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Genetic similarity among Tunisian cultivated olive estimated through SSR markers

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ABSTRACT: Olive (*Olea europaea* L. subsp. *europaea* var. *europaea*) is one of the oldest fruit tree in the Mediterranean basin, and is cultivated for oil and canned fruit. Part of this interest is driven by the economic importance of olive oil which is increasing throughout the world due to its beneficial effect to human health. In Tunisia, olive has great socio-economic importance, with more than 60 millions olive trees cultivated for olive oil production including a wide range of cultivars which are widely extended from the north to the south regions of the country for its high economic value. Here, we applied microsatellites (SSRs) molecular markers to assess the genetic variability of the most important Tunisian olive cultivars. In total, the 10 simple sequence repeats (SSR) loci revealed 73 alleles with a mean number of 07 alleles per locus were detected. The polymorphism index content (PIC) values were high (0.72) ranging from 0.86 at GAPU 103 to 0.56 at EMO 90. The analysis of the dendrogram showed six main separate groups.
Keywords: *Olea europaea*, genetic diversity, microsatellite, genetic relationships

Introduction

Tunisia is the fourth largest producer of olive oil country in the world and oil exports represent 40 % of the overall value of agronomic exports and 5.5 % of aggregate exports, making it the fifth largest source of foreign currency earnings for the country.

Olive (*Olea europaea* L. subsp. *europaea* var. *europaea*) is grown by almost two out of three farmers in the country. The crop is spread over areas from the northern to the southern regions, where a wide range of edaphoclimatic conditions prevail, from lower semi-arid to arid conditions and generally receiving less than 250 mm of rain-fall (IOOC, 2003). The main variety cultivated is 'Chemleli' in the south and the centre of the country and "Chetoui" in the north. These two varieties account for 95 % of the total olive tree orchards and contribute more than 90 % of the national production of olive oil. 'Chemleli' alone covers 60 % of the olive-growing surface. The recent boom in demand for olive oil around the world requires Tunisian producers to improve fruit and oil quality to maintain their competitiveness on the international oil market and to meet consumer demands.

The distribution of *Olea* varieties in the Mediterranean basin gave rise to a very complex and highly articulated structure of olive culture which was marked by the existence of a considerable number of different olive cultivars (Bartolini et al., 2005). The great number of existing varieties led to the need of a powerful method of genetic analysis for the development of conservation management strategies for the genetic resources and for the protection of the commercial varieties quality label. In addition, there is the problem arising from the existence of homonyms and synonyms (Poljuha et al., 2008). This makes cultivar identification very difficult and complex.

The identification of olive tree cultivars has been traditionally carried out by morphological markers (Trigui and Msallem, 2002; Hannachi et al., 2007) and by biochemical markers that are allelic variants of proteins based on iso-enzymes and alloenzyme markers. The necessity to target olive cultivar identification led researchers to undertake new studies of varietal identification based on genetic markers such as DNA.

In order to exploit genetic diversity for targeted olive cultivar improvements, several Tunisian research teams have used PCR-based markers for basic and applied research to assess the genetic diversity of Tunisian olive cultivars. These markers types include RAPD (Zitoun et al., 2008), AFLP (Grati-Kamoun et al., 2006; Taamalli et al., 2006), SSR (Taamalli et al., 2007; Hannachi et al., 2008; Rekik et al., 2008) and SNP (Rekik et al., 2010).

In this study, we have made an attempt to systemically test the suitability of microsatellites for Tunisian cultivar identification and genetic similarity studies.

Materials and Methods

Plant material and DNA extraction

Molecular analysis was performed on 33 trees belonging to 12 Tunisian cultivars collected from groves located in various agroecological areas of Tunisia which are characterised by very high oil content in their fruits. More than one accession per cultivar was included in some cases such as the cultivar 'Chemleli', 'Zarrazi', 'Meski', 'Chetoui' and 'Nabli Zalmati'. The cultivar analysed and their origins are reported in Table 1.

