark) in TBE 1× buffer in the presence of ethidium bromide (1 $\mu g \cdot mL^{-1}$) at 100 V for 5 h. Amplified bands were cut out of the gel and purified using the QIAquick Gel Extraction kit (Qiagen Spa, Milan, Italy).

The SSR analyses were checked all alleles by sequencing all the SSR amplification products. In fact, all the samples examined in this article were sequenced and the allele size and the number of repeat units was established for every sample. The procedure for sequence analysis was carried out as described by Muzzalupo et al. (2006b).

Data analysis. The alleles detected for each microsatellite were recorded into a data matrix of presence (1) and absence (0) of bands (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 overall genotypes. The combined power of discrimination overall loci was then calculated as $1 - \prod_{l=1}^{L}(1 - PD_l)$ where index l is relative to the L loci and the product is taken for all loci. The probability of null alleles was estimated according to the formula of Brookfield (1996): $r = (He - Ho)/(1 + He)$.

The data matrix was converted into a matrix of similarity (S) values using Jaccard 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 i and absent in 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 cultivars i and j.

A tree was then inferred using the unweighted pair group method using an arithmetic average clustering algorithm. All analyses were done using NTSysPc program version 2.1 (Rohlf, 1999).

A likelihood-based parentage analysis was performed using FaMoz software (Di Vecchi Staraz et al., 2007; Gerber et al., 2003) to find potential parent–offspring or sibling relationships among cultivars. We calculated exclusion and identity probabilities based on the allele frequencies of the 10 microsatellites. The most likely parents were identified based on the log of the odds ratio scores (LOD scores) and 10,000 offsprings were simulated to determine the threshold value of the LOD score for parentage assignment. Simulations, calculations, and tests were done considering no genotyping errors. Because genotypes represent adult trees growing in different regions, the parent–offspring relationship can be difficult to distinguish from sibship. The relationship between individuals was further analyzed using ML-Relate software (Kalinowski et al., 2006). For each pair of individuals, maximum likelihood estimates of relatedness (r) were computed (Wagner et al., 2006). The log likelihood of four relationships (unrelated, half sibs, full sibs, and parent–offspring) was then calculated for all pairs of individuals and 10,000 random genotypes were used to test for the significance of the relationship at the 5% level.

Results and Discussion

Marker characteristics. Ten microsatellite markers were used to assess the genetic diversity of the 20 Tunisian varieties. We improved the specificity of this analysis by sequencing of all amplicons so that the exact number of repeat units was established. A total of 43 alleles were found for the 10 loci with an average of four alleles per locus ranging from three for DCA18 to six for UDO39 (Table 2). The observed heterozygosity ranged from 0.30 to 0.95 (average, 0.625). The PD ranged from 0.63 (GAPU71B) to 0.82 (UDO03) with an average value of 0.71. This is higher than that found by Cipriani et al. (2002) in 12 Italian cultivars (0.44) and by Muzzalupo et al. (2006b) on 39 Italian cultivars (0.38). Markers UDO12, UDO28, and UDO39 have high levels of heterozygosity and a PD value consistent with that found in other studies (Cipriani et al., 2002; Muzzalupo et al., 2006b). Among the GAPU markers, GAPU71B has a very high heterozygosity, whereas the two DCA markers exhibit low heterozygosity (Table 2). This is in contrast with results reported by Sefc et al. (2000), probably because it includes a lot of different olive cultivars, mostly of Iberian origin.

The combined PD is 0.9999995, which means that the probability of finding two cultivars with the same genotype combination for the 10 SSR markers is over one million, indicating the high discrimination of the marker system used.

The frequency of null alleles varied from 0 to 0.17. Note that the frequency of null alleles could not be estimated for marker GAPU71B because there was a high excess of heterozygotes.

The genotypic profiles for all loci are reported in Table S1*. The shortest allele among these 10 loci was 108 base pairs (bp) for UDO39, whereas the longest allele was 232 bp for UDO39. As mentioned previously, all alleles were checked by DNA sequence analysis, but the samples examined showed no difference of the number repeat units in alleles of the same total length and not increasing the number of polymorphism present in microsatellite loci. Sequence data from all alleles were deposited to GenBank (Table 2). The lowest allelic frequency (0.025) was observed for alleles 208 bp of GAPU59, 220 bp of UDO39, and 232 bp at UDO39. These three alleles were observed in

Table 2. Repeat motif and sequence size of the simple sequence repeat amplification products observed among the 20 Tunisian olive accessions[z].

