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Molecular characterization and genetic relationships of cultivated Tunisian olive varieties (*Olea europaea* L.) using SSR markers

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Abstract - We studied 58 trees belonging to 13 Tunisian cultivated olive varieties (*O. europaea* L.) from 3 regions of origin (north, center and south) evaluated by means of simple sequence repeat (SSR) markers. In total, 7 simple sequence repeats (SSR) loci used in this study and revealed 51 alleles with a mean number of 7.29 alleles per locus and a mean polymorphic information content (PIC) of 0.693 showing a high genetic diversity of Tunisian olive cultivars. A larger variability than expected was found in the analyzed genotypes.
Clustering of individual accessions based on UPGMA dendrogram separated accessions out to four major clusters. The analysis of molecular variance (AMOVA) indicated that genetic differentiation occurred mainly within populations. Bayesian model-based clustering method show inference of relationships between cultivars according to geographic criteria and allowed us to identify putative admixed cultivars.
Hence, the study revealed a homogenous genetic structure of Tunisian olive cultivars, probably due to the geographical origin of the genus *olea*. The information collected in this study can be used for olive breeding strategies and for the management and the conservation of Tunisian olive germplasm.

Keywords: *Olea europaea* L.; Genetic structure; Microsatellites; Genetic diversity; Olive

1. Introduction

Olive tree (*Olea europaea* L. subsp *europaea*) is one of the oldest and interest fruit species cultivated in Mediterranean basin. It is a traditional tree crops with significant cultural and economic interest in all the Mediterranean countries.
In Tunisia, the olive is an important crop mainly cultivated for olive oil production and widely extends from the north to the south regions of the country for its high economic value, where the two third are localized in arid and semi-arid conditions. Tunisia olive germplasm is characterized by a large number of varieties with about 200 cultivars. The major variety cultivated is the Chemlali in the south and the centre of the country and Chetoui in the north (Trigui 1996). These varieties account for 95 % of the total olive tree orchards and contribute more than 90 % of the national production of olive oil.
Although olive varieties in Tunisia is considered very complex and varies greatly in its growth habit, size of fruit, oil content and exhibits great morphological differentiation and phenotypic plasticity to environmental factors which marked by the existence of a considerable number of different olive cultivars. Morpho-pomological traits related to the pit, fruit, leaf and tree led to the publication of catalogue entitled "olives of Tunisia: autochthonous varieties and local types" (Trigui and Msallem 2002).
It is evident that understanding the extent of natural variation and determining the genetic diversity at molecular level is essential to develop new strategies for olive improvement and to preserve and to promote the potential value of this resource. The valorization of interesting Tunisian cultivars should improve the competitiveness of olive oil in international market and protect the commercial varieties quality label to develop typical olive oil products such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG).
Since 1989, breeding and improvement of olive varieties are drawing great attention from breeders and researchers in the olive institute to improve the quality of olive oil of Chemlali and Meski varieties and other local types by self- and controlled crossing which is a frequent mode of reproduction using foreign

and local varieties as pollinators. The National Olive Genetic Resources Conservation located in Boughrara-Sfax, Tunisia, collected and maintained diverse germplasm resources of olive worldwide, which constitute available sources of plant genetic material for breeding and improvement of the quality and oil content of varieties.

The identification of olive germplasm is complicated and remains a hard task due to the great number of varieties described in the species with many synonymous and clonal differences within the same named cultivar (Abdessemed et al. 2015; Mnasri et al. 2017). The increasing interest in cultivation of olive in Tunisia requires much effort in varietals identification. Neither chemical analysis of different clusters of compounds, nor the analysis of morphological traits has led to cultivar identification, due to environmental effects on the phenotype (Ouazzani et al. 1995).

Efficient and robust molecular markers are increasingly needed for improving varieties, for establishing a molecular database for tree identification and for determining the extent methods for genetic analysis to evaluate their utility in large-scale genotyping applications in olive. At present Simple Sequence Repeats (SSRs) or microsatellites offers the opportunity to evaluate many potentially polymorphic sites and are the markers of choice for olive DNA fingerprinting and cultivar identification (De la Rosa et al. 2002)

Many works was accumulated in application of SSRs method within the species *Olea*, to study the genetic variation in natural populations (Belaj et al. 2007; Brito et al. 2010; Erre et al. 2010; Besnard et al. 2012) and the genetic diversity of cultivated varieties (Taamalli et al. 2008; Linos et al. 2014; Mnasri et al. 2017).

