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Exploring olive trees genetic variability in the South East of Tunisia

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ABSTRACT. Tunisia is one of the most important olive growing countries in the Mediterranean basin and it is classified in fourth rank on world scale in terms of olive oil production. Since the emergence of the modern oliviculture, the identification, the evaluation and conservation of the local genetic resources have been considered as a priority in countries like Tunisia. Molecular studies for genetic characterization allowed to enhance knowledge about the autochtonous olive germplasm, but currently it lacks a systematic and extensive work to compare and validate Tunisian olive varieties. This work focused on the molecular characterization and identification of 21 olive accessions grown mainly in the South East of Tunisia using a set of 16 microsatellite molecular markers. An extent of authentication process was also carried out by comparison with molecular profiles from Tunisian cultivars already characterized from official collections obtained from a set of 12 commonly shared macrosatellite markers by different authors. Population structure analysis of the Tunisian olive germplasm was also conducted using structure software. The results highlighted that no correlation between geographical and genetic origin occurred. New alleles were individuated, and two new genotypes were characterized contributing to enhance the Tunisian germplasm biodiversity. This work

allowed individuating a few olive accessions that should be considered as reference cultivar in the authentication process and contributed to clarify the genetic pattern of the Tunisian germplasm.

Key words: *Olea europaea;* Genetic diversity; Microsatellite; Genetic relationships;South-Eastern Tunisia.

INTRODUCTION

Olive tree (*Olea europaea.* L), is a characteristic species of the Mediterranean region, with a high degree of genetic diversity. This species belongs to the family of Oleaceae which contains approximately 30 genera and 600 species (Bracci et al. 2011). It is assumed that first farmers began to select empirically wild olive trees, in particular those individuals most outstanding by traits such as fruit size, productivity and fat content and propagated them by vegetative propagation. The first olive varieties obtained in this way, were subsequently submitted to a progressive incorporation of genes by introgression from populations of wild olives present throughout the Mediterranean Basin. This phenomenon of natural hybridization allowed the continuous appearance of new varieties of olive (Fendri 2008). Tunisia is one of the most important olive growing countries in the Mediterranean basin and it is the fourth greatest olive oil global producer. The olive plantations are spread almost on the entire territory totaling approximatively 82425 million olive trees covering 1835 million hectares (Tunisian Agriculture Ministry 2016). It is the first producer and exporter of olive oil in the southern Mediterranean and it is ranked directly after the European Union with 20% world olive area growing (IOC 2016).

Tunisian olive orchards are spread over all agricultural land from North to South and East to West. The major cultivated varieties are ‘Chemlali Sfax’ in the South and the Centre and ‘Chetoui’ in the North representing the 95% of the olive germplasm and more than 90% of the olive oil national production (Abdelhamid et al. 2013; Hannachi et al. 2010; Grati-Kamoun et al. 2006).

Recently, horticulture-based agricultural systems need a continuous supply of new cultivars and improved varieties, drawn from the available gene pool adapted to drastic climate changes and sustain production systems on marginal lands, in order to enhance food security and obtain the product of quality which is demanded by producers and consumers consequently achieve balanced nutrition. Genes for desirable traits are embedded in biodiversity and as such crop genetic diversity has a critical role to play in increasing and sustaining production levels and nutritional diversity throughout the full range of different agro ecological conditions (Lutaladio et al. 2010).

Before the 90s of the previous century, no notable efforts has been made to study olive cultivars in Tunisia. However, since the emergence of the modern oliviculture, the identification, the evaluation and conservation of the local genetic resources have been considered as a priority in countries like Tunisia (Fendri 2008). Evaluation and characterization of olive genetic resources is crucial, since identification of olive cultivars is complicated by the large number of varietal synonyms and homonyms, the intensive exchange of plant material, the presence of varietal clones and problems of varietal certification in nurseries (Taamalli et al. 2006). Trigui and Msallem (2002) identified fifty-six varieties while Khlif Grati and Kamoun (2001) reported more than seventy varieties. Molecular studies for genetic characterization of Tunisian germplasm allowed to enhance knowledge about the autochthonous olive germplasm. Recently Abdelhamid et al. (2017) reported about 200 known varieties. In Tunisia there are 11 olive collections registered to the Fao website (FAO 2017) but the main collection is the Bank of germplasm ‘Boughrara’ from Sfax where more than 120 varieties are maintained (Fendri 2008). Other official collections are present where most of the worldwide olive germplasm is held: the olive collection from the Council for Agricultural research and Economics - Research Centre for Olive, Citrus and Tree Fruit (CREA-OFA) located in Mirto Crosia, Cosenza, Italy, the olive world germplasm banks (OWGB) in Cordoba, Spain and in Marrakech, Morocco (Haouane et al. 2011). Varieties are propagated asexually and sometimes through the process of exchanging plant materials, they have been inadvertently renamed. This had lead to the misidentification of genotypic diversity of intra- and inter-varietal cultivated olive trees, also within official collections (Zelasco et al. 2014). However, currently it lacks a systematic and extensive work aimed to authenticate olive accessions by comparing varieties in collections with genotypes collected in field.

In general, the important number of existing varieties and unknown accessions still to characterize, led to the need for a highly efficient technique of genetic analysis for the management of genetic resources in the success of breeding programs and for the protection of the commercial varieties quality label (Abdelhamid et al. 2013).

Molecular DNA markers reflect the variety of nucleotides in the genome of genotypes and are used to assess the genetic relationships among olive genotypes and cultivars in many recent research of the genetic diversity (Consolandi et al. 2007; Dìaz et al. 2006; Kaya et al. 2013). Simple sequence repeat (SSR) is one of the most recently molecular markers used in different studies to reveal the genetic variability between olive genotypes (Ipek et al. 2009; Işık et al. 2011; Linos et al. 2014; Abdessemed et al. 2015; Zhan et al. 2015). Microsatellites represent a powerful method for varietal characterization and have also been used to assess inter and intra-varietal variability (Roubos et al. 2010; Ercisli et al. 2011) and genetic population structure studies (Marra et al. 2013; Diez et al. 2015; Albertini et al. 2011). Currently, methods are being standardized by using genetic analyzers, producing a lot of reference alleles and adopting a common set of SSR markers leading to results more and more comparable (Salimonti et al. 2013).

This work focused on the molecular characterization and identification of 21 olive accessions grown mainly in the South East of Tunisia. Recently, a set of these olive accessions has been preliminary characterized (Ben Mohamed et al., 2017) using a set of 7 SSR markers. In this work. The main objectives of this study were: (i) to evaluate the genetic diversity of 21 local accessions grown in the South East of Tunisia (ii) to validate the olive accessions in comparison with cultivars from official collections (iii) to evaluate the overall genetic structure of Tunisian olive germplasm currently characterized.

MATERIALS AND METHODS

Plant material and local conditions

A total of 21 olive accessions Table 1 grown under different local areas in olive orchards from the South East of Tunisia were collected for this study (Figure 1). This region is characterized by an arid to semi-arid climate. The Beni khedache-Médenine and Toujane Matmata-Gabès regions are mountain areas while the other ones are mainly characterized by flat areas. The samples of young leaves were collected from 30-40 years-old olive trees spaced about 2-3m each other.