Locus	Accession numbers	Size of amplicon in bps	*Na*	*Ho*	*He*	*PD*	*r*
GAPU59	EU376393	214 (208–218)	4.0	0.450	0.583	0.725	0.084
GAPU71A	EU376392	212 (210–228)	4.0	0.550	0.665	0.770	0.069
GAPU71B	EU376384	124 (121–144)	4.0	0.950	0.665	0.630	—
GAPU103A	EU376386	150 (136–184)	5.0	0.700	0.771	0.720	0.039
UDO03	EU376385	135 (135–202)	4.0	0.600	0.719	0.825	0.069
UDO12	EU376389	166 (166–193)	4.0	0.850	0.616	0.705	—
UDO28	EU376387	154 (143–210)	5.0	0.800	0.801	0.815	0.000
UDO39	EU376388	164 (108–232)	6.0	0.750	0.724	0.755	—
DCA09	EU376390	172 (172–206)	4.0	0.550	0.637	0.750	0.053
DCA18	EU376391	177 (174–190)	3.0	0.300	0.562	0.675	0.168

[z]For each locus, the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the total nucleotide sequences, the size range in base pairs, the number of alleles (*Na*), the observed heterozygosity (*Ho*), the expected heterozygosity (*He*), the power of discrimination *(PD*), and the probability of null alleles (*r*) are reported.

Table S1. Genotypic profiles for 10 simple sequence repeat markers of the 20 olive cultivars arranged in the order of their grouping in Figure 2.

Accessions name	GAPU 59	GAPU 71A	GAPU 71B	GAPU 103A	UDO 03	UDO 12	UDO 28	UDO 39	DCA 09	DCA 18
Chem-Sfax	212-212	212-214	124-144	159-159	135-182	166-182	182-210	205-205	182-182	174-174
Chemdok	212-212	212-214	124-144	159-159	135-182	166-182	182-210	205-205	182-182	174-174
Zalmati	212-212	212-214	124-144	159-159	135-135	166-182	182-210	205-205	182-182	174-174
Chem-ontha	212-212	212-212	126-144	159-159	182-202	166-182	182-210	213-232	182-194	174-190
Fougi	212-218	214-228	121-144	157-184	135-182	166-182	182-210	108-205	182-182	174-174
Chem-Zarzis	214-214	212-212	124-144	159-159	202-202	166-182	154-210	108-213	182-182	174-174
Beldi	212-212	212-212	144-144	150-157	166-166	166-193	182-182	108-108	172-182	174-190
Lguim	212-212	212-212	124-144	150-157	166-166	166-166	182-182	108-213	182-206	174-174
Toffehi	212-212	212-212	126-144	150-157	166-182	166-177	154-205	108-205	182-182	174-190
Chemcheli-G	218-218	212-228	124-144	150-157	166-182	166-177	154-205	108-205	182-206	177-177
Jemri-Bou	218-218	212-228	124-144	150-157	182-182	166-177	154-205	108-205	182-182	177-190
Chemcheli-S	212-218	210-210	124-144	150-157	166-166	166-177	154-205	108-205	182-182	177-177
Horr	212-218	210-214	124-144	150-157	182-182	166-177	154-210	165-165	182-206	174-177
Meski	214-218	212-212	124-144	136-150	166-182	166-193	154-154	108-213	194-206	177-177
Zarrazi	214-218	212-212	126-144	159-159	182-182	166-166	154-205	108-213	172-206	174-174
Chet-ML	212-218	210-228	121-144	136-157	135-182	166-177	143-205	108-213	194-206	174-174
Chetoui-Sfax	212-218	212-228	121-144	136-157	135-182	166-177	143-205	108-213	194-206	174-174
Chetoui-BA	212-218	212-228	121-144	136-157	135-182	166-177	143-205	108-213	194-206	174-174
Besbessi	208-212	212-212	124-144	136-184	182-202	166-193	182-182	108-220	182-206	177-190
Tounsi	212-212	210-214	121-126	150-157	166-202	166-166	143-154	108-205	172-172	177-177

one copy in the whole set of studied cultivars analyzed (Table S1). Alleles 212 bp of GAPU59 and 166 bp of UDO12 showed the relatively highest frequency (0.575).