The aim of the present study is to determine the extent of allelic diversity at the microsatellite loci and for evaluating their utility in large-scale genotyping and genetic structure studies applications for Tunisian olive cultivars.

2. Materials and methods

2.1. Plant material and DNA extraction

A total of 58 accessions belonging to 13 Tunisian cultivars were analysed from different geographical regions. More than one accession per cultivar was included in some cases such as Zarrazi (9), Zarrazi Injassi (6), chemlali (18), Zalmati (3), Picholine (3), Marsaline (3), Nabli Zalmati (2), Chetoui (3) and Meski (7). The locations of the studied individuals are mentioned and listed in Table 1and Figure 1.

DNA was extracted from young leaflets of single plants, using a procedure previously (phenol-chloroform method) described by Doyle and Doyle (1990). DNA quality and quantification were checked by electrophoresis on agarose gel 0.9% (w/v).

2.2. SSR analysis

Three sets of primers were used to perform molecular analysis and were used for their high levels of polymorphism: 5 DCA (Sefc et al. 2000), 1 GAPU (Carriero et al. 2002) and 1 EMO (De la Rosa et al. 2002).

Amplification reactions were carried out in final volumes of 10-µl. Reaction contained 1 x *Taq* polymerase reaction buffer, 0.75 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of each primer , 0.5 U/µl *Taq* DNA polymerase (Gotaq, Promega) and 50 ng/µl template DNA. The forward primer was 5′ labeled with one of the two fluorophores (6FAM or HEX).

The reactions were performed on a thermocycler (PE Applied Biosystems) using the following steps: initial denaturing (3 min at 94°C) followed by 35 cycles of (1 mn at 93 °C) denaturation, 1 mn at optimal temperature ranging from 50 °C to 57 °C (annealing) and (90 sec at 72 °C) extension; and a final extension step at 72 °C for 10 min was followed.

PCR products were mixed with a 12 µl volume 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 before sequencing. In order to establish the size of the different alleles at each locus, each sample was loaded and run on the ABI-310 automated DNA sequencer (capillary electrophoresis). The Gnenescan v.4 software (PE Applied Biosystems) was employed for a result analysis.

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

Number of accession	Denomination cultivars in targeted locations	Locality
1	Zarrazi Zarzis01	South-west
2	Zarrazi Zarzis02	South-west
3	Zarrazi Zarzis03	South-west
4	Zarrazi Tataouine01	South
5	Zarrazi Tataouine02	South
6	Zarrazi Tataouine03	South
7	Zarrazi Gafsa01	South
8	Zarrazi Gafsa02	South
9	Zarrazi Gafsa03	South
10	Zarrazi Injassi Matmata01	South
11	Zarrazi Injassi Matmata02	South
12	Zarrazi Injassi Matmata03	South
13	Zarrazi Injassi Mareth01	South
14	Zarrazi Injassi Mareth02	South
15	Zarrazi Injassi Mareth03	South
16	Chemlali Sfax01	South
17	Chemlali Sfax02	South
18	Chemlali Sfax03	South
19	Chemlali Matmata01	South
20	Chemlali Matmata02	South
21	Chemlali Matmata03	South
22	Chemlali Zarzis01	South-west
23	Chemlali Zarzis02	South-west
24	Chemlali Zarzis03	South-west
25	Chemlali Jerba01	South-west
26	Chemlali Jerba02	South-west
27	Chemlali Jerba03	South-west
28	Chemlali Tataouine01	South
29	Chemlali Tataouine02	South
30	Chemlali Tataouine03	South
31	Meski Gafsa	South-west
32	Meski Sfax	South
33	Meski Zarzis	South
34	Zalmati Zarzis01	South-west
35	Zalmati Zarzis02	South-west
36	Zalmati Zarzis03	South-west
37	Picholine du Languedoc 01	South
38	Picholine du Languedoc 02	South
39	Picholine du Languedoc 03	South
40	Chemchali	South-west
41	Fouji	South
42	Fokhari	South
43	Chemlali Tibar01	North
44	Chemlali Tibar02	North
45	Chemlali Tibar03	North
46	Marsaline01	North-west
47	Marsaline02	North-west
48	Marsaline03	North-west
49	Chetoui01	North
50	Chetoui02	North
51	Chetoui03	North
52	Meski Borj el Amri	North
53	Nabli Zalmati01	North-east
54	Nabli Zalmati02	North-east
55	Meski Enfidha	Centre-east
56	Meski Sidi Bouzid	Centre-west
57	Meski Kairouan	Centre
58	Ouislati	Center-west

