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Genetic Diversity of Major Olive Varieties from Southern Tunisia

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ABSTRACT

Considering the importance of olive-growing in Tunisia, microsatellite (SSR) analysis was used to study the genetic variation among twenty olive accessions from southern Tunisia. This set of olive microsatellites showed potential utility for genetic studies and it could contribute to the development of strategies for Tunisian germplasm conservation and breeding. Unweighted pair group method cluster analysis was performed and cultivars separated in three main groups. Five polymorphic simple sequence repeats (SSR) loci were employed and they revealed 38 alleles with a mean number of 7.6 alleles per locus. Genetic variability was wide as indicated by high values of both observed heterozygosity (mean value = 0.79) and PIC values (average value = 0.60). Cultivars formed 3 distinct and clear groups. Var. 'Chemlali' was grouped with the others cultivars and showed low genetic diversity. We hypothesize that this variety is a population of cultivated varieties, with the presence of different clones of the same cultivar.

Keywords: genetic characterization, *Olea europaea*, olive, SSRs

INTRODUCTION

The olive tree (*Olea europaea* L.) is a woody species specific to the Mediterranean region and is one of the oldest fruit species cultivated for oil and canned fruit in Tunisia (Abdelhamid *et al.* 2010). Southern Tunisia covers an area of 9.8 million ha, representing about 60% of the total land area (16.18 million ha). Tunisia is an important olive oil-producing country from the north to the south with a large variety of olive plantation and different densities whish are generally very low (<20 trees ha^{-1}) in southern and high (>100 trees ha^{-1}) in northern of the country (Hannachi *et al.* 2007; Oueslati *et al.* 2009).

Olive growing constitutes one of the principal economical and agricultural sectors and plays an important role in natural resources conservation in many semi-arid and arid regions in southern Tunisia, whish represent at least 75% of the total number of trees. Drought is a permanent feature of all these regions. Nevertheless, the average annual rainfall received is 90 mm. Traditional diet for people in the region is based specially on cereals, palm fruits and olive oil whish is considered the main fat source (Oueslati *et al.* 2009).

Suitable crops for cultivation in arid and semi-arid areas depend generally on the topography of the area and different biotopes are defined. Numerous species are encountered and are either drought-resistant annuals with a short growing period like wheat and barley or perennial crops as fruit species which can survive dry spells (such as olive, grape, pomegranate, almond, apple, fig and date palm). Crop diversification results improved economic status of the rural southern population and reduced dependence on state support in particular olive has become valued alternative sources of income (Available online: http://www.asnaped.org.tn).

The olive tree is known for its resistance to drought and for its well adapted to arid conditions with a limited annual number of water supply events. The olive oil production is very closely related to the improvement of conditions for olive culture and must increasingly be used to provide a balanced diet, protect the farm ecosystem, and improved economic status of the rural population (Bosabalidis and Kofidis 2002).

In general, genetic variability is a factor taken into account and mentioned in the conservation and the management of biodiversity in wild and cultivated species. Sustainable agriculture, led to find a powerful genetic analysis method based on DNA for the development and the conservation of management strategies for the genetic resources of olive varieties and the protection of the commercial varieties quality label to develop typical southern olive oil products such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG) (Giménez *et al.* 2010). In addition, there is the problem arising from the existence of homonyms and synonyms of cultivars. This makes cultivar identification very difficult and complex.

Several previous studies were performed to characterize and study the genetic diversity of olive (Abdelhamid *et al.* 2010). The identification has been carried out with reference to morphological traits such as character related to the fruit shape, leaves, etc. and by biochemical methods based on iso-enzymes markers (Ouazzani *et al.* 1995). However, these markers are too limited because in general, depend on environmental factors and their numbers are reduced.

The necessity to target olive cultivar identification led researchers to undertake new methods of genetic studies such as microsatellites or SSR (simple sequence repeats) molecular markers which represent a powerful method to assess the polymorphism of olive cultivars. These markers are highly polymorphic, co-dominant and reproducible. SSRs are used in several studies to investigate the genetic diversity and identification of genotypes in the olive tree in different Mediterranean countries such as olive cultivars from Italy (Bracci *et al.* 2009), Spain (Belaj *et al.* 2011), Tunisia (Taamalli *et al.* 2008), Portugal (Brito *et al.* 2008) and Morocco (Khadari *et al.* 2007).

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Short Communication

Table 1 List of studied accessions and their location origin.