Genetic diversity levels. The similarity degree between the 20 olive tree cultivars based on SSR markers (Table 2) range from 0.148 to 1.000 with an average value of 0.574 showing the high degree of intervarietal genetic diversity at the DNA level. The smallest similarity value of 0.148 was observed between 'Tounsi' and 'Chemlali', two cultivars that differ greatly for their end use, as well as agromorphological and chemical characteristics. The maximum genetic similarity (GS) between nonsynonymous varieties (GS = 0.933) was found between 'Chemlali Sfax' and 'Zalmati'. These two cultivars, growing in the south of Tunisia, exhibit very similar morphological and chemical characteristics. It is likely that they have a common genetic origin. The same result was reported by Grati Kamoun et al. (2006) based on AFLP markers.

The average GS between two ecotypes of the same cultivar is higher for 'Chemlali' (1.00) than that for 'Chetoui' (0.90).

Patterns of genetic diversity. The GS matrix was used to obtain a cluster diagram of cultivars based on SSR markers (Fig. 2). The dendrogram in Figure 2 depicts the pattern of relationships between the studied cultivars. There is no clear clustering of cultivars in relation with their growing area or end use. Nevertheless, three major clusters can be defined by cutting the dendrogram at a GS value of 0.32. The first group corresponds to the six cultivars of the top of the dendrogram composed of the two 'Chemlali' from the Sfax region and 'Zalmati', 'Fougi', 'Chemlali-ontha', and 'Chemlali Zarzis'. These six cultivars, except 'Fougi', have many common features including small-sized fruits (0.8 to 1.3 g), their use for oil production, and similar agromorphological and chemical characteristics (Grati Kamoun et al., 2000); 'Fougi' seems to be misclassified because it is used for both for oil and table and has medium-sized fruits (4.0 g; Grati Kamoun et al., 2000). The second group contains 'Beldi', 'Lguim', 'Toffehi', 'Chemcheli-G', 'Jemri Bou', 'Chemcheli-S', and 'Horr', all having small- to medium-sized fruit (1.2 to 3.9 g) and dual end use (oil and table olive). It is interesting to find that the three 'Chetoui' cultivars cluster together in the third group, where we also find 'Meski' and 'Zarrazi'. These varieties have medium- to large-sized fruits (2.8 to 6.5 g) but are from different areas (Grati Kamoun et al., 2000). 'Besbessi' and 'Tounsi' were well separated from the other groups. This is probably the result of a different genetic background as might be expected from their very big fruit size (9.0 and 10.0 g, respectively; Grati Kamoun et al., 2000).

Taamalli et al. (2006) studied 25 olive cultivars using 10 SSR markers and AFLP. Only eight cultivars are in common with our study so a direct comparison between our results is not possible.

If one looks globally at the dendrogram, there seems to be no correlation between SSR polymorphism and fruit weight and growing area. However, the representatives of the two major cultivars, Chemlali and Chetoui, showed high similarity among them. This indicates that there is a sound genetic basis to the homonymy issue. All the cultivars with 'Chemlali' denomination seem to have a common ancestor. This also holds for 'Chetoui', in which 'Chetoui' grown in Sfax showed perfect similarity for the 10 SSRs with that grown in Northern Tunisia. This is also supported for 'Chemlali' by our previous result with AFLP markers (Grati Kamoun et al., 2006). Taamalli et al. (2007) found, using 11 SSR markers, three different profiles for Chemlali cultivars and showed that Chetoui cultivars are genetically very close differing by only one locus.