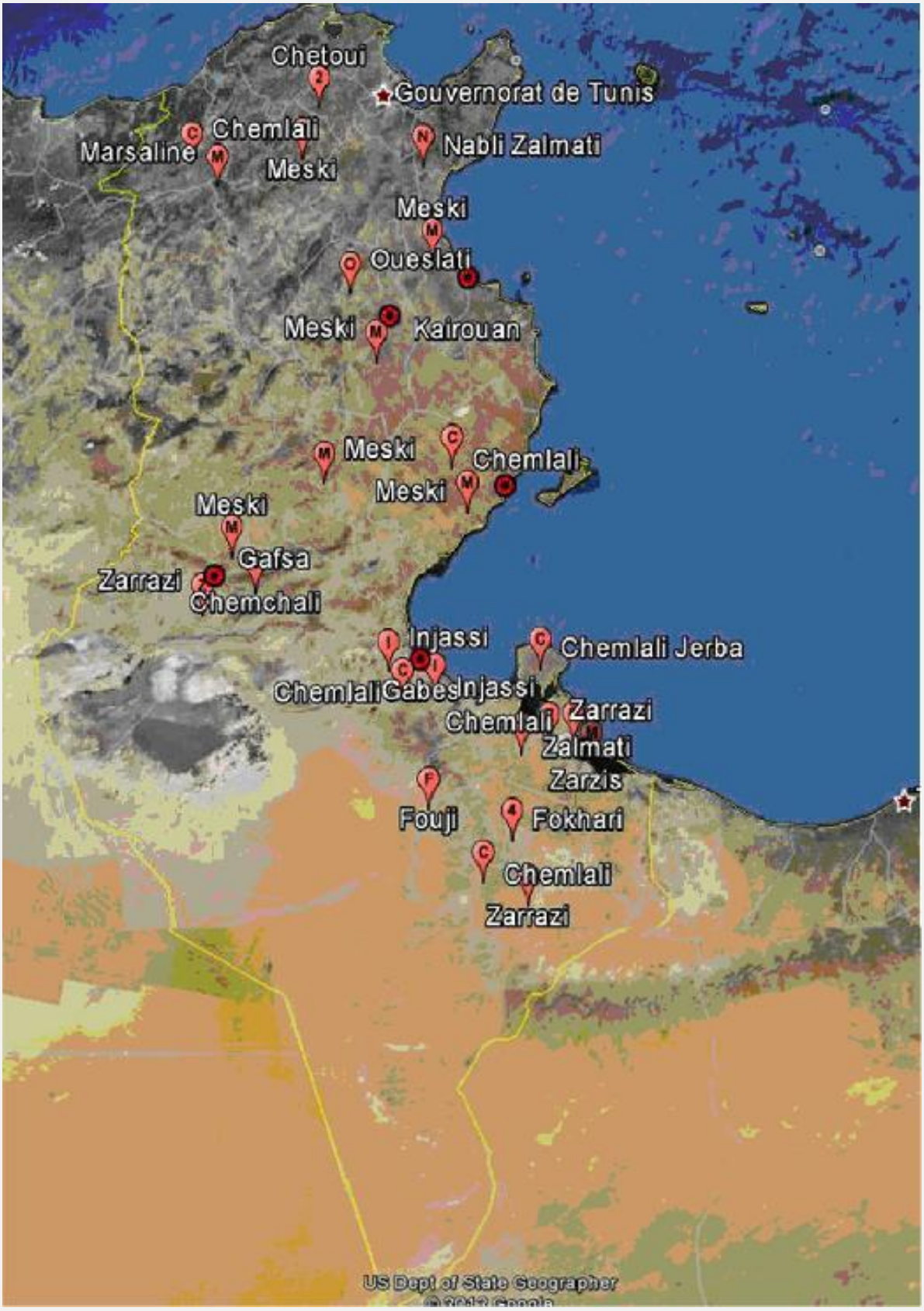

Figure 1. Geographic distribution of Tunisian variety accessions included in the study.