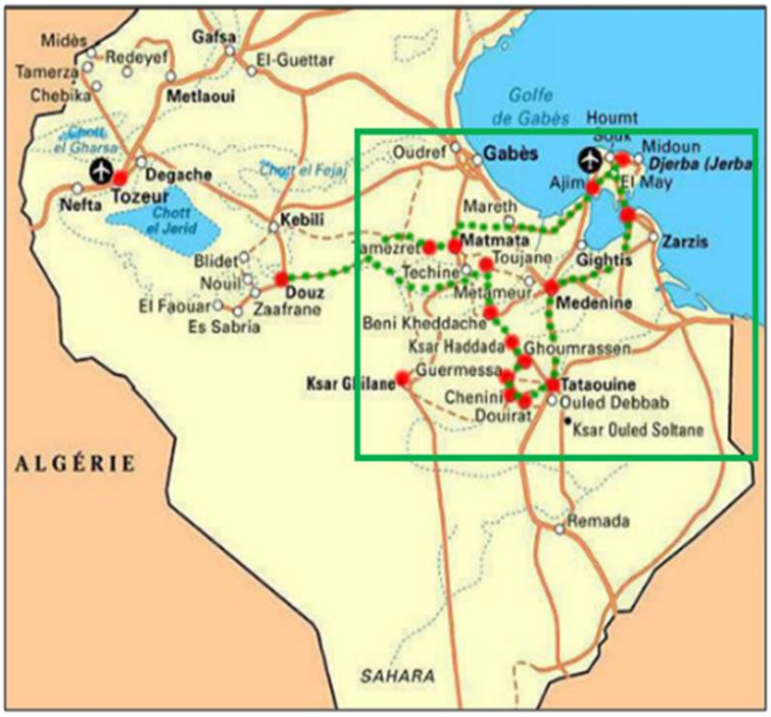

Figure 1. Geographical localization of sampled olive trees

Table 1. List of the olive accessions analyzed and their growing area

Code	Cultivar name	Growing area
1	Chemlali Abyath	Mareth-Gabès
2	Chemlali Djerba	El May-Djerba
3	Chemlali Douiret	Douirat-Tataouine
4	Chemlali Ontha	Douirat-Tataouine
5	Chemlali Zarzis	Zarzis
6	Dokhar el Gorthab	Tataouine
7	Fakhari	Tataouine
8	Fougi	Toujane Matmata-Gabès
9	Gousalani	Toujane Matmata-Gabès
10	Jemri Ben Guardène	Beni khedache-Médenine
11	Jemri Beni Khedache	Beni khedache-Médenine
12	Jemri Bouchouka Beni Khedache	Beni khedache - Médenine
13	Kemri Bouchouka Matmata	Toujane Matmata-Gabès
14	Neb Jmel	Médenine
15	Nourgou	Toujane Matmata-Gabès
16	Toffahi Mareth	Mareth-Gabès
17	Toffahi Matmata	Toujane Matmata-Gabès
18	Zalmati Zarzis	Zarzis
19	Zarrazi Zarzis	Zarzis
20	Zarrazi Injassi Mareth	Mareth-Gabès
21	Zarrazi Injassi Matmata	Matmata-Gabès

Microsatellite analysis

Sampled leaves were washed with distilled water, lyophilized, grinded to a fine powder and stored at -80°C until analysis. Total genomic DNA was extracted from leaves using a commercial kit (Plant DNA Mini Kit, Qiagen, Germany). The DNA quality was checked on 0.8% agarose gel and DNA concentration was determined by NanoDrop 2000 spectrophotometer (Thermo scientific). A set of sixteen labelled microsatellites (SSRs) were used. Different combinations of three SSR loci were used in multiplex PCR amplification strategy. DCA3-6Fam, DCA18-6Fam, DCA11-HEX, DCA16-6Fam, DCA13-Hex, DCA9-NED and DCA4-6Fam (Sefc et al. 2000), GAPU71B-6Fam, GPAU101-6Fam, GAPU59-6Fam, GAPU71A-6Fam (Carriero et al. 2002), UDO12-6Fam, UDO28-6Fam, UDO39-6Fam and UDO43-6Fam (Cipriani et al. 2002) and EMO090-6Fam (De La Rosa et al. 2002) loci were used in this work. These SSR markers were very informative and characterized by their highly polymorphic degree in Tunisian olive trees (Taamalli et al. 2006; Rekik et al. 2008; Ben-Ayed et al. 2009; Abdelhamid et al. 2013).

Multiplexed PCRs were carried out in 15 µL final volume using a thermal cycler (GeneAmp PCR System 9700 Applied Biosystems). Reaction mixture was composed of 10 ng of template DNA, 10X PCR buffer, 25 mM MgCl2, 2.5 mm dNTPs, 10 µM of forward and reverse primers and 5U/µl, Taq polymerase. PCR thermal profile was programmed as follows: a first step at 94 °C for 5 min, 35 cycles at 94 ºC for 30s, 60 ºC for 30s and 72 ºC for 40s. The last step included 7 min of incubation at 72 ºC.

PCR products (1 µL) were mixed with 12 µL of formamide and 0.4 µL of Gene Scan 500 internal standard (ROX). Denaturation was conducted at 95 °C for 5 min and amplification products were separated on an ABI PRISM Genetic Analyzer 3130xl (Applied Biosystems Inc., Foster City, CA, USA). Frantoio and Leccino authenticated cultivars were included into the analysis as internal reference to verify the correctness of molecular data. SSR fragments were analyzed by Gene Mapper 3.7 software (Applied Biosystems, USA).

The obtained data by scoring of SSR profiles were used to calculate a similarity matrix using Dice's coefficient (Sneath and Sokal 1973). The similarity values were utilized to determine the cluster analysis based an unweighted pair group method with arithmetic mean (UPGMA). Dendrogram, cophenetic correlation coefficient and principal coordinate analysis in according with Gower genetic distance were conducted using PAST software v.2.12. Cervus v.3.0.7 (Marshall 1998) software was used to analyze the number of alleles detected (Na), observed (Ho) and expected (He) heterozigosity, polymorphism information content (PIC), the probability of null alleles (r) and the exact tests for Hardy-Weinberg equilibrium corrected using Bonferroni method. The fixation index (F_{is}) was calculated according to the formula of Wright (1968):

F_{is}= 1- (Ho/He)

Authentication process and genetic structure analysis of the olive accessions

In order to carry out the authentication of Tunisian accessions here analyzed, molecular data obtained in this study, were compared with those from the internal CREA-OFA standardized database. It includes molecular data from the following authors: Trujillo et al. 2014; Fendri 2008; Besnard et al. 2014; Sarri et al. 2006, Diez et al. 2011 and oleadb database. Molecular data from four Tunisian cultivars (Bidh El Hammam, Chetoui, Yacauti, Zarrazi) held at the CREA-OFA olive tree collection previously analyzed using a set of 7 SSR markers (GAPU71b, DCA3, DCA18, DCA8, DCA5, UDO15, EMO090 and DCA9) in according the protocol above described were also used. Olive varieties included in the comparison and their provenience were shown in Table 2. Cluster analysis was conducted using molecular data selected among the most shared SSR markers used in literature. Molecular profiles coming from twelve SSR markers (GAPU71b, DCA3, DCA18, DCA8, DCA5, DCA15, UDO15, UDO12, EMO090, DCA11, GAPU59 and DCA9) were compared after harmonization. Standardized data were obtained by shifting of one or more single repeat for each allele in comparison with reference one. For the loci SSR GAPU71b, DCA3, DCA5, DCA9, DCA18, DCA15, GAPU71b and EMO090, reference alleles were taken from oleadb database; reference alleles for the loci SSR DCA11 and UDO12 came from Sarri et al. (2006) while for the locus GAPU59 were used those from Diez et al. (2011).