Total genomic DNA was isolated from young leaves according to the protocol of Doyle and Doyle (1990). DNA so prepared was further purified and resuspended in Tris-EDTA of pH 8.0. The quality of the DNA was assessed by agarose gel electrophoresis and the quantity by measuring optical density at 260 and 280nm.

SSR markers

A great effort has been conducted during the last

Table 1 – Studied accessions of olive genotypes and their origin.

Denomination cultivars in targeted locations	Geographic region of production origin	Denomination cultivars in targeted locations	Number of accessions
Chemleli Sfax 01	South	Sfax	01
Chemleli Sfax 02	South	Sfax	02
Chemleli Sfax 03	South	Sfax	03
Chemleli matmata	South	Matmata	-
Chemleli Zarzis 01	South	Zarzis	01
Chemleli Zarzis 02	South	Zarzis	02
Chemleli Jerba 01	South	Jerba	01
Chemleli Jerba 02	South	Jerba	02
Chemleli Jerba 03	South	Jerba	03
Chemleli Tataouine	South	Tataouine	-
Chemleli Thibar 01	South	Thibar	01
Chemleli Thibar 02	South	Thibar	02
Chemleli Thibar 03	South	Thibar	03
Zarrazi Zarzis	South	Zarzis	-
Zarrazi Tataouine	South	Tataouine	-
Zarrassi Injassi Matmata 01	South		01
Zarrassi Injassi Mereth 02	South		02
Zarrazi Gafsa	South	Gafsa	-
Zalmati Zarzis	South	Zarzis	-
Meski 01	Centre	Sidi Bou Zid	01
Meski 02	Centre	Kairouan	02
Meski 03	North	Borj El Amri	03
Chetoui 01	North		01
Chetoui 02	North		02
Chetoui 03	North		03
Nabli Zalmati 01	North East		01
Nabli Zalmati 02	North East		02
Fouji	South West		-
Fokhari	South		-
Chemcheli	South West		-
Picholine	South		-
Marsaline	North West		-
Ouislati	Center West	Siliana	-

decade towards the identification of olive cultivars using molecular markers. At present SSR are the markers of choice for cultivar identification (Alba et al., 2009; Muzzalupo et al., 2010).

Ten microsatellite primers labelled with one of two fluorescent dyes 6-FAM or HEX (Sigma) (Table 2). ssrOeUA-DCA 3, ssrOeUA-DCA 7, ssrOeUA-DCA 9, ssrOeUA-DCA 14, ssrOeUA-DCA 16, ssrOeUA-DCA 17, ssrOeUA-DCA 18 (Sefc et al., 2010), ssrOeUA-EMO 90 (De la Rosa et al., 2002), ssrOeUA-GAPU 101 and ssrOeUA-GAPU 103 (Carriero et al., 2002).

Amplification reactions were carried out in final volumes of 10 µL using a thermal cycler (PE Applied Biosystems). The reaction contained 1X PCR buffer, 0.75 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of forward and reverse primers, 0.5 units μL^{-1} Taq DNA polymerase (Gotaq, Promega) and 50 ng μL^{-1} template DNA.

PCR amplification was completed at the following profile with one cycle of 94 °C of initial denaturation for 3 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s, and 72 °C for 30 s followed by a final extension for 20 min at 72 °C. The amplified products were tested on 1.2 % agarose gel to check for the amplification of the PCR products.

PCR product (0.5 µL) was mixed with a 12 µL of deionised formamide and 0.5 µL Gene Scan 500 (LIZ) size standard marker. The resulting mixture was heated for 2 min at 95 °C and then quickly cooled on ice. Each sample was loaded and run on an ABI-310 automated DNA sequencer (Applied Biosystems, USA). Allele sizing of electrophoretic data was done using Genotyper 3.1 software (Applied Biosystems, USA).