2.3. Data analysis

For each individual and for each primer that yielded a clear pattern, polymorphic DNA pics for SSR markers were scored as present (1) or absent (0). For studying the informative potential of the microsatellites and parameters of diversity, the number of alleles per locus (Na), the observed (H_o) and expected (H_E) heterozygosity, the total number of null alleles (Fnull), the polymorphic index content (PIC), and the deviation from the Hardy–Weinberg equilibrium (HWE), which was inferred by sequential Bonferroni correction, were calculated using the data obtained in the set of cultivars by the software CERVUS v.2 (Marshall et al. 1998). For the data set, dendrograms was constructed by UPGMA (Unweighted Pair-Group Method with Arithmetic Averages) cluster analysis according to Jaccard's coefficient (Sneath and Sokal 1973) using the NTSYS software ver. 2.11a analysis and grouping (Rohlf 1998). Bootstrapping with 1,000 replicates and no resampling was conducted to determine support for each node. To estimate the genotypic variations at hierarchical level between individuals from different geographic provenances (north/center/south), we employed the ARLEQUIN 2. 000 software package (Schneider et al. 2000) by means of analysis of molecular variance (AMOVA, Excoffier et al. 1992). The significance of the resulting variance components and inter-population genetic distances were tested using 5 000 random permutations. Bayesian model-based clustering approach using the SSR data to identify the genetic structure and to infer relationships in Tunisian olive cultivars from three sites of origin was performed using the software package Structure 2.3.4 (Pritchard et al. 2000). The 'admixture model' of Structure algorithm was run with 10 independent replicate per K value ranging from 2 to 10. Burning period of 100,000 iterations, followed by 200,000 post burning simulation length of 200,000 runs at each k was calculated. The validation of the most likely number of clusters K was performed with the Structure Harvester as suggested by Linos et al. (2014).

3. Results

3.1. SSR genetic diversity

The 7 microsatellite loci were polymorphic in examined Tunisian olive cultivars (Table 2). A total of 51 putative alleles were obtained from the 7 microsatellite markers and used to assess the genetic relationships among 58 examined genotypes. The number of alleles ranged from 4 to 14, with an average of 7.29 alleles per putative locus. Alleles sizes vary among the seven loci, differences between the longest and shortest allele ranged from 20 to 80 bp.

Genetic variability was wide as indicated by the high values of observed heterozygosity (Ho) that ranged between 0.96 at locus DCA17 and 0.43 at DCA16, with a mean value of 0.77. The PIC values of the 7 microsatellite loci ranged from 0.58 to 0.81 (average = 0.69). The highest PIC value was found in Gapu103 (0.81). SSR loci DCA14, DCA16, DCA17 and EMO90 showed a significantly estimated null allele frequency.

Table 2. Locus name, product size range (bp), N_a: number of alleles, H_e: Expected Heterozygoty, H_o: Observed Heterozygosity, PIC: polymorphism index content, *F:* null alleles, *PI:* probability of identity, NE-I: non-exclusion probability (identity) and NE-SI: non-exclusion probability (sib identity) of the 7 SSR Loci studied for all genotypes.

Locus	Size range (bp)	Na	H_o	H_E	PIC	*F* (null)	PI	NE-I	NE-SI
ssrOeUA-DCA09	169-193	4	0.845	0.721	0.664	-0.085	0.361	0.132	0.426
ssrOeUA-DCA14	166-188	6	0.793	0.713	0.662	-0.083 [a]	0.364	0.131	0.429
ssrOeUA-DCA16	121-176	8	0.431	0.650	0.650	+0.233 [a]	0.388	0.168	0.470
ssrOeUA-DCA17	105-185	7	0.966	0.793	0.757	-0.118 [a]	0.219	0.074	0.376
ssrOeUA-DCA18	167-187	8	0.724	0.803	0.769	+0.051	0.207	0.069	0.369
EMO-90	126-188	4	0.931	0.638	0.582	-0.241 [a]	0.438	0.185	0.480
GAPU103	127-187	14	0.724	0.842	0.817	+0.076	0.141	0.045	0.344
Mean		7.29	0.773	0.737	0.693				
Combined							$1.3x10^{-5}$	$1.2x10^{-7}$	$1.9x10^{-4}$

[a] Significant and deviating from Hardy-Weinberg equilibrium.

The probability of identity (PI) which indicates the probability that two unrelated genotypes chosen at random from all assessed genotypes have the same profile was calculated and showed low combined or total value 1.3×10^{-5}.

The probability that genotypes at a single locus do not differ between two randomly chosen genotypes was calculated by the mean of the non-exclusion probability between two unrelated individuals (NE-I) and two hypothetical full siblings (NE-SI) as shown by Marra et al. (2013), ranged from 0.045 for GAPU103 to 0.185 for EMO90 and from 0.344 for GAPU103 to 0.480 for EMO90, respectively.

