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Morphological and molecular characterization of minor olive (*Olea europaea*. L) cultivars in Tunisia

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Abstract

Management of *ex situ* olive collection is crucial for suitable genetic resources conservation. This study aims to complement characterization of olive accessions belonging to the National olive germplasm collection located in the south of Tunisia. The morphological characterization of endocarp of 22 Tunisian cultivars was made. Multiple component analysis (MCA) showed 5 different groups of cultivars and one case of synonymy. A set of six Simple Sequence Repeat markers (SSR) were used to fingerprinting the cultivars studied. The total number of alleles was 44 with a mean of 7.33 alleles/locus. The polymorphism information content (PIC) varied from 0.45 for DCA 03 to 0.85 for DCA 16 with a mean of 0.7. The Principal Component Analysis (PCoA) was used to cluster cultivars. We assume the power of the microsatellite markers and the phenotypic richness of our local germplasm in Tunisia.

Keywords: *Olea europaea*, endocarp characterization, microsatellite.

Résumé

La gestion de la collection d'oliviers *ex situ* est cruciale pour une bonne conservation des ressources génétiques. Cette étude vise à compléter la caractérisation des accessions d'oliviers appartenant à la Collection nationale de ressources génétiques oléicoles située dans le sud de la Tunisie. La caractérisation morphologique de l'endocarpe de 22 cultivars tunisiens a été faite. L'analyse en composantes multiples (AMC) a montré 5 groupes différents de cultivars et un cas de synonymie. Un ensemble de six marqueurs de répétition de séquence simple (SSR) a été utilisé pour identifier les cultivars étudiés. Le nombre total d'allèles était de 44 avec une moyenne de 7,33 allèles/locus. Le contenu en informations sur le polymorphisme (PIC) variait de 0,45 pour le DCA 03 à 0,85 pour le DCA 16 avec une moyenne de 0,7. L'analyse en composantes principales (PCoA) a été utilisée pour regrouper les cultivars. Nous supposons la puissance des marqueurs microsatellites et la richesse phénotypique de notre matériel génétique local en Tunisie.

Mots-clés: *Olea europaea*, caractérisation de l'endocarpe, Microsatellite

1. Introduction

The cultivated olive tree (*Olea europaea* subsp. *europaea*, var. *europaea* L.) is one of the most important oil crops in the world being (Green, 2002). The Mediterranean basin produces near by the 95% of total olive oil production (Marra *et al*., 2013; Trujillo *et al*., 2014). The cultivated olive (subsp. *europaea*) is an evergreen tree with extreme longevity adapted to Mediterranean climates. The olive species counts a very rich varietal heritage. It is represented by more than 1200 named cultivars, over 3000 minor cultivars and an uncertain number of genotypes including pollinators, local ecotypes and centennial trees (El Bakkali *et al*., 2013; Hosseini-Mazinani *et al*., 2014; Mazzitelli *et al*., 2015; Laroussi-Mezghani *et al*., 2016, Mousavi *et al*., 2017).

This great genetic diversity is the result of empirical and local selection of exceptional trees since the olive has been domesticated about 6000 years ago in the Middle East (Zohary and Spiegel-Roy, 1975). The intensive exchange and dissemination of plant material throughout the Mediterranean basin with similar climatic conditions is the origin of the presence of synonyms and homonyms. Tunisia is formerly a major producer of olive oil in North Africa with about 102 millions olive trees, which occupy the third part of cultivated areas (DGPA, 2023). Most of the producing areas are represented by two prevalent oil cultivars 'Chétoui' and 'Chemlali', while the rest is represented by several minor cultivars. As most of traditional olive-producing countries (Rallo *et al*., 2005), Tunisia includes a broad diversified genetic resources, which are mainly formed by small distributed autochthonous cultivars. Genetic erosion and loss of biodiversity seem to be major issues for olive germplasm due to the absence of turnover of new genotypes that do not occur as fast as in other woody crops. *Ex situ* conservation of genetic resources is important to preserve adaptive characters, to prevent genetic erosion and extinction of local varieties as well as to enable the use of outstanding accessions with interesting genes in breeding and selection programs. The species *Olea europaea* includes several subspecies with different morphological traits and geographical origins (Green, 2002). Morphological and biometric characters have been widely used to describe olive germplasm (Cantini *et al*., 1999). Traditionally, these traits are used to identify olive cultivars (Mehri and Hellali, 1995; Barranco *et al*., 2000; Trigui and Msallem, 2002). However, this is a slow process due to the long juvenile period of the trees besides the morphological and agronomic traits are subjected to environmental influences. Molecular studies offer complementary data to characterize germplasm. At a later stage DNA markers such as Restriction Fragment Length Polymorphisms (RFLP) (Besnard *et al*., 2001), Random Amplified Polymorphic DNAs (RAPD) (Vergari *et al*., 1998; Hess *et al*., 2000; Belaj *et al*., 2001) and Inter Simple Sequence Repeats (ISSR) (Hess *et al*., 2000) have been carried out and used for olive cultivar identification. Microsatellite or Simple Sequence Repeat (SSR) are currently used for characterization of different plant species because of their abundance, high polymorphism content, codominance, ease of detection and transferability across studies. These positive features make microsatellites the markers of choice in studies on population genetics and for the fingerprinting of individuals (Rafalski *et al*., 1996). More recently, several microsatellites have been isolated from olives (Rallo *et al*., 2000, Sefc *et al*., 2000, Carriero *et al*., 2002).