Data analysis

SSR fragments data were scored using Genescan and Genotyper softwares. The expected heterozygosity (He) of each microsatellite was calculated according to the formula $He = 1- \sum(p_i)^2$ (Nei, 1979) using the GDA program (Weir, 1996). Polymorphic information content (PIC) values was calculated according to the formula $1- \sum(p_i)2 - \sum\sum 2 (p_i)^2 (p_j)^2$ using the CERVUS v.2 software (Marshall et al., 1998).

Genetic relationships among accessions were calculated on the basis of a similarity matrix analysis according to Jaccard's coefficient (Sneath and Sokal, 1973). A dendrogram was generated based on the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis and grouping using NTSYS software ver. 2.11a (Rohlf, 1998).

Results

SSR polymorphism and alleles detected

The SSR method used in this study was able to uniquely fingerprint each of the 33 cultivated olive accessions. All SSR loci amplified consistently a 73 total number of amplified alleles with an average number of 7 alleles per locus. DCA 09 and EMO 90 showed the lowest number of alleles (4) whereas GAPU 103 presented the highest number of alleles (14). Alleles sizes vary among the ten loci, differences between the longest and shortest allele ranged from 20 to 91 bp (Table 2). Genetic variability was wide as indicated by the very high values of observed heterozygosity that ranged between 1.00 at locus ssrOeUA-DCA17 and 0.51 at ssrOeUA-DCA16, with a mean value of 0.84. The PIC values were high (0.72) ranging from 0.86 at GAPU 103 to 0.56 at EMO 90.

Cultivar identification

Among accessions, the average number of unique alleles per locus was 2.1 depending on the locus, between one (markers ssrOeUA-DCA14, ssrOeUA-DCA16 and GAPU 101) and eight unique alleles for GAPU 103. The identification key for the studied accessions is shown in Table 3. Specific allele profiles at locus GAPU 103 were first assigned to six varieties ('Meski', 'Chemleli', 'Chetoui', 'Chemchali', 'Fokhari' and 'Nabli Zalmati'). Specific allele profiles at locus ssrOeUA-DCA07 were assigned to four varieties ('Ouislati', 'Meski', 'Zarrazi' and 'Chemleli'). These cultivars 'Ouislati', 'Meski' and 'Zarrazi' were differentiated by ssrOeUA-DCA03 while differences at a single locus were found among varieties.

Genetic relationships and differentiation among olive cultivars

Affinities among cultivars and accessions are shown in the obtained dendrogram (Figure 1), forming six groups by cutting the dendrogram at a Genetic Similarity (GS) value of 0.35. In the first group, most accessions were of 'Chemleli' variety from the north and the south of Tunisia included only one accession of 'Zalmati' from Zarzis. The second main group included 'Zarrazi' olive variety from different geographic origin from the south of Tunisia. The others groups were formed by 'Meski, 'Chetoui', 'Nabli Zalmati' and 'Ouislati' while the other included 'Fouji', 'Fokhari' and 'Chemcheli' and the latest was constructed by 'Picholine' and 'Marsaline'.

Discussion

Based on a combination of scores for individual plants, all accessions can be distinguished using the ten markers employed here. However, not all individual plants can always be identified, as some varieties are mixtures of genotypes. Nevertheless, the value of the olive genotyping lies not only in shaping the continued use of microsatellite data in olive research but also

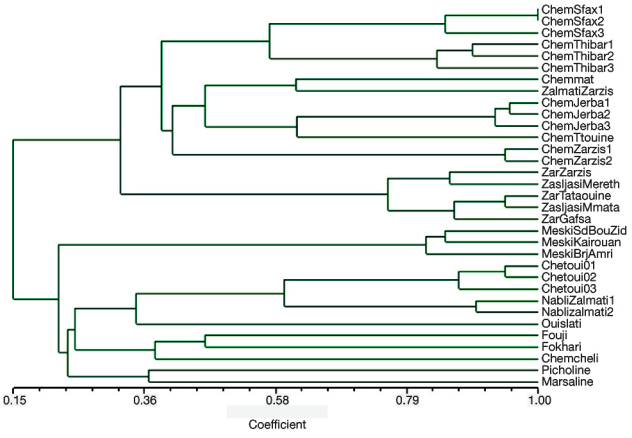

Figure 1 – SSR dendrogram based on Jaccard's genetic distance.