In fact, we studied, using AFLP technique, 29 olive cultivars among which 14 cultivars are considered in the current study

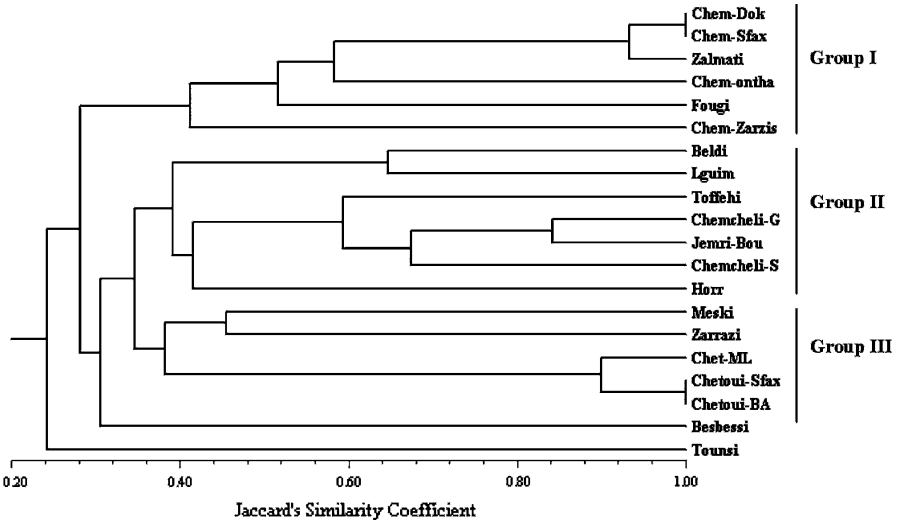

Fig. 2. Dendrogram of 20 olive cultivars generated by unweighted pair group method using an arithmetic average cluster analysis using Jaccard similarity coefficients from simple sequence repeat markers.

(Grati Kamoun et al., 2006). When comparing distances calculated using the two marker systems, a small and nonsignificant correlation was found (r = –0.17; P = 0.07). However, some common features were found between the two dendrograms, particularly the close proximity of the Chemlali cultivars and 'Zalmati' and the distinctiveness of 'Besbessi' and 'Tounsi' from the other cultivars.

Parentage and relationship between cultivars. Giving the high power of discrimination of the 10 SSRs, parentage analysis is justified. It is clear from Figure 2 that 'Chem-Dok' and 'Chem-Sfax' on one side and 'Chetoui-Sfax' and 'Chetoui-BA' on the other side are clones (have identical genotypes; see supplementary electronic material).

Offspring simulations based on genotypes and allele frequencies indicated a LOD threshold of 1.2 for single parents and 5.8 for parent pairs. With this setting for assignment of parent–offspring relations, the type I error (assign no parent when the true parent is in the data set) is 0.045 and the type II error (assign a parent when the true parent is not sampled) is 0.072.

Parent–offspring relations were found for 'ChemSfax' and 'Zalmati', 'ChetouiML' and 'ChetouiBA', and these two cultivar pairs differ by only one allele. However, given the high polymorphism of our markers and the reproductive biology of the olive tree, rather than parent–offspring, it is far more probable that those individuals are clones that have recently diverged by mutation or that the divergent allele is the result of genotyping error. Another interesting parent–offspring relation was found between 'Beldi' and 'Lguim'.

Relatedness estimations between individuals give values ranging from 0.00 to 0.93 (Table 3). The highest relatedness value (0.93) was found for cultivars Chetoui-Sfax and Chetoui-BA followed by 'Chemlali' and 'Zalmati' (0.92). 'Zalmati' is closely related to 'Chemlali' both at the genetic level as seen here and in previous studies and at the phenotypic level. There seems to be a synonymy relationship between this cultivar and the Chemlali cultivars from Sfax.

Cultivars with relatedness value of 0.00 are unambiguously assigned as unrelated. This concerns 128 pairs among the 190 cultivar pairs (67%). Except for these extreme values of relatedness, it is very often difficult to assign pairs of genotypes to a single relationship.

The general picture from the parentage analysis is that we can distinguish mainly two groups corresponding to those identified by cluster analysis (groups I and II in Fig. 2). The first one includes the Chemlali cultivars, Zalmati and Fougi. The second group corresponds to four full sibs ('Toffehi', 'Chemcheli-G', 'JemriBou', and 'Chemcheli-S').