Microsatellite markers are widely used for the study of genetic diversity of olive (Saddoud Debbabi *et al*., 2020; 2021). Belaj *et al*. (2012) joined molecular marker (SSR, SNP and DArT) data and agronomical traits to analyze 361 olive accessions of the germplasm bank in Cordoba (Spain), defining their genetic diversity and structure. This study aims the identification of olive genotypes collected in the national collection of olive tree at Boughrara, Sfax. This collection is considered one of the most important in the Mediterranean region. It is currently composed of 200 genotypes and varieties, of which 146 accessions have Tunisian origin and 54 accessions have foreign origin (Ben Amar *et al*., 2019). The correct characterization of this diversity is essential for the better management of the national germplasm and for genetic breeding programs. In this study, we used the six markers that have been described by Trujillo *et al*. (2014). The present study aims to identify and classify 22 olive accessions obtained by a national exploration survey and introduced in the "Boughrara"-Sfax olive germplasm collection (South of Tunisia).

2. Material and Methods

2.1. Plant material

We have studied twenty-two accessions of cultivated olive (*Olea europaea* L.) belonging to the national olive tree collection (Table 1): "Conservatoire National de l'Olivier de 'Boughrara'-Sfax" South of Tunisia (belonging to Olive Institute of Tunisia). The collection was maintain under rainfed condition with planting distance 8×12 m, and regular agricultural practices are applied to the olive plants, without irrigation. As a reference, we choose 06 foreign varieties that located at the Aula Dei Experimental Station-CSIC in Zaragoza (Spain).

Table 1. Codes, names and origin of 22 local olive cultivars collected from different regions in Tunisia and preserved at the national collection of Boughrara-Sfax.

Code	Cultivar Name	Region
BG1	Mlouki Blettech	Sfax
BG2	Mfartah Blettech	Sfax
BG3	K 15	Sfax
BG4	K 55 pg	Sfax
BG5	K 11 pg	Sfax
BG6	K 6	Sfax
BG7	Bl 6	Sfax
BG8	Bl 17	Sfax
BG9	Bl 4	Sfax
BG10	Bl 28	Sfax
BG11	Sig 11	Sfax
BG12	Sig 17	Sfax
BG13	Jemri dhokar	South (Medenine)
BG14	Zarrazi injassi	South (Tataouine)
BG15	Dhokar BG	South (Medenine)
BG16	Fouji Asli	Gafsa
BG17	Zarrazi Sned 5	Gafsa
BG18	Baldi Gtar	Gafsa
BG19	Zarrazi Sned 4	Gafsa
BG20	Sayali	North (Nabeul)
BG21	Meski	North (Manouba)
BG22	Barouni	North (Nabeul)

2.2. Endocarp characterization

For endocarp description, 9 characters were selected from the pomological pattern widely used for olive cultivar characterization (Fendri *et al*., 2010).