Allele frequencies ranging from 0.008 for allele 169 bp at locus DCA18 to 0.534 for allele 176 bp at locus DCA16. In this study rare alleles were also found: allele 166 bp of the locus DCA14 was present in Picholine and allele 186 bp was present in Chetoui, allele 121 bp in Zalmati, allele 128 bp and 132 bp in Picholine and in Marsaline and allele 130 bp in Chemlali Djerba at the locus DCA16. At locus DCA18 we found the allele 169 bp in Chemchali and allele 185 bp in Zalmati Zarzis. The locus GAPU103 has a high number of alleles and has low allelic frequency. In locus GAPU103, the allele 133 bp and 169 bp were found in Chetoui, 147 bp was present in Meski, 167 in Ouislati and Fouji, 175 bp in Fokhari, 183 bp in Chemchali and 187 bp in Nabli Zalmati. On the contrary, some accessions (Chetoui vs Nabli Zalmati, or Chemlali Sfax vs Zalmati) already reported by other authors as synonyms, were discriminated in our analysis.

Specific allele profiles at locus GAPU103A were first assigned to 6 varieties (Meski, Chemlali, Chetoui, Chemchali, Fokhari and Nabli Zalmati). Specific allele profiles at locus DCA9 were assigned to 4 varieties (Ouislati, Meski, Zarrazi and Chemlali). These cultivars Ouislati, Meski and Zarrazi were differentiated by DCA17 while differences at a single locus were found among varieties. As already stated by Marra et al. (2013) these differences seemed of somatic origin and can be considered insufficient to have originated by sexual reproduction in a species such as olive, predominantly allogamous and with a high degree of heterozygosity at the genomic level. As shown by Taamalli et al. (2006) the locus DCA09 indicate the highest discriminating capacity among studied locus and differentiate some Tunisian varieties such as Kbiret Louzir and ElHorr.

At locus DCA17 we observed the lowest size range of alleles and the highest observed heterozygosity. Earlier analyses revealed by Poljuha et al. (2008) show the same phenomenon refers to the fact that long alleles may have problems to be amplified at target DNA region relative to loci with short alleles leading to the apparent reduction of heterozygosity. The molecular analysis of different accessions of the same cultivar such as cv Chemlali led to obtain different SSR profiles. DCA-16 has the highest discriminating capacity among locus that differentiated Chemlali accessions. The cv Chemlali is widespread in all the country from the north to the south, is characterized by a smaller fruit size 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. Chemlali accessions could be derived from common ancestors and from seedling, the cv Chemlali is considered as cultivar population which is usually propagated vegetatively (Trigui 1996).

3.2. Genetic structure and relationships among Tunisian olive accessions

Genetic analysis of 58 genotypes of olive revealed four distinct clusters (Fig. 1). The examined genotypes were differentiated based on similarity coefficients. Genotypes were separated into four clusters (I, II, III and IV). Cluster I consisted of two subclusters: contained (Ouislati, Nabli Zalmati, Chetoui, Chemcheli, Fouji and Fockari). Cluster II contained only the genotype of olive table (Picholine and Marsaline). The cluster III contained only the Meski (olive table) genotypes and the fourth cluster contained just the main varieties for oil production such as Chemlali, Zarrazi and Zalmati which were collected in the south of the country. The Southern Tunisia cultivar group was found to be more inferred, which reflected the exchange of plant material within the area.

Hierarchical analysis and genetic differentiation of groups determined by AMOVA (Table 3) shows that 96.29% of the percentage of genetic variation was attributable to variation within accessions and 7.52% among regions and 21.25 % to variation among populations within regions. Therefore, the highest proportion of genetic variation is within populations rather than between regions.

Table 3. Table 3 Hierarchical analysis of molecular variance (AMOVA) of microsatellite loci allele frequencies among accessions within and among different geographical origins..

Source of variation	d.f.[a]	Sum of squares	Variance components	% of variation	P-value[b]
Among regions	1	21.084	0.210	7.52	<0.001
Among populations/Within regions	10	59.801	0.595	21.25	<0.001
Within populations	58	156.5	2.698	96.29	<0.001

[a] The degree of freedom.
[b] The probability of null hypothesis

In order to further inquire genetic diversity, the pattern of genotypic relationships among genotypes, was calculated with a Bayesian-based approach by using the programme *Structure* 2.3.1. We analyze the Bayesian cluster analysis of the optimum K cluster. In our study, the K range from K = 1 to K = 10 and the probability was calculated for each value of K using the estimated log likelihood of K. The optimum clustering of membership of genotypes based on rate change of the likelihood function with respect to K was inferred at K = 3 (Fig. 3). Structure analysis divided the Tunisian olive genotypes in three groups which group I was marked (red), group II (green) and group III (blue). Theses clusters contained admixed genotypes. Furthermore, the close relationship among Chemlali cultivars was once again confirmed. The majority of the Southern cultivars was found in the red and green group and has a membership value close to 1.00, which appeared very structured and contained some of the most important cultivars which have admixed genotypes.
Most individuals from the group II were admixed linearly linked and had partial interference to entries from the blue group.