For group II cultivars, results in Table 3 indicate that 'Chemcheli-G', 'Toffehi', 'Jemri-Bou', and 'Chemcheli-S' are very likely full sibs. The homonymy between the two Chemchali cultivars seems to be problematic because they were found to differ for four markers. However, in all cases, except for GAPU71A, the genotype is heterozygous for one cultivar and homozygous for the other with one shared allele between them. So, one cannot exclude a genotyping problem (no amplification of one of the alleles). Another interesting relationship (0.61) is found between 'Beldi' and 'Lguim' that are more likely full sibs than parent–offspring as previously stated. In group I cultivars (Table 3), many half sib relations are identified, particularly among 'Zalmati', 'Chemontha', and 'ChemZarzis' as well as 'ChemZarzis' and 'ChemSfax', indicating that all these cultivars derive very likely from a common ancestor. However, as mentioned by Taamalli et al. (2007), there is at least three different genetic profiles within the cultivars named Chemlali. Chemlali cultivars of the Sfax region very likely derive from a single clone, but those of Zarzis and ontha seem to be synonymous but distinct cultivars because they both differ by five loci from 'Chemlali-Sfax' and also differ from each other by six loci.

In all the other cases, the assignment to one single relationship is not probable and all relationships are unrelated as a possible relationship. However, an interesting result is that the Chetoui group of cultivars does not have any significant relationship with any other cultivar.

All these results indicate that SSR markers are extremely useful tools for addressing the issue of homonymy and synonymy in olive cultivars. Particularly, we were able here to establish that 'Chetoui' are very likely to derive from a single clone, whereas Chemlali and Chemchali cultivars show some degree of heterogeneity. The high similarity between 'Zalmati' and 'Chemlali-Sfax' is troubling and should be addressed in deeper detail using a larger number of markers. The combination of molecular information from SSR markers and agrochemical features to establish a fingerprint of each variety is the major goal of this work. Now we have all the necessary tools to achieve this goal.

Table 3. Pairwise matrix of maximum likelihood estimation of relatedness (top half of table) and the most likely relationship (bottom half of table) between individuals[z].

	Cultivars corresponding to Group I in Figure 2				
	ChemSfax	Chemo.a	ChemZarzis	Zalmati	Fougi
ChemSfax	—	FS	HS	CL	FS
Chemo.a	0.30	—	HS	HS	U
ChemZarzis	0.36	0.33	—	HS	U
Zalmati	0.93	0.30	0.36	—	FS
Fougi	0.46	0.00	0.00	0.40	—

	Cultivars corresponding to Group II in Figure 2						
	ChemcheliG	ChemcheliS	Toffehi	JemriBou	Beldi	Lguim	Horr
ChemcheliG	—	FS	FS	FS	U	U	HS
ChemcheliS	0.59	—	FS	FS	U	U	HS
Toffehi	0.39	0.38	—	FS	HS	U	U
JemriBou	0.78	0.49	0.41	—	U	U	HS
Beldi	0.00	0.00	0.31	0.00	—	FS	U
Lguim	0.00	0.00	0.00	0.00	0.61	—	U
Horr	0.27	0.29	0.00	0.25	0.00	0.00	—

[z]We report relationships that gives the highest likelihood.
U = unrelated; HS = half sibs; FS = full sibs; PO = parent–offspring; CL = clones.

Literature Cited

Angiolillo, A., M. Mencuccini, and L. Baldoni. 1999. Olive genetic diversity assessed using amplified fragment length polymorphisms. Theor. Appl. Genet. 98:411–421.

Bandelj, D., J. Jakše, and B. Javornik. 2002. DNA fingerprinting of olive varieties by microsatellite markers. Food Technol. Biotechnol. 40:185–190.

Bandelj, D., J. Jakše, and B. Javornik. 2004. Assessment of genetic variability of olive varieties by microsatellite and AFLP markers. Euphytica 136:93–102.

Belaj, A., G. Cipriani, R. Testolin, L. Rallo, and I. Trujillo. 2004. Characterization and identification of the main Spanish and Italian olive cultivars by simple-sequence-repeat markers. Hort. Sci. 39:1557–1561.

Belaj, A., Z. Satovic, G. Cipriani, L. Baldoni, R. Testolin, L. Rallo, and I. Trujillo. 2003. Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and their effectiveness in establishing genetic relationships in olive. Theor. Appl. Genet. 107:736–744.

Belaj, A., Z. Satovic, L. Rallo, and I. Trujillo. 2002. Genetic diversity and relationships in olive (*Olea europaea* L.) germplasm collections as determined by randomly amplified polymorphic DNA. Theor. Appl. Genet. 105:638–644.