Table 2 – SSR locus, allelic number, Ho, He, PIC and product size range of the 10 SSR loci studied.

SSR locus	N° alleles	Observed heterozygosity	Expected heterozygosity	PIC	Range size
ssrOeUA-DCA-07	9	0.879	0.848	0.815	127-183
ssrOeUA-DCA- 09	4	0.879	0.740	0.680	169-193
ssrOeUA-DCA-03	11	0.879	0.851	0.820	161-252
ssrOeUA-DCA-14	5	0.758	0.721	0.667	168-188
ssrOeUA-DCA-16	6	0.515	0.739	0.684	122-176
ssrOeUA-DCA-17	7	1.000	0.792	0.752	105-185
ssrOeUA-DCA-18	7	0.818	0.800	0.755	167-187
EMO-90	4	0.939	0.628	0.560	123-200
GAPU-101	6	0.879	0.719	0.657	183-216
GAPU-103	14	0.909	0.893	0.868	127-199
Total	73				
Mean value	7.30	0.84	0.77	0.72	

in the realm of evolution and domestication of natural and wild olive (Rubio de Casas et al., 2006; Besnard et al., 2008; Belaj et al., 2010; Erre et al., 2010). The olive is not only an economically important species for Mediterranean basin countries where it represents the most important oil-producing crop but also as a model species to initiate conservation programmes that need to integrate genetic variability, diversity, cultural and economic aspects. The olive oil has value as a model therapy of a spectrum of human diseases (cardiovascular disease, obesity) and considered as source of foreign currency earnings for Mediterranean countries.

In this study, we have made an attempt to characterize Tunisian olive using nuclear microsatellites and to identify individuals that would represent suitable progenitors for a breeding programme to increase the quality of olive oil (Casagrande et al., 2009). The analyses of our marker data revealed that the Tunisian germplasm contains substantial diversity, which could support the national programme's breeding objectives as well as allow participation in international programmes aiming at olive improvement and conservation.

The microsatellite markers used in this study were polymorphic and demonstrated their utility in discriminating between Tunisian olive accessions. Our present study is largely in accordance and comparable with previous studies (Baldoni et al., 2009; Bracci et al., 2009; Muzzalupo et al., 2010) using SSRs as genetic analysis method to assess the genetic variability of Italian olive cultivars, including some minor exceptions. In our analysis, the total number of alleles amplified was 73 alleles with an average number of 7 alleles per locus (Table 2). This is comparable to the 2-7 alleles found by Soleri et al. (2010) with a total of 62 alleles identified for fourteen microsatellite markers in a set of American olive trees. The values of heterozygosity found at each locus was comparable with those reported in several studies, performed by SSR markers, such as on Moroccan olive germplasm (Khadari et al., 2007; Charafi et al., 2008).

PIC values for the landraces were quite high (Table 2), in accordance with many previous studies (Taamalli et al., 2008), showing that these accessions are a good source of diversity and the ten loci used are suitable for mapping (Poljuha et al., 2008). The ten primer pairs produced simple banding patterns, showing a low degree of differential amplification and easy scorability. They were used for genotyping and will therefore be useful to identify olive varieties and to perform genetic studies what is of particular interest to conduct programme breeding and conservation. It was possible to choose only seven loci (ssrOeUA-DCA03, ssrOeUA-DCA07, ssrOeUA-DCA14, ssrOeUA-DCA16, ssrOeUA-DCA 17, GAPU 101 and GAPU 103) for rapid identification (Table 3).