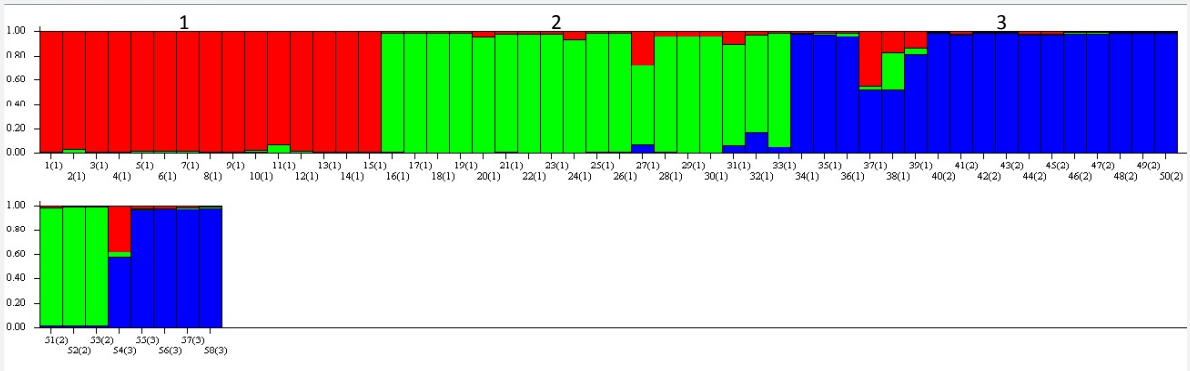

Figure 3. Bayesian cluster analysis of Tunisian olive accessions, as inferred at K = 3. Each olive accession is represented by a vertical bar.

The blue group (III) which appeared more admixed and contained cultivars from all the geographic area, containing 2 admixed cultivars Picholine and Meski. This cluster containing accessions according to their use. Cultivars from this group had complex genetic relationships which are in accordance to the dendrogram clustering (Fig. 2).

4. Discussion

Our study targeted cultivated olive trees growing in traditional groves of different main production zones in Tunisia and displaying different agro-ecological conditions.
Our results show that SSR markers can be successfully used on genetic variability of some Tunisian olive cultivated varieties. The seven primer pairs 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.
Our present study is largely in accordance and comparable with previous work (Bracci et al. 2009; Abdelhamid et al. 2012) using SSRs as genetic analysis method to assess the genetic variability of olive cultivars, including some minor exceptions

