Characterization and Identification of Tunisian Olive Tree Varieties by Microsatellite Markers

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Additional index words. cultivar genotyping, SSR markers, Olea europaea, genetic diversity

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) markers 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 amplified fragment length polymorphism markers (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 (RAPD) or amplified fragment length polymorphism markers (Grati Kamoun et al., 1999; Bandelj et al., 2004; Belaj et al., 2002; Bogani et al., 1994; Bronzini de Caraffa et al., 2002; Fabbrì 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 markers (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, more than 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., 2005; 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; Sajjad et al., 2006; Taamalli et al., 2006, 2007). More recently, it was reported that DNA sequence analysis of microsatellites enhances the characterization of cultivars by SSR markers.

Received for publication 30 Jan. 2008. Accepted for publication 23 Apr. 2008. This research was supported by the Italian Ministry of Agriculture, Food and Forestry Policies and Consiglio per la Ricerca e la Sperimentazione in Agricoltura and by the Tunisian Ministry of Agriculture and the Tunisian Ministry of Higher Education, Research and Technology.

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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).

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

| Cultivar name | Abbreviation | Growing region | End use | Avg fruit wt (g) |
|---------------|--------------|----------------|---------|-----------------|
| Beldi         | Beldi        | Gafsa          | Both    | 3.90            |
| Besbessi      | Besbessi     | Tunis          | Table olive | 10.00        |
| Chemcheli     | Chemcheli-S  | Sfax (Taous)  | Both    | 3.00            |
| Chemcheli     | Chemcheli-G  | Gafsa          | Both    | 3.00            |
| Chemlali ontha | Chem-ontha   | Zarzis         | Oil     | 1.00            |
| Chemlali      | Chemdok      | Sfax (Dokhane) | Oil     | 1.07            |
| Chemlali      | Chem-Sfax    | Sfax (Sfax)   | Oil     | 1.07            |
| Chemlalazarzis| 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            |
| Jenribouchouka| Jenri-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            |

*Department from which the cultivar tree was sampled.
*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. The markers were in fact highly polymorphic and very informative in Italian olive cultivars (Muzzalupo et al., 2006a, 2006b, 2008a; 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.

Sequence of polymerase chain reaction products. The PCR product were run in 4% agarose gels (FMC BioProducts, Valensschaedt, Denmark) in TBE 1× buffer in the presence of ethidium bromide (1 μg·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):

\[
PD = 1 - \sum_{i=1}^{g} p_i \cdot 1 - (1 - PD) \times \prod_{j=1}^{n} (1 - 1 - PD),
\]

where \( p_i \) is the frequency of the \( i \)th genotype for the locus and the sum is overall genotypes. The combined power of discrimination overall loci was then calculated as 1 – ∑ \( i=1 \) to \( g \) \( p_i \) \( (1 - PD) \) where index \( i \) 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:

\[
S_{ij} = \frac{n_{ij}}{n_i + n_j - n_{ij}},
\]

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 NTYSysPC 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 relatendedness (\( r \)) 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 in 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.

| 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 (130–202)           | 4.0  | 0.600 | 0.718 | 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 |
| DAC18     | EU376391          | 171 (174–190)           | 3.0  | 0.300 | 0.562 | 0.675 | 0.168 |

For each locus, the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the total nucleotide sequenced, 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.
one copy in the whole set of studied cultivars analyzed (Table S1). Alleles 212 bp of GAPUS9 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’, ‘Toufhi’, ‘Chemcheli-G’, ‘Jenri 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...
(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 ‘ChemDok’ and ‘ChemSfax’ 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.** 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 ‘Chetoui’ (0.92). ‘Zalmati’ is closely related to ‘Chemlali’. Chemlali cultivars of the Sfax region very likely 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.

|                          | ChemSfax | Chemo.a | ChemZarzis | Zalmati | Fougì | ChemcheliG | ChemcheliS | Toffehì | JenmiBow | Beldì | Lguim | Horì |
|--------------------------|----------|---------|------------|---------|------|------------|------------|---------|----------|------|------|------|
| **ChemSfax**             |          |         |            |         |      |            |            |         |          |      |      |      |
| **Chemo.a**              | 0.30     |         |            |         |      |            |            |         |          |      |      |      |
| **ChemZarzis**           | 0.36     | 0.33    |            |         |      |            |            |         |          |      |      |      |
| **Zalmati**              | 0.93     | 0.30    | 0.36       |         |      |            |            |         |          |      |      |      |
| **Fougì**                | 0.46     | 0.00    | 0.00       | 0.40    |      |            |            |         |          |      |      |      |

| **ChemcheliG**           |          |         |            |         |      |            |            |         |          |      |      |      |
| **ChemcheliS**           | 0.59     |         |            |         |      |            |            |         |          |      |      |      |
| **Toffehì**              | 0.39     | 0.38    |            |         |      |            |            |         |          |      |      |      |
| **JenmiBow**             | 0.78     | 0.49    | 0.41       | 0.61    |      |            |            |         |          |      |      |      |
| **Beldì**                | 0.00     | 0.00    | 0.31       | 0.61    | 0.61 |            |            |         |          |      |      |      |
| **Lguim**                | 0.90     | 0.00    | 0.00       | 0.25    | 0.00 | 0.00       |            |         |          |      |      |      |
| **Horì**                 | 0.27     | 0.29    | 0.29       | 0.00    | 0.00 | 0.00       | 0.00       |         |          |      |      |      |

*We report relationships that gives the highest likelihood.

U = unrelated; HS = half sibs; FS = full sibs; PO = parent–offspring; CL = clones.

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