Table 2. List of olive accessions compared in this study: accession code, accession, author and provenience

Code	Author	Accession name	Provenience
1	Fendri 2008	Autre Zarrazi	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
2	Fendri 2008	Besbessi	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
3	Original data	Bidh El Hammam	CREA-OFA collection, Mirto Crosia, Italy
4	Fendri 2008	Chahla	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
5	Fendri 2008	Chemchali Gtar	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
6	Besnard et al. 2014	Chemchali-Jemri	World Olive Germplasm Bank, Cordoba, Spain
7	Sarri et al. 2006	Chemlali	World Olive Germplasm Bank, Cordoba, Spain
8	Baldoni et al. 2009	Chemlali	World Olive Germplasm Bank, Cordoba, Spain
9	Besnard et al. 2014	Chemlali	World Olive Germplasm Bank, Cordoba, Spain
10	Trujillo et al. 2014	Chemlali-744	World Olive Germplasm Bank, Cordoba, Spain
11	Original data	Chemlali Abyath	Field, Mareth-Gabès, Tunisia
12	Oleadb	Chemlali de Sfax	World Olive Germplasm Bank, Cordoba, Spain
13	Fendri 2008	Chemlali de Sfax	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
14	Fendri 2008	Chemlali Bahli	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
15	Fendri 2008	Chemlali Barrani	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
16	Fendri 2008	Chemlali Bouchouka	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
17	Fendri 2008	Chemlali Chouamekh	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
18	Original data	Chemlali Djerba	Field, El May-Djerba, Tunisia
19	Original data	Chemlali Douiret	Field, Douirat-Tataouine, Tunisia
20	Fendri 2008	Chemlali Ontha Tataouine	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
21	Original data	Chemlali ontha	Field, Douirat-Tataouine, Tunisia
22	Fendri, 2008	Chemlali Sig	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
23	Fendri, 2008	Chemlali Zarzis	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
24	Original data	Chemlali Zarzis	Field, Zarzis, Tunisia
25	Fendri 2008	Chetoui	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
26	Original data	Chetoui	CREA-OFA collection, Mirto Crosia, Italy
27	Trujillo et al. 2014	Chetoui	World Olive Germplasm Bank, Cordoba, Spain
28	Fendri 2008	Dhokar Nafti	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
29	Original data	Dokhar el Gorthab	Field, Tataouine, Tunisia
30	Fendri 2008	Ech -Chahla	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
31	Original data	Fakhari	Field, Tataouine, Tunisia
32	Fendri 2008	Fakhari tataouine	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
33	Original data	Fougi	Field,Toujane Matmata-Gabès, Tunisia
34	Trujillo et al. 2014	Gerboui	World Olive Germplasm Bank, Cordoba, Spain
35	Original data	Gousalani	Field, Toujane Matmata-Gabès, Tunisia
36	Fendri 2008	Horr Louzir	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
37	Fendri 2008	Injassi Hcichina	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
38	Fendri 2008	Jeddaria Chaal	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
39	Original data	Jemri Ben Guardène	Field, Ben Guerdane-Médenine, Tunisia
40	Original data	Jemri Beni Khedache	Field, Beni khedache-Médenine, Tunisia
41	Original data	Jemri Bouchouka Beni Khedache	Field, Beni khedache -Médenine, Tunisia
42	Original data	Jemri Bouchouka Matmata	Field, Toujane Matmata-Gabès, Tunisia
43	Fendri 2008	Lattout sned	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
44	Fendri 2008	Mallahi el Moummar	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
45	Fendri 2008	Marsaline	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
46	Besnard et al. 2014	Meski	World Olive Germplasm Bank, Cordoba, Spain
47	Trujillo et al. 2014	Meski	World Olive Germplasm Bank, Cordoba, Spain
48	Original data	Neb jmel	Field, Médenine, Tunisia
49	Fendri 2008	Neb Tataouine	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
50	Original data	Nourgou	Field, Toujane Matmata-Gabès, Tunisia
51	Sarri et al. 2006	Ouslati	World Olive Germplasm Bank, Cordoba, Spain
52	Oleadb	Ouslati	World Olive Germplasm Bank, Cordoba, Spain
53	Trujillo et al. 2014	Ouslati	World Olive Germplasm Bank, Cordoba, Spain
54	Fendri 2008	Semni Jbeniana	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
55	Sarri et al. 2006	Toffahi	World Olive Germplasm Bank, Cordoba, Spain
56	Oleadb	Toffahi	World Olive Germplasm Bank, Cordoba, Spain
57	Trujillo et al. 2014	Toffahi	World Olive Germplasm Bank, Cordoba, Spain
58	Original data	Toffahi Mareth	Field, Mareth-Gabès, Tunisia

59	Original data	Toffahi Matmata	Field, Toujane Matmata-Gabès, Tunisia
60	Original data	Yacauti	CREA-OFA collection, Mirto Crosia, Italy
61	Sarri et al. 2006	Zalmati	World Olive Germplasm Bank, Cordoba, Spain
62	Oleadb	Zalmati	World Olive Germplasm Bank, Cordoba, Spain
63	Besnard et al. 2014	Zalmati	World Olive Germplasm Bank, Cordoba, Spain
64	Trujillo et al. 2014	Zalmati	World Olive Germplasm Bank, Cordoba, Spain
65	Original data	Zalmati Zarzis	Field, Zarzis, Tunisia
66	Fendri 2008	Zarrazi	Bank of Germplasm ' Boughrara' , Sfax, Tunisia
67	Besnard et al. 2014	Zarrazi	World Olive Germplasm Bank, Cordoba, Spain
68	Original data	Zarrazi	CREA-OFA collection, Mirto Crosia, Italy
69	Original data	Zarrazi Injassi Mareth	Field, Mareth-Gabès, Tunisia
70	Original data	Zarrazi Injassi Matmata	Field, Matmata-Gabès, Tunisia
71	Original data	Zarrazi Zarzis	Field, Zarzis, Tunisia
72	Fendri 2008	Zeitoun Boubazzoula COI	Bank of Germplasm ' Boughrara' , Sfax, Tunisia

The genetic structure of population was determined using the same set of molecular data and STRUCTURE v.2.3.4 (Pritchard et al. 2000) software with K ranging from 1 to 10. The admixture model with correlated allele frequency, a burn-in length of 100.000 followed by 100.000 runs at each K, with three iterations for very K, were used. The true value of K was determined by Structure Harvester web version 0.6.93 (Earl and Vonholdt 2012).