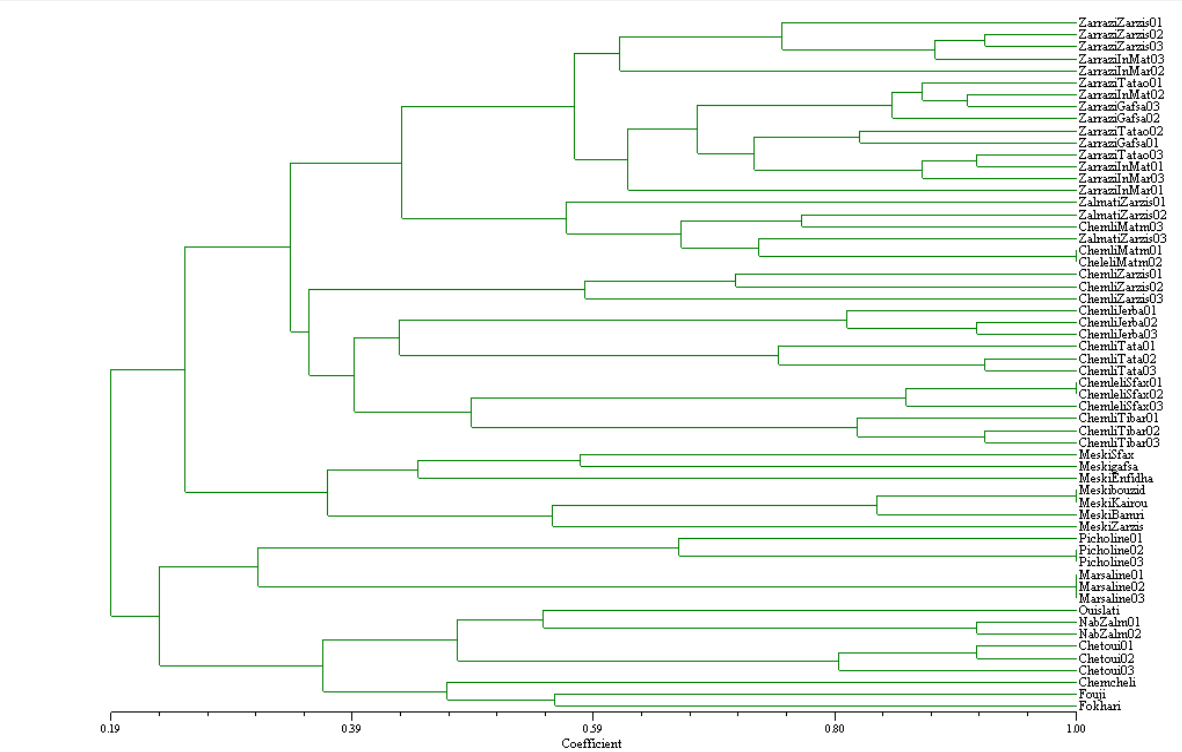

Figure 2. SSR dendogram of relationships among Tunisian cultivars.

For the 58 trees microsatellites displayed an average 7.29 alleles per locus. This is comparable to the 4-14 alleles found by Alba et al. (2009) for their microsatellite markers in a set of olive distributed in southern Italy. The average number of allele generated by loci found up to 13.5 alleles than has been reported by Belaj et al. (2011) for Spain wild olive. We can assume that olive trees have high genetic polymorphism and have undergone a different selection/domestication process in the Mediterranean regions. The five microsatellites developed by Sefc et al. (2000), DCA9, DCA14, DCA16, DCA17 and DCA18, gave from four (DCA9) to eight (DCA16 and DCA18) alleles, with an average of 6.6 alleles per locus (Table 2) which is similar to values reported by Alba et al. (2009).

Microsatellites Gapu 103 detects the highest number of alleles (14) than the others primers probably due the lower number of analysed samples. The co-dominant nature of SSR markers permitted the detection of high levels of expected heterozygosity (H_e) that ranged between 0.96 at locus DCA17 and 0.43 at DCA16, with a mean values of 0.77 consistent to those reported in literature (Diaz et al. 2006). These detailed analyses are important for establishing the fundamental utility of marker profiles for the purpose of cultivar identification and general genetic analysis resources. These results were in agreement with the results from earlier SSR markers applied to olive analyses witch scored high values of observed (H_o) and expected heterozygosity (H_e) (Ganino et al. 2007), indicating large genetic variation among Tunisian studied cultivars.

At loci DCA 17 we observed the lowest size range of alleles and the highest observed heterozygosity. This results could be explain by the fact that multiple unspecific products were amplified with high frequency by SSRs markers probably as a result of multiple priming sites along the genome (Rallo et al. 2000) and as possible complete genome duplication (Cipriani et al. 2002).

The Polymorphism Index Content (PIC) values were quite high ranging from 0.58 to 0.81 among loci (Table 2), in accordance with many previous studies (Taamalli et al. 2008), showing that these accessions are good source of diversity and these loci are suitable for mapping.

The total probability of identity (PI) value was very low (1.3×10^{-5}), representing that the 7 SSR markers used were useful for genotyping olive cultivars. The presence of "rare alleles" in our study showed great genetic variability within the studied cultivars.

The dendrogram revealed a clear separation of most Tunisian olive cultivars (Fig. 2). For instance, four main groups were observed. In the first group, most accessions were of Chemlali variety from the north and the south of Tunisia and contain all Zalmati and Zarrazi accessions. Chemlali genotypes are clustered in the same group reflecting the low degree of variability within the most common and oldest variety in the country and adjacent areas.

Actually, olive trees were assigned to each cultivar based on information obtained from farmers, on morphological analysis and on previous academic study. The denomination Chemlali is commonly used with the geographic area of origin from the south to the north (Table 1) and attributed to varieties with similar phenotypic characteristics such as the leaf, the shape of inflorescences and the shape of fruit. This classification may correspond to a bounding category of genetic material including clones and different genotypes. Hence, rather than a common variety, these local genetic material can be classified within a single polyclonal landrace variety called Chemlali as indicated by Chalak et al. (2015) in Lebanese olive genetic study by mean of SSRs and plastid DNA analysis for the Baladi cultivar.