2.3. DNA extraction and molecular assay

DNA was extracted from frozen leaves in liquid nitrogen and grinding the leaves using the Qiagen DNeasy Plant Mini Kit following manufacturer's instructions. A set of six microsatellite markers (ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA15, ssrOeUA-DCA16, ssrOeUA-DCA18 (Sefc et al., 2000) and GAPU 101 (Carriero et al., 2002) were used for fingerprinting the olive cultivars. For each primer pair, the sequence of the original published forward primer was redesigned by adding a universal M13 (-21) tail (5'-TGT AAA ACG ACG GCC AGT-3') to their 5' ends (Schuelke, 2000). The third, universal M13 (-21) primer was labelled with Cy5, allowing fluorescence detection. Amplification reactions were carried out in a total volume of 50 µl, containing 50 ng genomic DNA, 5X supplied PCR buffer (BIOLINE), 2 mM of dNTP, Taq DNA polymerase 5 unit (BIOLINE), 2 pmoles of sequence-specific reverse primer, 8 pmoles of fluorescence labelled universal M13(-21) sequence and 8 pmoles of forward primer with M13(- 21) tail. The amplification protocol for loci ssrOeUA-DCA3, ssrOeUA-DCA10, and ssrOeUADCA16 was carried out according to Bandelj *et al*. (2002), with some modifications, and performed in a C 1000 thermal cycler (Bio-Rad). The conditions of the two-round PCR amplification were as follows: 94 °C (5 min), then 30 cycles at 94 °C (30 s)/ 52°C (45 s) / 72 °C (45 s). Followed by 8 cycles at 94 °C (30 s) / 53 °C (45 s) / 72 °C (45 s). A final extension step at 72 °C for 10 min. PCR products were separated in an automatic capillary sequencer, ABI PRISM 3100-AVANT GENETIC ANALYSER, using a definite size marker: GeneScan-500 ROX Size Standard.

2.4. Data analysis

Estimation of genetic indices was estimated using GenAlEx software v.6.5 (Peakell and Smouse, 2012). We have estimated number of alleles (Na), effective number of alleles (Ne), Shannon's diversity index (I) (Shannon and Weaver, 1948), observed heterozygosity (Ho), expected heterozygosity (He) (Nei 1978), fixation index (F) and the Polymorphic Information Content (PIC) (Botstein et al., 1980). The genetic relationships between the 22 accessions were estimated and principal coordinates analysis (PCoA) was carried out using GenAlEx. Using R software 3.6.1 (2019). Nei genetic distance was estimated. The genetic distances between the 22 genotypes were constructed based on UPGMA. To determine the support for each node a 100 replicate bootstrapping was performed.

3. Results

3.1. Morphological characterization

We measured 09 traits regarding endocarp (Table 2). Results showed a large variability for all parameters except for the shape and the base which were rounded for almost the endocarps.

Table 2. Morphological characteristics of 22 endocarp of Tunisian olive cultivars according to the International Olive Council.

Origin	Cultivars	Weight	Shape	Sy A	PMD	Apex	Base	Surface	NVB	DVB
Sfax	Mlouki Blettech	VH	EP	SA	C	R	R	S	H	R
	Mfartah Blettech	H	EL	A	C	P	P	S	H	G
	k6	M	EP	SA	C	R	R	S	H	R
	k15	M	EP	SA	C	R	R	S	M	R
	k11pg	M	EP	SA	C	P	R	S	H	R
	K55pg	M	EP	SA	C	R	R	R	M	G
	Bl 6	M	EP	A	C	P	R	S	H	G
	Bl 17	M	EL	A	A	R	P	R	M	G
	Bl 4	M	EL	A	C	P	R	S	H	R
	Bl 28	M	EP	A	B	P	R	S	H	G
	Sig 17	M	EP	SA	C	R	R	S	H	R
	Sig 11	M	EP	SA	A	R	R	R	M	R
Sud	Zarrazi injassi	VH	EL	SA	C	R	R	R	H	G
	Jemri dhokar	H	EL	SA	C	R	R	R	H	G
	Dhokar BG	H	EP	SA	A	R	R	S	H	R
Nord	Sayali	H	EL	A	B	P	R	S	H	R
	Meski	VH	EP	S	C	R	R	R	M	R
	Barouni	VH	EP	SA	C	R	R	R	H	G
Gafsa	Zarrazi Sned 4	VH	O	SA	C	R	R	R	H	G
	Zarrazi Sned 5	H	O	S	C	R	R	R	H	R
	Fouji Asli	VH	EL	A	A	R	R	R	H	G
	Baldi Gtar	VH	O	SA	C	R	R	R	H	R