RESULTS

Genetic diversity of Tunisian accessions

All markers produced polymorphic and reproducible amplification fragments which allowed the discrimination of the 21 studied olive accessions (Figure 2). Genetic diversity parameters were reported in Table 3. A total of 121 alleles over 16 loci was detected. The number of alleles at each locus (Na) varied from 4 for both loci Gapu71B and DCA16 to 12 for locus UDO43 with a mean of 7.6 alleles per locus. A total of 142 distinguished allelic combinations was detected ranging from 5 to 13 for GAPU71a and DCA4, UDO28, UDO43 loci, respectively. The loci SSR GAPU101 and UDO 43 allowed to distinguish the highest number of olive accessions discriminating 10 accessions Table 3.

Table 3. Parameters of genetic diversity for each SSR marker: size range in base pairs; allele number (Na); Number of discriminated: accession (NDA); range of allele frequency (F); observed heterozygosis (Ho); expected heterozygosis (He); fixation index (Fis); significativity of HWE test***p<0,01; *p<0,05; polymorphism information content (PIC); probability of null alleles (r).

Locus	Size range (bp)	Na	NDC	F	Ho	He	HWE	Fis	PIC	r
DCA3	232-253	6	3	0.023-0.357	1	0.756	NS	0.322	0.696	-0.163
GAPu71b	124-144	4	1	0.119-0.381	0.81	0.733	NS	-0.105	0.664	-0.058
DCA18	171-189	8	5	0.023-0.381	0.524	0.768	NS	0.317	0.713	0.198
DCA11	127-157	8	5	0.023-0.309	0.857	0.834	NS	-0.027	0.791	-0.02
EMO090	186-198	5	1	0.047-0.428	0.571	0.697	NS	0.18	0.625	0.068
DCA16	150-174	4	2	0.023-0.404	0.81	0.67	NS	-0.208	0.584	-0.112
DCA13	120-138	5	2	0.047-0.261	0.81	0.786	NS	-0.03	0.727	-0.026
UDO039	108-232	10	5	0.023-0.238	0.381	0.878	NS	0.566	0.842	0.376
UDO028	150-250	11	7	0.023-0.238	0.667	0.882	NS	0.243	0.846	0.127
DCA4	145-220	9	8	0.023-0.261	0.524	0.836	NS	0.373	0.793	0.215
DCA9	162-210	7	2	0.047-0.404	0.286	0.751	***	0.619	0.696	0.463
GAPU101	184-208	9	10	0.023-0.238	1	0.889	NS	0.124	0.835	-0.075
UDO12	150-166	8	9	0.023-0.261	0.857	0.873	NS	0.018	0.81	-0.009
GAPU59	203-223	8	3	0.023-0.238	0.952	0.857	NS	-0.11	0.812	-0.069
UDO43	172-220	12	10	0.023-0.19	0.905	0.92	NS	0.016	0.84	-0.003
GAPU71A	205-229	7	4	0.023-0.428	1	0.75	*	-0.333	0.67	-0.185
Total		121	77							
Average		7.6	4.812		0.747	0.805			0.75	

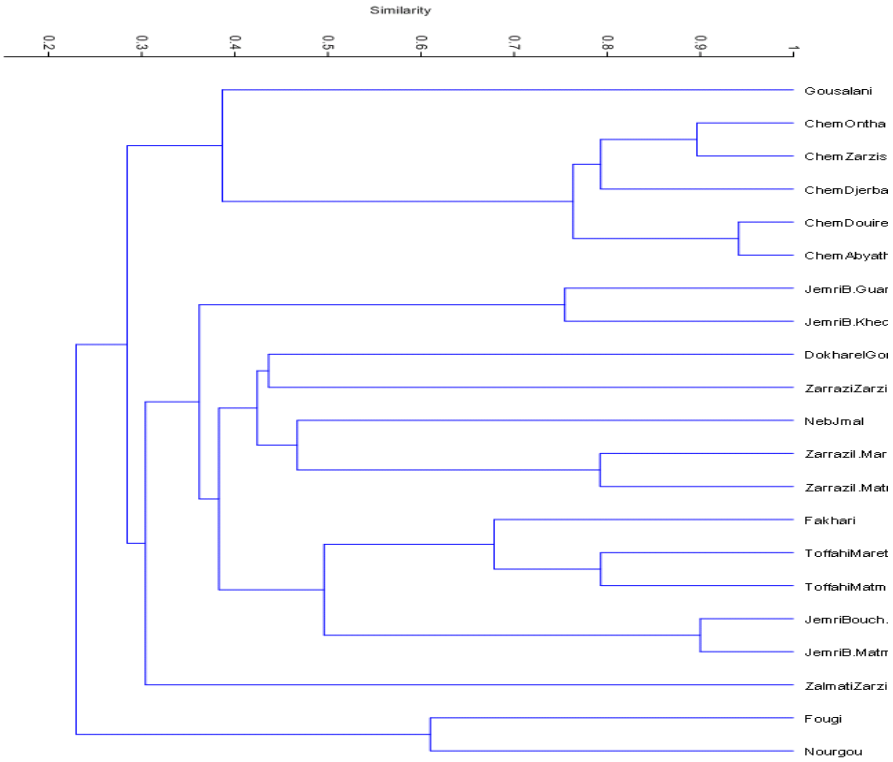

Figure 2. UPGMA dendrogram showing genetic similarity among the 21 olive accessions

To evaluate the ability of each locus to discriminate among accessions the polymorphism information content (PIC) was calculated. PIC ranged from 0.584 for the locus DCA16 to 0.846 for the locus UDO28 with an average of 0.75, confirming its ability to distinguish among the accessions (Table 3). Allele frequencies ranged from 0.023 to 0.43. The most frequent alleles were 188 bp and 215 bp alleles at the loci EMO090 and GAPU71a respectively, followed by 150 bp allele at the locus DCA16 and 182 bp allele at the locus DCA9 with a frequency of 0.404. The lowest allele frequency (0.024) was found in almost all the loci SSR analyzed indicating the presence of 19 unique alleles. Unique alleles sizing was shown in Table 4. The highest number of unique alleles was detected at the UDO43 locus, confirming its high polymorphism, and discriminating ability (Table 3). No unique alleles were found at the GAPU71b, EMO090, DCA9, DCA13 and GAPU59 loci but they showed however a low allele frequency. The observed heterozigosity (Ho) varied from 0.286 at DCA9 locus to 1 at DCA3, GAPU101 and GAPU71a loci with a lower average (0.747) than the expected heterozigosity (He) value that showed an average of 0.805 ranging from 0.67 to 0.88 respectively at DCA16 and UDO28 loci (Tab.3). The fixation index (Fis) ranged from-0.333 at the GAPU71a locus to 0.619 at the DCA9 locus with a positive mean value (0.123) that could be attributed to the null alleles presence. The probability of occurrence of null alleles values ranged from -0.009 to 0.46. Values > 0.20 of null allele frequency (r) were considered as a threshold over which a significant overestimation of He due to null alleles can be found (Chapuis and Estoup 2007). For all tested SSR markers, the r value was below this threshold except for the UDO039, DCA4 and DCA9 loci (Tab.3). Almost all SSR loci did not show significant deviation from the Hardy-Weinberg equilibrium except DCA9 and GAPU71a loci. The DCA9 locus showed the highest distorsion ($p<0.01$) from HW equilibrium showing an excess of homozygous with the lowest value of observed heterozigosity (0.286) and the highest positive fixation index value (0.619). On the contrary, the GAPU71a locus showed a weak excess of heterozygosity ($p<0.05$) showing the lowest negative value of fixation index (-0333) and the highest value of observed heterozigosity (1) together DCA3 locus and GAPU101 (Table 3).