Besnard, G., P. Baradat, and A. Bervillé. 2001. Genetic relationships in the olive (*Olea europaea* L.) reflect multilocal selection of cultivars. Theor. Appl. Genet. 102:251–258.

Bogani, P., D. Cavalieri, R. Petruccelli, L. Polsinelli, and G. Roselli. 1994. Identification of olive tree cultivars by using random amplified polymorphic DNA. Acta Hort. 356:98–101.

Brenner, C. and J. Morris. 1990. Paternity index calculations in single locus hypervariable DNA probes: Validation and other studies, p. 21–53. In: Proc. for the International Symposium on Human Identification. Promega Corporation, Madison, WI.

Breton, C., M. Tersac, and A. Bervillé. 2006. Genetic diversity and gene flow between the wild olive (*oleaster, Olea europaea* L.) and the olive: Several Plio-Pleistocene refuge zones in the Mediterranean basin suggested by simple sequence repeats analysis. J. Biogeogr. 33: 1916–1928.

Bronzini de Caraffa, V., J. Giannetti, C. Gambetti, and L. Maury. 2002. Genetic relationship s

between cultivated and wild olives of Corsica and Sardinia using RAPD markers. Euphytica 123:263–270.
Brookfield, J.F.Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Mol. Ecol. 5:453–455.
Burns, M., D. Shanahan, H. Valdivia, and N. Harris. 2003. Quantitative event-specific multiplex PCR detection of round up ready Soya using LabChip technology. Eur. Food Res. Technol. 216:428–433.
Carriero, F., G. Fontanazza, F. Cellini, and G. Giorio. 2002. Identification of simple sequence repeats (SSRs) in olive (*Olea europea* L.). Theor. Appl. Genet. 104:301–307.
Cipriani, G., M.T. Marrazzo, R. Marconi, A. Cimato, and R. Testolin. 2002. Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. Theor. Appl. Genet. 104:223–228.
De La Rosa, R., C.M. James, and K.R. Tobutt. 2004. Using microsatellites for paternity testing in olive progenies. HortScience 39:351–354.
Di Vecchi Staraz, M., M. Boselli, S. Gerber, V. Laucou, T. Lacombe, P. This, and D. Varès. 2007. FaMoz: A software for large scale parentage analysis in *Vitis vinifera* L. Species. Acta Hort. 754:79–84.
Díaz, A., R. De la Rosa, A. Martín, and P. Rallo. 2006. Development, characterization and inheritance of new microsatellites in olive (*Olea europaea* L.) and evaluation of their usefulness in cultivar identification and genetic relationship studies. Tree Genetics and Genomes 2:165–175.
Fabbri, A., J.I. Hormaza, and V.S. Polito. 1995. Random amplified polymorphic DNA analysis of olive (*Olea europaea* L) cultivars. J. Amer. Soc. Hort. Sci. 120:538–542.
Gerber, S., P. Chabrier, and A. Kremer. 2003. FaMoz: A software for parentage analysis using dominant, codominant and uniparentally inherited markers. Mol. Ecol. Notes 3:479–481.
Grati Kamoun, N., M. Ayadi, M. Khlif, A. Tridui, B. Karray, H. Rekik, B. Rekik, T. Hamdi, and M.N. Arous. 2000. Pomological and chemical characterization of Tunisia olive tree (*Olea europaea* L.), p. 25–30. In: Proc. of the 4th International Olive Growing Symposium CIHEAM-IAM, Bari, Italy, September.
Grati Kamoun, N., F. Chibani, A. Chaari, and M. Khlif. 1992. Etude de la variabilité isoenzymatique chez l'olivier. In: Proc. of the 32nd Science week Symposium, Damas, Syria, 7–11 Nov. 1992, Book 3. 1:407–420.
Grati Kamoun, N. and M. Khlif. 2001. Caractérisation technologique des variétés d'olivier cultivées en Tunisie. Ezzaitouna, Special Number. p. 1–74.
Grati Kamoun, N., M. Khlif, and M.T. Hamdi. 1999. Evolution of oils characteristics with maturity of olives in Sfax: Chemleli variety. Acta Hort. 474:701–704.