Weight= low = L (Less than 0.3 g); medium = M (0.3 to 0.45 g); high = H (0.45 to 0.7 g); very high = VH (greater than 0.7 g).
Shape= spherical = S (length/ width less than 1.4); ovoid = O (length/ width 1.4 to 1.8); elliptic = EP (length/ width 1.8 to 2.2); elongated = EL (length/ width greater than 2.2).
Sy A= Symmetry of position A: symmetric = S; slightly asymmetric = SA; asymmetric = A
PMD= Position of the maximum transversal diameter: toward the base = B; centered = C; toward the apex = A.
Apex: pointed = P; rounded = R.
Base: pointed = P; rounded = R.
Surface= Roughness of the surface: smooth = S; rough = R
NVB= Number of vascular bundles over the surface: low = L (Less than 7); medium = M (7 to 10); high = H (greater than 10).
DVB=Distribution of vascular bundles over the surface: regular = R; grouped together in the suture = G.

3.2. Molecular characterization

All accessions were fingerprinted for the six selected microsatellite markers. Microsatellites showed a high level of polymorphism throughout the analyzed accessions and revealed the existence a total of 44 alleles. As reported in Table 3, the number of alleles per locus ranged from 03 for DCA03 to 10 alleles for DCA16 and DCA09 with a mean of 7.33 alleles per locus. Overall, the size of alleles ranged from 122 bp for DCA16 to 268 bp for DCA15. The mean value of the observed

heterosygosity (Hobs) for all loci was 0.71, varying between 0.5 (DCA15) and 0.9 (DCA16). Based on PIC values (0.45 (DCA 3) to 0.85 (DCA 16)), five loci were classified as informative markers (PIC > 0.5). For the Wright's fixation index, the F value calculated at the loci assayed ranged from -0.41 (DCA3) to 0.28 (DCA9), with an average of -0.04 (Table 3).

Table 3. Genetic diversity indices number of alleles (Na), effective number of alleles (Ne), Shannon's diversity index (I), observed heterozygosity (Ho), expected heterozygosity (He), fixation Index (F) and PIC values revealed on 22 olive accessions analyzed with 06 SSR primers.

	Na	Ne	I	Ho	He	F	PIC
DCA03	03	1.810	0.714	0.632	0.447	-0.412	0.45
DCA16	10	6.453	2.048	0.909	0.845	-0.076	0.85
DCA15	05	3.143	1.289	0.500	0.682	0.267	0.69
DCA18	08	3.951	1.641	0.864	0.747	-0.156	0.75
DCA09	10	3.753	1.765	0.524	0.734	0.286	0.74
GAPU101	08	3.486	1.571	0.857	0.713	-0.202	0.72
Average	7.33	3.76	1.50	0.71	0.69	-0.04	0.7
Total	44	22.59	9.02	4.28	4.16	-0.29	

These results suggested the usefulness of SSR markers to enhance molecular diversity to identify olive genotypes.

To elucidate genetic relationships among Tunisian olive cultivars, a dendrogram was constructed using the UPGMA analysis over all 06 SSR loci (Figure 1).

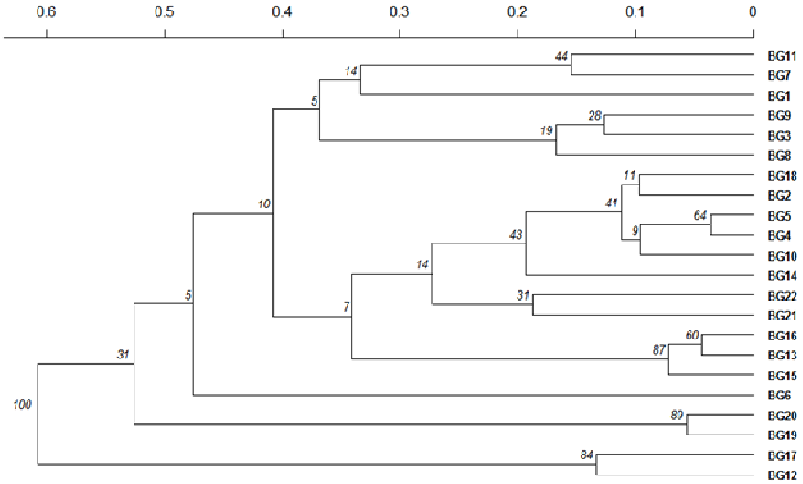

Figure 1. Dendrogram of the 22 olive cultivars based on genetic relationships.