The principal coordinate analysis of the 21 olive genotypes (Figure 3) generated a total variation of 46.42%; the first and the second principal coordinates explained 30.65 and 15.77 of the molecular variance respectively. ‘Nourgou’ (10) and ‘Fougi’ (16) cultivars appear as two genetically distinct genotypes as observed in cluster analysis. The ‘Gousalani’ (14) genotype was also separated from the rest of the cultivars in the PCoA scatter plot. All ‘Chemlali’ accessions (8,9,11,13 and 21) were discriminated along the coordinate 1 and placed in the plot area very close each other, indicating no significant variability among ‘Chemlali’ accessions. The remaining genotypes were included in a same big group in this analysis.

Table 4. Number of unique alleles found for each SSR locus and size in base pair

Locus	Unique alleles	Locus Size (bp)
DCA3	1	253
GAPu71b	-	
DCA18	3	179-181-189
DCA11	1	149
EMO090	-	
DCA16	1	156
DCA13	-	
UDO039	1	232
UDO028	3	180-220-250
DCA4	2	145-198
DCA9	-	
GAPU101	1	208
UDO12	1	164
GAPU59	-	
UDO43	4	182-192-212-218
GAPU71A	2	205-213
Total	19	

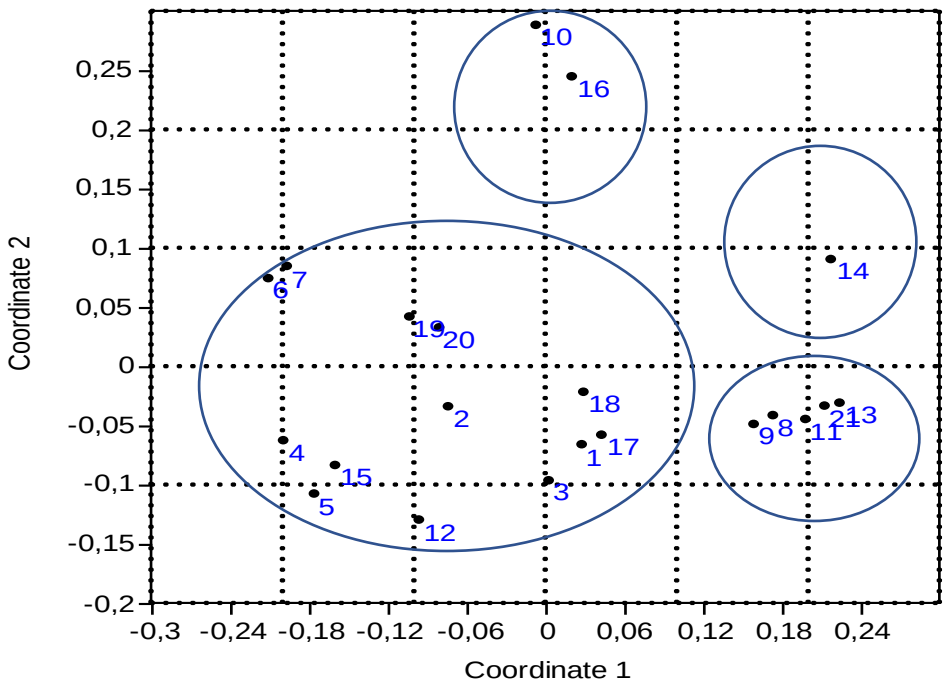

Figure 3. Zalmati Zarzis (1), Zarrazi injassi Matmata (2), Zarrazi injassi Mareth (3), Toffahi Mareth (4), Toffahi (5), Jemri bouchouka Matmata (6), Jemri bouchouka (7), Chemlali Douiret (8), Chemlali abyath (9), Nourgou (10), Chemlali ontha (11), Neb jmal (12), Chemlali Djerba (13), Gousalani (14), Fakhari (15), Fougi (16), Dokhar el Gorthab (17),Zarrazi Zarzis (18), Jemri Beni khedache (19), Jemri BenGuardène (20), Chemlali Zarzis (21)

The UPGMA cluster in Figure 2 showed two main groups with a wide genetic diversity. The first group included some well differentiated sub-groups. The first one included all the 'Chemlali' accessions and 'Gousalani' genotype. This last accession showed a genetic similarity index in comparison with 'Chemlali' accessions ranging from 0.31 to 0.5. The second sub-group included all the accessions named 'Zalmati', 'Zarrazi', 'Jemri', 'Toffahi', 'Fakhari', 'Neb Jmal' and 'Dhokar' with a wide range of genetic similarity ranging from 0.18 to 0.75. Two accessions 'Nourgou' and 'Fougi' clustered in the second group. The cluster and PCoa analysis lead to obtain a similar genetic pattern of the biodiversity found in the 21 olive accessions here

analyzed. No correspondence was found between geographical provenience and genetic origin among the analyzed olive accessions under local level.

The similarity degree between the 21 olive accessions using Dice's coefficient ranged from 0.07 to 0.94 showing a very high degree of inter-varietal genetic diversity. The minimum genetic similarity value was 0.07, observed between 'Toffahi', 'Matmata' and 'Gousalani' accessions. Although these accessions were collected under the same local growing area (Toujane Matmata-Gabès), they clustered in two well differentiated groups. High genetic dissimilarity was also observed between 'Gousalani' and 'Jemri beni khedache' with 0.11 genetic similarity value. Strict correlations were instead observed among 'Chemlali' accessions where genetic similarity index ranged from 0.72 to 0.94 revealing a putative clonal origin. The strictest correlation was observed between 'Chemlali Douiret' and 'Chemlali Abyath' accessions (0.94). Cophenetic correlation coefficient was 0.87 showing a good fit of the cluster analysis to the similarity matrix.