Results concerning cultivar identification suggest that genotypic and genetic polymorphism were originated from different geographic areas. Tunisian olive groves are essentially dominated by two olive oil varieties: 'Chetoui' in the north and 'Chemleli' in the center and south of the country. Furthermore, those two varieties have been the most multiplied by olive growers (Grati-Kamoun et al., 2006). Indeed, 'Chemleli' was widely cultivated (two-third of total olive area) and is vigorous and well adapted to arid regions (Trigui, 1996).

Allelic differences have been reported in olive using SSR (Cipriani et al., 2002). Undoubtedly, olive (*Olea europaea* L.) is an ancient crop cultivated since the Roman period, and there may have been occurred some somatic mutations in long-lived trees (Khadari et al., 2007). This is in agreement with the complexity of the history of olive domestication. Smulders et al. (2010) thought it may be related to the multi-allelic nature of SSR loci that is considered to derive principally from errors occurring due to slipped-strand mispairing during DNA replication, via retrotransposition events, otherwise to interhelical junctions forming during chromosome alignment, unequal crossing over, or gene conversion.

The results obtained by microsatellite DNA analysis revealed a clear separation of most Tunisian olive cultivars and showed a significant degree of inter-varietal genetic diversity. A dendrogram was made using the pairwise genetic distances between accessions to visualise the genetic similarity between accessions (Figure 1). A high range of similarity was found among accesses ranging from 0.15 to 1. For instance, six main groups were observed. In the first group, most accessions were of 'Chemleli' variety from the north and the south of Tunisia included only one accession of 'Zalmati' from Zarzis. The second main group included 'Zarrazi' olive variety from different geographic origin from the south

Table 3 – Distinct SSR private alleles among Tunisian varieties.

	ssrOeUA-DCA-07	ssrOeUA-DCA-03	ssrOeUA-DCA-14	ssrOeUA-DCA-16	ssrOeUA-DCA-17	GAPU-101	GAPU-103
Ouislati	153	243	-	-	-	193	-
Meski	173	193-183	-	-	-	-	147
Zarrazi	177	161	-	-	-	-	-
Chemleli	179	-	-	130	-	-	153-171
Chetoui	-	-	186	-	-	-	133-169
Picholine	-	-	-	-	177	-	-
Chemchali	-	-	-	-	169	-	183
Fokhari	-	-	-	-	-	-	175
Nabli Zalmati	-	-	-	-	-	-	187

of Tunisia. The others groups were formed by 'Meski, 'Chetoui', 'Nabli Zalmati' and 'Ouislati' while the other included 'Fouji', 'Fokhari' and 'Chemcheli' and the latest was constructed by 'Picholine' and 'Marsaline'.

The high utility of SSR markers in providing varieties clustering is in overall accordance with previous studies carried out on a large number of Tunisian olive cultivars (Rekik et al., 2008; Taamalli et al., 2006). There is no clear structuring of the variability relative to the end use of olive cultivars. In fact, 'Meski' accessions form a distinct single group and were not clustered with 'Picholine' and 'Marsaline' that are the most important Tunisian table cultivars used for canning and have a high average fruit weight (6 g). This result is consistent with previous findings indicating that cultivated olive cultivars were characterised by the same origin from the wild population of the Eastern Mediterranean and was spread to the Western part through cultivar dispersal by humans (Besnard et al., 2002).

The developed set of markers demonstrated its performance in discriminating 'Chemleli' and 'Zalmati' accession from different regions of Tunisia and is clustered in a uniform group that shared many of the phenotypic characteristics. This study has shown that, among Tunisian varieties, the 'Chemleli' accessions are the most important targets for preservation based on its contribution to diversity and particularly those from southern Tunisia appear to represent important reservoirs of genetic diversity and have been considered as a polyclonal cultivar (Trigui, 1996). It is possible that this variety provides evidence for the movement and exchange of olive cultivars through different regions and is a mixture of closely related genotypes (Charafi et al., 2008).

As a conclusion, microsatellite markers used in this study may be used for establishing a molecular database for Tunisian olive identification and to construct a molecular catalogue that can compare the molecular pattern of cultivars as well as to avoid redundant genetic entities to make a reference collection.

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