The dendrogram in Figure 2 showed the presence of 2 main groups: BG 12 (Sig 17 from Sfax) and BG 17 (Zarrazi Sned 5 from Gafsa) (group 1) and all the remained groups (group 2).

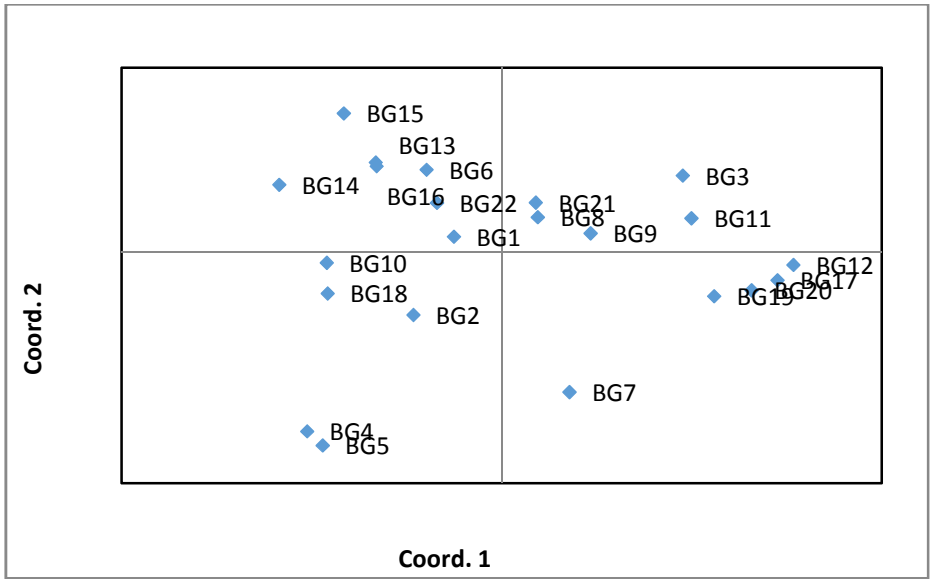

Figure 2. Principal coordinate analysis (PCoA) of the 22 olive accessions from the national olive collection of Boughrara.

There is no clear separation of cultivars indicating a high level of gene exchanges that could be due to either the relative proximity of geographical areas of the studied accessions in Tunisia or the human impact.

3.3. Discussion

In this study, the application of SSR markers was successful in characterizing the olive collection and proved to be an efficient tool for genotyping cultivars. It represents a detailed fingerprinting by means of SSR of "Boughrara" olive collection from different sites in Tunisia. It was done in order to complete the genotypes identification. It is complementary to previsious molecular characterization realized by Fendri *et al*. (2010) and Saddoud Debbabi *et al*. (2020). The overall allelic diversity displayed by the 06 SSR loci revealed a high genetic variation in the olive germplasm included in the study. Regarding average allele number per locus and observed heterozygosity, we assume the presence of high and consistent diversity in Olea europaea L. as reported in other studies (Breton *et al*., 2006; Baldoni *et al*., 2006; Belaj *et al*., 2007; Erre *et al*., 2010; Saddoud Debbabi *et al*., 2020). In agreement with previous studies (De la Rosa *et al*., 2002; Sarri *et al*., 2006; Fendri *et al*., 2010), observed heterozygosity values (Ho = 0.71) were higher than expected (He = 0.69). In this study, a total of 44 alleles for the 22 cultivars studied were identified (Table 3). From the same collection, Fendri *et al*. (2010) found 40 alleles for 84 Tunisian cultivars using the same primers. A negative value of F (Fixation index) indicating an excess in heterozygotes in comparison with the Hardy-Weinberg equilibrium expectations which can be explained by the use of a reduced number of cultivars. Although the relatively high similarity among cultivars, we assume that the studied

genotypes are all different. The results obtained in this work regarding to the total number of alleles, the number of allele per locus and the total number of genotype showed the presence of high genetic diversity in the "Boughrara"-Sfax germplasm collection. It confirms the effectiveness for the use of SSR markers of the olive genetic diversity (Saddoud Debbabi *et al*., 2021). Results of this study will be very useful in order to dress the final catalog of olive cultivars of the National collection and to identify later a core collection. These results will be of great interest for olive germplasm management and for olive breeding (Saddoud Debbabi *et al*., 2022). At the same time, the authors recognized the necessity of developing more SNPs for a better exploration of Tunisian olive germplasm.

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