Authentication process and genetic structure of olive accessions

Cluster analysis was conducted using molecular data from 12 loci SSR widely shared in literature (see materials and methods) and present within the CREA-OFA internal database after a standardization procedure (Table S1). The dendrogram showed also in this case two main groups (Figure 4). The first group showed a wide genetic variability including a lot of differentiated sub-groups while the second one showed a restricted number of cultivars characterized by wide genetic diversity as well. The 'Zarrazi-Zarzis' (71), 'Zarrazi injassi Matmata' (70) and 'Zarrazi injassi Mareth (69)' accessions clustered with 'Zarrazi' coming from the Bank of germplasm 'Boughrara'(66), the Olive World Germplasm Bank of Cordoba (67) and the CREA-OFA collection (68). Dice's similarity index values ranged from 0.55 to 0.91, where consistent genetic diversity (0.55) was found only for 'Zarrazi-Zarzis 'accession (71) A soma clonal variation distinguished the 'Zarrazi injassi' accessions (69,70) from 'Zarrazi' (66,67,68) coming from the official collections: allele differences were observed at the loci DCA18 and DCA9 (Table S1). The 'Zarrazi' accessions from the Bank of germplasm 'Boughrara' and the CREA-OFA collection (66,68) did not show any genetic divergence each other by comparing of this set of SSR markers, while the 'Zarrazi' (67) analyzed by Besnard et al. (2014) was slightly different for two alleles at the loci DCA8 and DCA15 (Table S1).

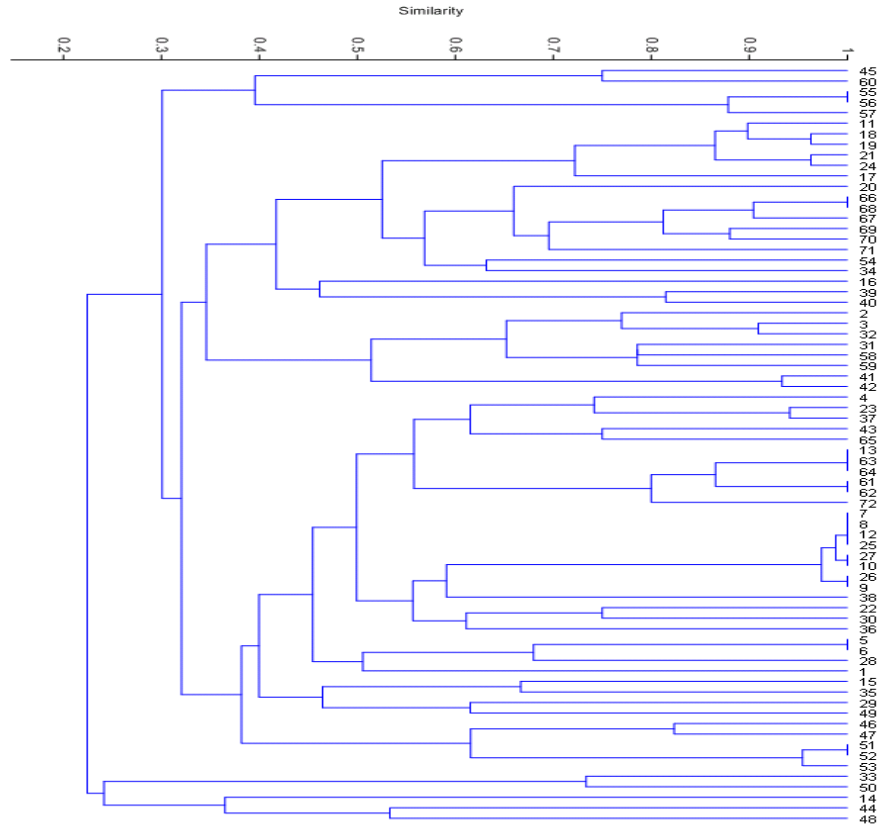

Figure 4. UPGMA dendrogram obtained using standardized molecular data from 12 SSR markers and comparing 72 Tunisian olive accessions

On the contrary, ‘Chemlali’ accessions (11,18,19,21,24) analyzed in this study clustered in a different group from the ‘Chemlali’(7,8,9,10,12,13,14,15,16,20,22,23) from the all olive collections, except ‘Chemlali Chouamekh’(17) from the Bank of germplasm ‘Boughrara’. However, all the other ‘Chemlali’(13,14,15,16,20,22,23) from the official collection of Sfax, clustered in different groups. The ‘Chemlali’(7,8,9) from the OWGB of Cordoba analyzed by different authors and ‘Chemlali-744’ (10) from the same collection, clustered very strictly with ‘Chetoui’ (25,26,27) from the all three official collections. Dice’s similarity index values ranged from 0.93 to 1. It is worthy to note that ‘Chemlali de Sfax’(12) from the OWGB of Cordoba clustered in the same group. Also in this case, ‘Chemlali’ (9) analyzed by Besnard et al. (2014) showed a difference for one allele at the locus DCA8 in comparison with the ‘Chemlali’ analyzed by Sarri et al. (2006) (Table S1).

The ‘Chemlali de Sfax’(13) from the Bank of germplasm ‘Boughrara’ showed a Dice’s similarity index values ranged from 0.14 to 0.67 in comparison with all the other ‘Chemlali’(7,8,9,10,11,12,14,15,16,17,18,19,20,21,22,23,24) and did not diverge from ‘Zalmati’ (61,62,63,64) from the Olive World Germplasm Bank of Cordoba, analyzed by different authors. The ‘Zalmati-Zarzis’ (65) accession here analyzed, clustered in a different sub-group than ‘Zalmati’ (61,62,63,64) from this collection.

The accessions ‘Toffahi Mareth’ (58) and ‘Toffahi Matmata’ (59) here analyzed, clustered in a different group than ‘Toffahi’ (55,56,57) from the OWGB of Cordoba, analyzed by different authors. These last two accessions showed indeed an association with ‘Fahkari’ (31) with a Dice’s similarity index value 0.79, indicating a enough strict relationship. The ‘Toffahi’ from the OWGB of Cordoba was analyzed by Sarri et al. (2006), Trujillo et al. (2014) and the allelic pattern is also published in the oleadb database indicated in Table 2 with corresponding code 55, 57 and 56. Although the same cultivar was analyzed, and data made comparable by standardization, a few weak allele differences were however observed at the loci DCA5 and DCA15 (TableS1).

The other accessions resulted well differentiated enhancing the biodiversity patrimony of Tunisian olive germplasm. In particular, from our knowledge, accessions such as those named ‘Nourgou’ and ‘Gousalani’ have never been described before within the Tunisian olive germplasm. The genetic structure of the Tunisian olive germplasm was determined by using structure software version 2.3.4 excluding synonymy cases. The smallest value of K found able to maximize the global like hood of data without overestimating population structure was K=2, indicating the presence of two big groups (Figure 5). The first group included ‘Chemlali’, ‘Fahkari’, ‘Toffahi’, ‘Zarrazi injassi’ and ‘Jemri’ here accessions analyzed and two accessions, ‘Fahkari tataouine’and ‘Zarrazi,’analyzed by Fendri (2008).