Serial No.	Denomination of cultivars	Region of origin	Number of accessions
1	Zarrazi	Zarzis	-
2	Zarrazi	Tataouine	-
3	Zarrassi Injassi	Matmata	-
4	Zarrassi Injassi	Mereth	-
5	Zarrazi	Gafsa	-
6	Chemlali	Sfax	01
7	Chemlali	Sfax	02
8	Zalmati	Zarzis	01
9	Zalmati	Zarzis	02
10	Chemlali	Matmata	01
11	Chemlali	Matmata	02
12	Chemlali	Zarzis	01
13	Chemlali	Zarzis	02
14	Chemlali	Jerba	01
15	Chemlali	Jerba	02
16	Chemlali	Tataouine	01
17	Chemlali	Tataouine	02
18	Chemcheli	Gafsa	-
19	Fouji	Sfax	-
20	Fakhari	Sfax	-

The molecular focus of this paper was encouraged by the fact that (semi-) arid regions in southern Tunisia show environmental problems such as climatic change (rise in temperature and low rainfall) associated with olive farming and that the olive germoplasm must be protected from disappearance. The practical objective is to provide a tool for accurate identification of major commercial varieties of the south zone.

In this paper, we have made an attempt to systemically test the suitability of microsatellites for southern Tunisian cultivars identification and study the genetic variability within and among them.

MATERIALS AND METHODS

Plant material and molecular methods

Sampling was restricted to the areas where olives have been cultivated traditionally for a very long time in the southern region of Tunisia. Young leaves of olive were collected on 20 accessions selected from a survey of 6 commercial autochthon varieties from farmers in southern region. **Table 1** shows the named varieties and identification numbers of the accessions tested.

Total genomic DNA was extracted from leaves according to the technique described by Doyle and Doyle (1990) with a CTAB buffer, and its concentration and quality were checked by standard spectrophotometry. The primers used in this study were originally developed by Sefc *et al*. (2000) labelled DCA and were selected for their usefulness in genotyping olive cultivars.

SSR amplifications were performed routinely using PCR mixture (10 µl) which contained 1 unit PCR buffer, 2.5 mM dNTPs, 0.5 unit (U) of *Taq* DNA polymerase (Gotaq, Promega) and 10 µM of each primer. PCR was performed using a thermal cycler (PE Applied Biosystems) at initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min denaturation at 93°C, 1 min annealing ranging from 50 to 57°C and 90 sec extension at 72°C with a final extension at 72°C for 10 min. SSRs at 5 loci were revealed on an ABI-310 automated DNA sequencer (capillary electrophoresis).

Fatty acids composition

Fatty acid components from fresh material from the six varieties mentioned above were performed as described by Grati-Kamoun and Khlif (2001) in the beginning of maturity for olive. The oil of different variety was extracted from 2.5 Kg of fresh olives and was crushed with a hammer mill (MC2 Ingenieria y Sistemas, S.L., Sevilla, Spain), slowly mixed for 30 min at ambient temperature, centrifuged without addition of water or chemicals, then was transferred into dark glass bottles. The fatty acid composition of the oil was determined as methyl ester by gas chromatography (GC) (ATI, UNICAM) equipped with a flame ionization detector using capillary column (15 m × 0.25 lm film thickness). The temperatures of the injector, detector and oven were 220, 250 and 180°C, respectively. 0.5 g of each olive oil sample was prepared by vigorous shaking of a solution of *n*-hexane (0.5 g in 5 mL) with 0.5 mL 2 N methanolic potassium hydroxide solution. Fatty acids were identified by comparing retention times with those of standard compounds according to the method of European Regulation 2568/91 (EEC 1991) and subsequent amendments.

Data analysis

The similarity coefficient among cultivars was computed and analysed with NTSYS (Rohlf 1998) using the Jaccard's coefficient (Sneath and Sokal 1973). The similarity matrix was used to construct a dendrogram by the un-weighed pair-group method with arithmetical averages (UPGMA). Polymorphism Index Content (PIC), observed and expected heterozygosity were calculated by the software CERVUS v.2 (Marshall *et al.* 1998). Average fruit and stone weight were determined according to the method indicated by IOOC (1997). At the mature stage, 100 fresh olives (3 replicates per genotype) were picked. Average fresh fruit weight was determined and, after removing and cleaning the stones, flesh and stone weights were also recorded.

RESULTS AND DISCUSSION

In this study, we used SSR markers to identify DNA fingerprints of 20 accessions of olive (*Olea europaea* L.) trees from the southern of Tunisia. A great effort has been conducted during the last decade towards the identification of olive cultivars using molecular markers. At present SSR are the markers of choice for cultivar identification allowed the detection of high level of polymorphism that is agreement with studies that investigated Tunisian olive cultivars with SSR markers (Rekik *et al.* 2008). These 5 SSR markers produced amplified fragments in all accessions that we included, and showed high transferability across accessions. Muzzalupo *et al*. (2010) reported that SSR DCA markers are sufficiently reliable to permit genetic diversity studies of olive. All five microsatellite markers were polymorphic, revealing a total of 38 alleles, ranging from 4 to 12 alleles per locus with a mean value of 7.6 alleles per locus (**Table 2**).

The mean H_o and H_e values for the Tunisian olive were 0.79 and 0.66, respectively (**Table 2**). The values of heterozygosity and number of alleles found at each locus was comparable with those reported in several studies, performed by SSR marker on olive genetic study (Poljuha *et al.* 2008).