Grati Kamoun, N., F. Lamy Mahmoud, A. Rebai, A. Gargouri, O. Panaud, and A. Saar. 2006. Genetic diversity of Tunisian olive tree (*Olea europaea* L.) cultivars assessed by AFLP markers. Genet. Resour. Crop Ev. 53:265–275.
Grati Kamoun, N., N. Ouazzani, and A. Trigui. 2002. Characterizing isozymes of some Tunisian olive (*Olea europaea* L.) cultivars. Acta Hort. 586:137–140.
Hess, J., J.W. Kadereit, and P. Vargas. 2000. The colonization history of *Olea europaea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and inter-simple sequence repeats (ISSR). Mol. Ecol. 9:857–868.
Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44:223–270.
Kalinowski, S.T., A.P. Wagner, and M.L. Taper. 2006. ML-Relate: A computer program for maximum likelihood estimation of relatedness and relationship. Mol. Ecol. Notes 6:576–579.
Khadari, B., C. Breton, N. Moutier, J.P. Roger, G. Besnard, A. Bervillé, and F. Dosba. 2003. The use of molecular markers for germplasm management in a French olive collection. Theor. Appl. Genet. 106:521–529.
Lombardo, N., G. Godino, M. Alessandrino, T. Belfiore, and I. Muzzalupo. 2004. Contributo alla caratterizzazione del germoplasma olivicolo pugliese. Ed. I.S.Ol., Rende, Cosenza, Italy.
Lombardo, N., E. Perri, I. Muzzalupo, A. Madeo, G. Godino, and M. Pellegrino. 2003. Il germoplasma olivicolo calabrese. Ed. Co.R.Ass.Ol., Regione Calabria, Lamezia Terme, CZ, Italy.
Lumaret, R., N. Ouazzani, H. Michaud, G. Vivier, M.F. Deguilloux, and F. Di Giusto. 2004. Allozyme variation of *Oleaster* populations (wild olive tree) (*Olea europaea* L.) in the Mediterranean Basin. Heredity 92:343–351.
Mehri, H., M. Msallem, and R. Kamoun Mehri. 1997. Identification des principaux cultivars d'oliviers cultives en Tunisie. Plant Genet. Resour. Newslett 112:68–72.
Montemurro, C., R. Simeone, A. Pasqualone, E. Ferrara, and A. Blanco. 2005. Genetic relationships and cultivar identification among 112 olive accessions using AFLP and SSR markers. J. Hort. Sci. Biotechnol. 80:105–110.
Muzzalupo, I., A. Fodale, N. Lombardo, R. Mulè, M.A. Caravita, A. Salimonti, M. Pellegrino, and E. Perri. 2007a. Genetic diversity and relationships in olive Sicilian germplasm collections as determined by RAPD markers. Ad. Hort. Sci. 21:35–40.
Muzzalupo, I., M. Pellegrino, and E. Perri. 2007b. Detection of DNA in virgin olive oils extracted from destoned fruits. Eur. Food Res. Technol. 224:469–475.
Muzzalupo, I., N. Lombardo, M. Pellegrino, and E. Perri. 2006a. Studio della variabilità genetica di ecotipi di olivo dell'Abruzzo e del Molise mediante l'uso di marcatori molecolari SSR, p. 31–35. In: Proc. of the Congress Maturazione e raccolta delle olive: strategia e tecnologie per aumentare la competitività in olivicoltura, Alanno (PE), Italy.
Muzzalupo, I., N. Lombardo, A. Musacchio, M.E. Noce, G. Pellegrino, E. Perri, and A. Sajjad. 2006b. DNA sequence analysis of microsatellite markers enhances their efficiency for germplasm management in an Italian olive collection. J. Amer. Soc. Hort. Sci. 131:352–359.
Muzzalupo, I., N. Lombardo, A. Salimonti, and E. Perri. 2008a. Molecular characterization of Italian olive cultivars by microsatellite markers. Ad. Hort. Sci. 22:144–150.
Muzzalupo, I., A. Salimonti, M.A. Caravita, M. Pellegrino, and E. Perri. 2008b. SSR markers for characterization and identification of cultivars of *Olea europaea* L. in the Abruzzo and Molise regions, in south-central Italy. Ad. Hort. Sci. 22:131–137.