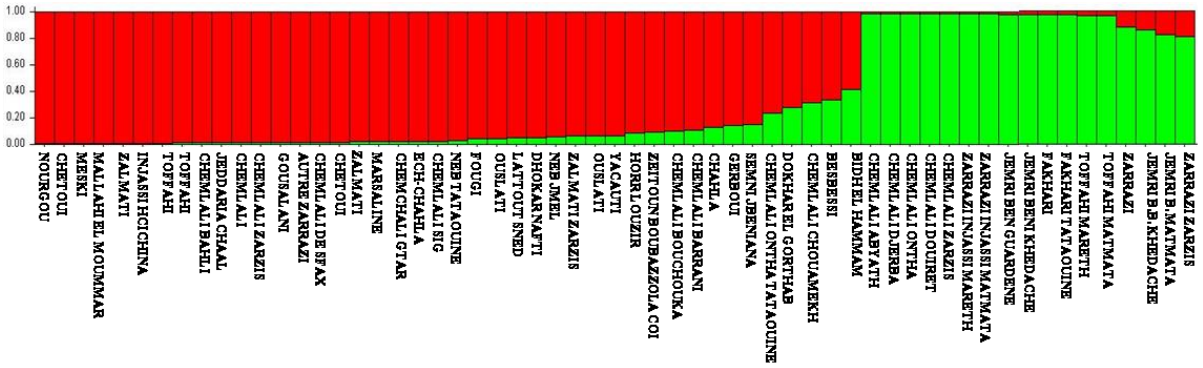

Figure 5. Genetic structure of the Tunisian olive accessions

The second group included all the other olive accessions analyzed by Fendri (2008), ‘Meski’ by Besnard et al. (2014); ‘Yacauti’ and ‘Chetoui’ from the CREA-OFA collection here analyzed, ‘Chetoui’,’ ‘Meski’, ‘Ouslati’, ‘Toffahi, ‘Gerboui’ by Trujillo et al. (2014), ‘Chemlali’,‘Ouslati’, Toffahi’, ‘Zalmati’ by Sarri et al. (2006) and, ‘Fougi’ ‘Gousalani’, ‘Neb jmel’, ‘Nourgou’, ‘Zalmati zarzis’ accessions here analyzed. A certain degree of admixture was found in ‘Dokhar el Gorthab’, ‘Zarrazi Zarzis’ and ‘Bidh El Hammam’ accessions here analyzed and ‘Besbessi’, ‘Chemlali Chouamekh’, ‘Chemlali Ontha Tataouine’ analyzed by Fendri (2008), but we attributed some admixed genotypes to low number of markers used. Cluster analysis results were in general confirmed by genetic structure analysis strengthening the thesis of absence of genetic structure within the Tunisian olive germplasm related to geographic provenience.

DISCUSSION

In Tunisia, olive (*Olea europaea* L.) is cultivated throughout the country where two major cultivars are present, 'Chemlali' in the South and in the Centre and 'Chetoui' in the North. However, Tunisia is rich in minor olive varieties and new olive genotypes are more and more individuated and characterized (Abdelhamid et al. 2017; Mnasri R.S. et al. 2014). This work represents a first investigation focused on the molecular characterization of 21 olive accessions collected in the Southeastern Tunisia with the main goal to evaluate their genetic diversity, population structure and attempt their authentication in comparison with Tunisian olive germplasm already characterized from official collections.

The results by SSR analysis of the 21 Tunisian olive accessions of this study, showed an important allelic variation over sixteen primer pairs tested and high overall genetic diversity, confirming that microsatellite markers can be effectively used to characterize and distinguish these olive accessions. The sixteen SSR markers showed a high polymorphic information content (PIC>0.5) which confirms that all tested SSR loci were polymorphic and suitable for genetic diversity analysis. The high polymorphism level was confirmed also by results reported in previous studies (Abdelhamid et al. 2013; Salimonti et al. 2013; Trujillo et al. 2014; Abdessemed et al. 2015; Zhan et al. 2015; Bahmani et al. 2016, Fendri et al. 2010). The genetic diversity indexes generated in this work, like the total number of alleles, the number of alleles per locus, the allele frequency, the high number of unique alleles, the expected heterozigosity level and the polymorphic information content, undoubtedly showed a high degree of genetic diversity of the Tunisian olive accessions here analyzed as reported by other authors (Fendri 2008; Abdelhamid et al. 2010). However, the expected heterozigosity was lower or comparable than to observed heterozigosity for the most of SSR markers used with a probability of occurrence of null alleles value negative or really close to zero, indicating a good reliability of the results.

Different authors reported the highest discriminating ability of the locus DCA9 to differentiate Tunisian olive accessions (Taamalli et al. 2006; Abdelhamid et al. 2017) showing a very high observed heterozigosity level. On the contrary the locus DCA9 showed a significant distorsion from HWE with a very high probability of occurrence of null alleles. Actually, we attributed this abnormal behavior to the methodology adopted, because when multiplex PCR amplification protocols are performed, allelic dropout may occur. However, we observed a certain degree of homozigosity at the DCA9 locus comparing the allele pattern of the Tunisian olive accessions here considered and those analyzed by Fendri (2008).

A lot of unique alleles were detected in this study confirming the results obtained by Abdelhamid et al. (2017). The 189 bp allele at the locus DCA18, the 180 bp and 220 bp alleles at the locus UDO028, the 192 bp allele at the UDO043 locus and the 205 bp allele at the locus GAPU71A not only resulted as new putative alleles within Tunisian olive germplasm but they were not found into the CREA-OFA database (data not shown). Two of these alleles were found exclusively in the 'Nourgou' accession, confirming the hypothesis that it could be a new genotype. The other unique alleles found within the 21 Tunisian olive accessions were also detected into the CREA-OFA database for known worldwide cultivars showing that they are to be considered true alleles (data not shown).

The cluster analysis showed no correspondence between geographical provenience and genetic relationships among the 21 analyzed olive accessions in the South East of Tunisia as expected, considering also the small extension of the area under study even though some geographic barriers can be detected. The high inter-varietal genetic variability was due probably to exchange of material under this local condition. Other authors (Abdemhamid et al. 2017, Fendri 2008, Ben Hayed et al. 2015a; Grati-Kamoun et al. 2006) asserted the absence of genetic structure within Tunisian olive germplasm related to geographic provenience.

High genetic inter-varietal diversity was also highlighted by PCoA analysis where three accessions were significantly different: 'Fougi', 'Nourgou', and 'Gousalani'. These two latter accessions were not ever described before and should be considered new candidate varieties. In general, almost all the accessions here analyzed showed a genetic diversity such to consider them as varieties coming from sexual reproduction.

In this study the Tunisian olive accessions from three official collections and analyzed by more than one author, were used as reference in order to attempt the authentication of 21 Tunisian olive accessions collected in the South East of Tunisia. Molecular data obtained in this study were compared with those found in literature after a harmonization procedure. In our study, weak allele differences were discovered when the molecular profiles obtained by different authors for the same olive accession from the official collection were compared. The standardization process was first used by Doveri et al. (2008) in order to compare molecular data coming from

different laboratories and they found little discrepancies among laboratories at one allele and at two alleles in 13.2% and 8.8% of cases, respectively.