The dendrogram obtained with SSR markers (**Fig. 1**) revealed three clear groups. Cluster I contained 'Chemlali' Matmata and Jerba, 'Zarrassi Injassi', 'Zarrassi' Tataouine and Gafsa and 'Zalmati' Zarzis. Cluster II contained 'Chemlali' Tataouine, the cultivar 'Fakhari' and 'Fouji' and the last cluster contained 'Chemlali' Sfax and Zarzis, 'Zalmati' Zarzis and 'Chemlali' Zarzis. The variety 'Chemchali' was clustered separately.

The clustering of varieties are enough reliable as the value because the Tunisian olive accessions used in this study were obtained from all over the south and cover a large proportion of the genetic resources in the cities of the

Table 2 Product size range, allelic number, He, Ho, and PIC of the 5 SSR Loci studied.

Locus	Size range (pb)	Number of alleles	Ho	He	PIC
DCA09	169-193	4	0.90	0.61	0.52
DCA14	168-188	4	0.90	0.67	0.60
DCA16	122-176	8	0.30	0.50	0.45
DCA17	105-177	10	1.00	0.76	0.70
DCA18	167-187	12	0.85	0.77	0.72
Mean	-	7.6	0.79	0.66	0.60

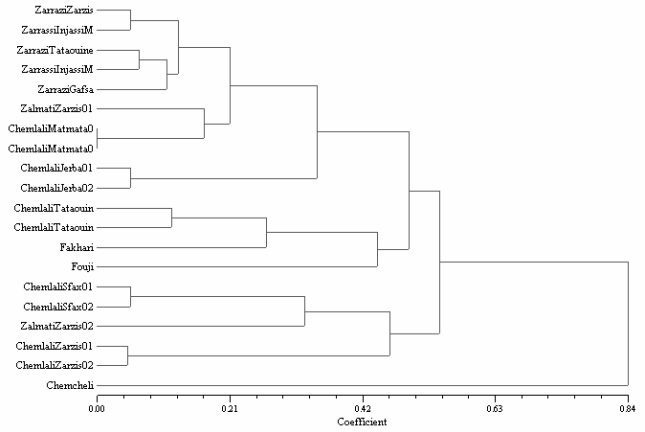

Fig. 1 UPGMA dendrogram obtained from microsatellite data.

Table 3 Weight of fruit, stone and fatty acid composition of virgin olive oil samples.

	Weight of fruit (g)	Weight of stone (g)	C16:0	C18:1	C18:2
Chemchali	2.26	0.28	18	62	15
Chemlali	0.76-1	0.17-0.23	20-22	53-65	9-20
Fakhari	1.35-2.5	0.38	16	65	12.7
Fouji	1.3	0.22	17	63	15
Zalmati	1.5	0.19	22	54	18
Zarrazi	3.9	0.37	12	70	14

C16:0 Palmitic acid ; C18:1 Oleaic acid; C18:2 Linoleic acid

south.

Many synonymous cultivars have been identified among olive trees by means of SSR analyses. Some synonym and homonym groups were also found in Mediterranean olive (Bracci *et al.* 2009). Here, we found 2 synonym accessions for 'Chemlali Matmata' by 05 SSR markers (**Table 3**). 'Chemlali' is widespread in all the country from the north to the south, is characterized by a smaller fruit size (**Table 3**) and high oil content in the mesocarp (Grati-Kamoun *et al*. 2006). This cultivar is a long-lived evergreen plant that adapts quite easily to many and varied environmental arid to semi-arid conditions and seems to be a population variety (Trigui 1996). 'Chemlali' showed similar nut ripening dates and morphological characteristics strongly suggesting synonymity (Rekik *et al.* 2008). This funding was supported by Taamalli *et al*. (2007) and showed that 'Chemlali' cultivars of the Sfax region are similar and could be derived from a single clone and was considered as variety population. Grati-Kamoun *et al*. (2006) also reported that 'Chemlali' and 'Zalmati' appeared to be synonyms because of the similar shape of their fruits, their leaf and similar harvesting dates. It was also reported that showed the same or very similar characteristics in fatty acid composition (**Table 3**), tree vigor, growth habit, branch development, leaf size, or harvest time (Trigui 1996). This result could explain the cluster which contains 'Chemlali' and 'Zalmati' accessions. This result was confirmed by Trigui and Msallem (2002) in the catalogue of Tunisian olive germplasm which was identified by the use of morpho-pomological traits.

Interestingly, many of these cultivars originated from different prefecture of the south showed low genetic variability. It was expected none or very little genetic variability among the 'Chemlali', 'Zarrassi' and 'Zalmati' olive accessions. Similar results on Moroccan germplasm were reported by Khadari et al. (2007) in 'Picholine Marocaine' where a unique genotype was found between 112 olive trees. The genetic homogeneity could be the result that analysed cultivars belonging to a limited geographical area and are the result of local selection, it suggests that the cultivars originated from the same genetic pool has been shown by Besnard *et al*. (2001).

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