Accessions from Chemlali (Zarzis, Tataouine, Sfax, Matmata, Jerba and Tataouine) and Zalmati were closely related as revealed in the dendrogramme. This clustering suggests that typical genotypes from the south may represent an ancestral shared origin and that sexual reproduction played a relevant role in shaping the genetic diversity of olive varieties in the early stages of olive tree spread (Albertini et al. 2011).

Southern accessions pairs displayed closely related genotypes which likely correspond to mutants of clones and confirm that these related genotypes may be derived from local selection with a narrow genetic base as these trees are very similar in fruit and leaf characteristics, as previously shown by Haouane et al. (2011).

The ambiguous genetic relationship present between and within Chemlali from the south and Zalmati, two cultivars grown in two narrow areas, are likely due to mutations on SSR alleles within a single clonal population for accessions belonging to the same original genotype, particularly when a clone is vegetatively multiplicated (Albertini et al. 2011; Haouane et al. 2011).

Our study show that cultivars used for oil and those used for table olive were significantly differentiated on the basis of SSRs markers. Clustering analysis among cultivars in the basis of their end use of olive for caning or olive oil production were in agreement with the results from earlier AFLP (Grati-kamoun et al. 2006) and RAPD (Besnard et al. 2001) markers analyses.

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. Currently, Meski is the most used cultivar for processing and is one of the most important and widely planted which covers more than two third of the area for table olives. Meski variety is known and appreciated for its taste and its technological traits. It is characterized by a spherical fruit with an excellent external appearance, very fleshy and tastes good.

The fourth group was formed by Chetoui, Nabli Zalmati, Ouislati, Fouji, Fokhari and Chemcheli suggest that olive cultivars have spread along wide agro-environments and are self-incompatible and usually propagated vegetatively. Therefore, it is evident that olive diversification has occurred in ancient times and is characterized by a wide genetic diversity that could be related to its domestication and spread, to some reproductive biological peculiarities as self-incompatibility, somatic mutations and sexual reproduction (Caruso et al. 2014). When the geographical criterion was tested, AMOVA analysis (Table 3) showed that most of total genetic diversity was attributable to differences within populations rather than between regions (96.29%). The proportion of diversity attributable to differences among regions had low values (7.52%), indicating a complete absence of genetic structure. AMOVA analyses showed similarities between cultivars from the north and the south of Tunisia and present clear geographic genetic distribution, it is likely that studied cultivars shared putative ancestors. The results agree with the general observation that olive maintains most of their variation within populations (Khadari et al. 2007). The correlation between genetic and geographic data was already stated by several authors and confirmed by our results, it is likely that studied cultivars share genetic relationships. It is possible that varieties with different genetic backgrounds tend to assume similar forms under the pressure exerted by human selection and agronomic conditions (Marra et al. 2013).

Moreover, Tunisian autochthon cultivars were clustered clearly in the dendrogram: a northern group and southern group suggesting that the locally cultivated olive trees are the result of local selection (Khadari et al. 2007). Tunisian olive groves are essentially dominated by two olive oil varieties: Chetoui in the north and Chemlali 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, Chemlali was widely cultivated (two-third of total olive area) and is vigorous and well adapted to arid regions (Trigui 1996). Furthermore, structure analysis, with a K value equal to 3, allowed inference of relationships among Tunisian cultivars. The STRUCTURE analysis, allowed us to obtain a clear inter and intra genetic relationships among studied accessions. The Southern and the Northern cultivars seemed to have a distinct genetic structure, more homogenous genetic makeup, possibly due to limited exchange or diffusion from the areas of origin. Eight cultivars: 4 accessions belonging to Zarrazi, 3 accessions belonging to Picholine and Meski Enfidha have a membership value lower than 0.1. These cultivars are known to be those having large fruits which are used as table olives. Similar results reported by Fendri et al. (2010) and may be explained by the fact that clustering of olive cultivars could be based on their usage and fruit size. Future studies could continue to explore and compare more Tunisian olive cultivars. In this study, microsatellite markers could potentially provide consistent information for cultivar identification and are valuable tools for studying the genetic relationships between olive cultivars. More primers preferably and others Tunisian or foreigner cultivars may be necessary for future researchers and should be compared with the present assay.

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