Genotyping errors could be due to different causes such as DNA variation in the sequence recognized by primer, artefacts due to the Taq polymerase errors (slippage, Adenine addition) and human errors (Pompanon et al., 2005). Di-nucleotide nature of the most of the micro satellite markers isolated from olive genome can lead to occur more frequently a stuttering phenomenon that usually complicates the correct sizing of alleles (Zelasco et al. 2014). In order to take under control, the stuttering phenomenon, the standardization is a crucial step, but it does not always guarantee the correct interpretation of the peaks. Baldoni et al. (2009) indicated that genetic profiles which differ by few alleles could be also assigned to a single genotype. We found only for the DCA8 locus a relatively high stuttering level, but we recommend excluding this SSR from the molecular analysis in order to avoid confusion in the genetic profile interpretation.

The variation in DNA flanking region of the repeated sequence leading to a failure of the PCR amplification and consequent occurrence of null alleles (Pompanon et al., 2005). The DCA15 locus showed to be less comparable than other SSR markers as well, because it failed, in some cases, the second allele amplification (allelic dropout). Even though the allelic dropout is usually attributed to a mutation in the flanking region of the SSR locus or a DNA degradation, we are more likely to believe, from our experience, that it is due to the multiplex amplification approach.

High correspondence was found for the following loci DCA3, GAPU71b, DCA9, DCA18 and EMO090 when genetic profiles from the same cultivar obtained in different laboratories were compared. Considering the high reproducibility of molecular data obtained after harmonization process, we speculate that this set of SSR markers could be reliable for genetic identification purposes within official protocols, confirming further the results obtained by Baldoni et al. (2009).
The Zarrazi Injassi accessions clustered with the ‘Zarrazi’ from official collections and should be considered as soma clonal variants of this cultivar. The ‘Zarrazi Zarzis’ accession has to be considered as admixed genotype confirmed also by genetic structure analysis. The ‘Zarrazi’ group in general clustered in the same group of the ‘Chemlali’ accessions here analyzed. These results are consistent with those from Abdelhamid et al. (2017).

The ‘Chemlali’ accessions here analyzed represented a small group with highly shared gene pool probably due to their clonal origin and we suggest naming this group of accessions, collected in the Southern eastern Tunisia (Zarzis, Mareth, Djerba, Douirat), with an unique denomination in order to avoid confusion afterwards. When ‘Chemlali’ accessions here studied were compared with ‘Chemlali’ group analyzed by the other authors, cluster analysis distinguished clearly each other’s, except for ‘Chemlali Chouamekh’ by Fendri (2008). Trujillo et al. (2014) reported that the accession Chemlali-744, after the identification process, was determined to be almost identical to cultivar Chetoui within the OWGB collection of Cordoba. Our results clearly showed that all the molecular profiles obtained before this report (Trujillo et al. 2014) from the accession named ‘Chemlali’ or ‘Chemlali de Sfax’ coming from the OWGB of Cordoba are now to refer to ‘Chetoui’ cultivar. This is clearly a case of synonymy because the two main Tunisian cultivars ‘Chemlali’ and ‘Chetoui’ results well differentiated at morphological (Trigui and Msallem 2002) and at molecular level by nuclear and plastid molecular markers (Hannachi et al. 2010, Ben Hayed et al. 2015). It is worthy to note that also the ‘Chetoui’ accessions from the CREA-OFA collection and Bank of germplasm ‘Boughrara’ from Sfax were almost identical to ‘Chetoui’ held in the OWGB of Cordoba.

The accession ‘Chemlali de Sfax’ by Fendri (2008), associated to ‘Zalmati’ from the OWGB of Cordoba or vice versa. This could be considered apparently a case of misnaming; actually, the accession ‘Chemlali de Sfax’ from the Bank of germplasm ‘Boughrara’ of Sfax, should be considered as the true cultivar to which it refers, because it was authenticated by an extensive morphological and molecular study in comparison with other local accessions (Trigui et al. 2002; Fendri 2008, Fendri et al. 2010). Ben Ayed et al. (2015b), Fendri (2008) and Rekik Hakim et al. (2010) showed the Zalmati variety was not genetically and morphologically different from ‘Chemlali’/’Chemlali de Sfax’ indicating a clear case of homonymy. Only Abdelhamid et al. (2017) distinguished these genotypes each other but attributing their genetic diversity to clonal differences.

The ‘Zalmati Zarzis’ accession here analyzed did not fit with ‘Zalmati’ from the OWGB of Cordoba or the ‘Chemlali de Sfax’ by Fendri (2008), suggesting that this accession could belong to ‘Zalmati’ cultivar. Similar results were obtained for the ‘Fakhari’ and ‘Toffahi’ accessions in comparison with the putative reference cultivars held in the official collections. From our results, these last two accessions showed a relationship as showed by Rekik Hakim et al. (2010) who analyzed olive accessions from four Tunisian collections.

Taking in account the olive germplasm held in the official collection, from our results, 'Chemlali de Sfax' from the Bank of germplasm 'Boughrara' of Sfax, 'Chetoui' and 'Zarrazi' from the OWGB of Cordoba, CREA-OFA collection of Mirto Crosia and Bank of germplasm 'Boughrara' of Sfax must be consider as reference cultivars. Genetic structure results were slightly different from those obtained by Abdelhamid et al. (2017). In this study a low number of SSR markers was used for the genetic structure analysis influencing the bayesan-approach (Porras-Hurtado et al. 2013). However, the structure analysis confirmed the genetic diversity observed for the 'Chemlali' accessions here analyzed in comparison with the 'Chemlali' from the official collections. The genetic pattern shown in this work did not allow us to individuate a unique molecular profile for 'Chemlali. Our results suggested a wide genetic diversity probably due to natural hybridization process. A similar wide genetic variability was also observed by Abdelhamid et al. (2013) using the same molecular markers and by Grati-Kamoun et al. (2006) who used AFLP markers. In the past, farmers used to name olive trees related to peculiar morphological traits. For instance, 'Chemlali' word means 'small fruit' in Old Spanish language and for this reason a lot of accessions were named 'Chemlali' (Fendri 2008). Furthermore, when geographic proveniences were added, the correct genetic identification of the Tunisian germplasm became more complicated.

CONCLUSION

In conclusion, our results did not allow us to authenticate the accessions here analyzed except for 'Zarrazi injassi' accession in comparison with those held in the official collections. More than one accession has to be collected under local condition in order to lead a crucial step of authentication. Furthermore, it raises more an more the issue about the need to include a reference cultivar to carry out a correct identification process using a robust set of SSR markers. However, this study contributed to individuate three reference cultivars, crucial step to carry out the authentication process.

Genetic structure of the Tunisian olive germplasm was also evaluated highlighting that no correlation between geographical and genetic origin was observed. New alleles and putative varieties were individuated contributing to enhance the Tunisian germplasm biodiversity. Although molecular information about Tunisian olive germplasm is not enough to carry out an exhaustive authentication process, this work allowed to individuate some olive accessions and cultivars that should be considered as reference and contributed to clarify the genetic pattern of the Tunisian germplasm.

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