Perri, E., I. Muzzalupo, and R. Siriani. 2002. RAPD-PCR amplification of DNA from virgin olive oil. Acta Hort. 586:583–586.
Perri, E., M.V. Parlati, A. Palopoli, and B. Rizzuti. 1995. Polimorfismo isoenzimatico del polline dell'olivo (*Olea europaea* L.). Italus Hortus 2:31–36.
Rallo, P., G. Dorado, and A. Martín. 2000. Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). Theor. Appl. Genet. 101:984–989.
Reale, S., S. Doveri, A. Díaz, A. Angiolillo, L. Lucentini, F. Pilla, A. Martín, P. Donini, and D. Lee. 2006. SNP-based markers for discriminating olive (*Olea europaea* L.) cultivars. Genome 49:1193–1205.
Rohlf, F.J. 1999. NTSYS-pc. Numerical taxonomy and multivariate analysis system. Version 2.1. Exeter Software, Setauket, NY.
Ruby, J. 1918. Recherches morphologiques et biologiques sur l'olivier et sur les variétés cultivées en France. Faculté des Sciences, Paris, France. Thesis.
Sajjad, A., I. Muzzalupo, E. Perri, and G. Sindona. 2006. Characterisation of olive germplasm from Abruzzo, Apulia, Molise, Tuscany and Sicily regions by microsatellite markers, p. 36–50. In: Sindona, G. and L. Maiuolo (eds.). The contribution of chemistry molecular biology and medicinal chemistry to life sciences. Aracne Editrice srl, Rome, Italy.
Sarri, V., L. Baldoni, A. Porceddu, N.G.M. Cultrera, A. Contento, M. Frediani, A. Belaj, I. Trujillo, and P.G. Cionini. 2006. Microsatellite markers are powerful tools for discriminating among olive cultivars and assigning them to geographically defined populations. Genome 49:1606–1615.
Sefc, K.M., M.S. Lopes, D. Mendonça, M. Rodrigues dos Santos, M. Laimer da Camara Machado, and A. Da Camara Machado. 2000. Identification of microsatellite loci in olive (*Olea europea*) and their characterization in Italian and Iberian olive trees. Mol. Ecol. 9:1171–1173.
Sensi, E., R. Vignani, M. Scali, E. Masi, and M. Cresti. 2003. DNA fingerprinting and genetic relatedness among cultivated varieties of *Olea europaea* L. estimated by AFLP analysis. Scientia Hort. 97:379–388.
Taamalli, W., F. Geuna, R. Banfi, D. Bassi, D. Daoud, and M. Zarrouk. 2006. Agronomic and molecular analyses for the characterisation of accessions in Tunisian olive germplasm collections. Electron J. Biotechn. 9:467–481.
Taamalli, W., F. Geuna, R. Banfi, D. Bassi, D. Daoud, and M. Zarrouk. 2007. Using microsatellite markers to characterise the main Tunisian olive cultivars Chemlali and Chetoui. J. Hort. Sci. Biotechnol. 82:25–28.
Trujillo, I. and L. Rallo. 1995. Identifying olive cultivars by isozyme analysis. J. Amer. Soc. Hort. Sci. 120:318–324.
Wagner, A.P., S. Creel, and S.T. Kalinowski. 2006. Estimating relatedness and relationship using microsatellite loci with null alleles. Heredity 97:336–345.
Weir, B.S. 1996. Genetic data analysis II. Sinauer, Sunderland, MA.
Wiesman, Z., N. Avidan, S. Lavee, and B. Quebedeaux. 1998. Molecular characterization of common olive varieties in Israel and the West bank using randomly amplified polymorphic DNA (RAPD) markers. J. Amer. Soc. Hort. Sci. 123:837–841.
Wu, S.B., G. Collins, and M. Sedgley. 2004. A molecular linkage map of olive (*Olea europaea*) based on RAPD, microsatellite, and SCARS markers. Genome 47:26–35.
Zhang, L., P.O. Hakins, G. Kochert, S. Kersovich, R. Dean, and W. Hanna. 1999. Differentiation of bermudagrass (*Cynodon* spp.) genotypes by AFLP analyses. Theor. Appl. Genet. 98:895–902.