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Morphological and molecular characterization of the main olive varieties cultivated in the region of Hbebsa (North West of Tunisia)

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Abstract

A group of seven olive varieties, commonly cultivated in the region of 'Hbebsa' localized in the North West of Tunisia were described using both morphological and molecular approaches. The morphological characters of each cultivar were collected according to the International olive council descriptor (1997). Biometric indexes of leaf, fruit, and endocarp were compared to the molecular data obtained on the same set of cultivars using ten SSR markers. We have noted a significant fluctuation of the flush percentage (70.68 to 84.82%), the fruit weight (from 0.58 to 4.48 g) and the endocarp weight (from 0.17 to 0.68g). The morphological study permitted a specific description of the characteristics for the tested varieties and their repartition into three groups according to their fruit and endocarp weight. Whereas, the molecular analyses based on SSR markers didn't present any clear segregation of the seven olive varieties relative to their fruit weight and their end-use. These result proved the insufficiency of the morphological parameters to discriminate the olive varieties and the importance of the SSR markers for studying variation between olive cultivars and for future breeding and olive germplasm management efforts.

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Introduction

Tunisia is the fourth largest producer of olive oil country in the world and oil exports represent 40 % of the overall value of agronomic exports and 5.5 % of aggregate exports, making it the fifth largest source of foreign currency earnings for the country (IOC, 1997). The distribution of *Olea* varieties in Tunisia gave rise to a very complex and highly articulated structure of olive culture which was marked by the existence of a considerable number of different olive cultivars. The main variety cultivated is 'Chemlali' in the south and the centre of the country and "Chetoui" in the north. These two varieties account for 95 % of the total olive tree orchards and contribute more than 90 % of the national production of olive oil (Trigui and Msallem 2002). Conversely, several minor varieties are maintained in restricted areas. The number is probably underestimated because of the scarce information on minor local varieties widespread in the different Tunisian olive growing areas. Thus, there is an urgent need to study and to inventory these traditional varieties before their lost (Abaza *et al.,* 2005; Baccouri *et al.,* 2007).

The region of Hbebsa localized in the North West of Tunisia is a rural area, which provides optimal growing conditions for most tree fruit crops, specially the olive trees. Our previous research on the morphological variability of the olive patrimony in this region (Mnasri *et al.,* 2013 a) was proved an important phenotypic variability of the analyzed olive cultivars for all the studied traits, especially for the fruit and endocarp parameters. However, these morphological markers have the disadvantage of the small number of polymorphism detected and of being environmentally dependent (Kamoun 1999 ; Trigui and Msallem, 2002) to overcome these problems several Tunisian research teams have used PCR-based markers for basic and applied research to assess the genetic diversity of Tunisian olive cultivars. These markers types include RAPD (Zitoun *et al.,* 2008), AFLP (Kamoun *et al*., 2006; Taamalli *et al*., 2006), SSR (Taamalli *et al*., 2010; Rekik *et al*., 2008) and SNP (Rekik *et al*., 2010).

Therefore, the objective of this analysis was to study for the first time in Tunisia the morphological and molecular parameters of five minor cultivars (Toufehhi, Besbessi, Meski, El Hor and Neb Jmel) and two major cultivars (Chetoui and Chemlali) cultivated in the region of Hbebsa. The molecular analysis was based on microsatellite markers which are more effective than others in estimating heterozygosity because of their codominant character (Carriero *et al.,* 2002). The major goal is to differentiate a number of Tunisian minor olive cultivars and to explore the genetic relationships among these genotypes, specially the autochthones varieties "Neb Jmel", "Besbssi", "Toufehhi" and "El Hor" which are characterized by a small geographic dispersion in the North West of Tunisia.

Materials and methods

Plant Material

The study was carried out during the growing season 2012-2013 localized in the region of Hbebsa (North West of Tunisia). Morphological and genotype description of the seven cultivated olive cultivars (Meski, Neb Jmel, El Hor, Chetoui, Chemlai, Touffehi and Tounsi) was carried out on three olive cultivars to a total of 21trees. The olive grove under study is not irrigated, pruned each 2 years and subject to the traditionally cultural practices in the area. This olive grove was selected due to the regularity of the productions of the last years and because all the accessions are presented, guaranteeing the homogeneity of the pedologic and climate conditions.

Morphological characterization

The morphological analysis was carried out by using the methodology for primary characterization of olive varieties, proposed by the International Olive oil Council (IOC, 1997). This investigation include the analysis of 29 distinct characters: four related to the leaf (length "V1", width "V2", shape "V3" and Longitudinal curvature of the blade "V12") , 12 related with the fruit (length "V4", maximum diameter "V5", shape "V6", weight "V7", symmetry in position (A) "V13", position of maximum transversal diameter "V14", apex "V15", base "V16", nipple

presence “V17”, presence of small lens “V18”, dimension of small lens “V19” and the localization of initial turning “V20”), and 13 related to the endocarp (length “V8”, maximum diameter “V9”, shape “V10”, weight “V11”, symmetry in position (A) “V21”, symmetry in position (B) “V22”, position of maximum transversal diameter “V23”, apex “V24”, base “V25”, surface “V26”, number of grooves “V27”, distribution of grooves “V28” and the mucro presence “V29”).

Molecular Characterization

DNA extraction

Total genomic DNA was extracted from young leaf tissue following the method described by (Angiolillo *et al.,* 1999) using a CTAB buffer with a concentration measured on agarose gel by lambda ladder.

SSR markers

Ten microsatellite (SSR) markers were used in this study. Four markers (GAPU59, GAPU71A, GAPU71B, GAPU103A) from the primer set designed by Carriero *et al.* (2002), four markers (UDO03, UDO12, UDO28, UDO39) from Cipriani *et al.* (2002) and two markers (DCA9, DCA18) from Sefc *et al.* (2000) were selected for their high polymorphism among olive cultivars, their easily scored patterns and their small-scale stuttering (Table 3). The 20-µl reactions contained 50 ng template DNA, 1.5 mM MgCl2, 0.3 mM dNTP, 10 pmol of each primer, and 1.5 U Taq DNA polymerase (Gibco-BRL) in 1X PCR buffer. The cycling regime consisted of 94°C for 4 min, followed by 34 rounds of 94°C for 30 s; 50-60°C (primer pair dependent; Sefc *et al.,* 2000; Cipriani *et al.*, 2002) for 45 s and 72°C for 60 s, with a final step of 72°C for 10 min.

Data analysis

An average value for each trait and accession was calculated. The value of the quantitative and qualitative morphological traits was standardized and subject to a Principal Component Analysis (PCA). Each trait was also subject of one-way analysis of variance (ANOVA) at a significant level of P\0.05. All calculations were done by the using of XLSTAT software (2010).

SSR data were analyzed using several genetic parameters such as: number of alleles per locus; observed heterozygosity (H_o, calculated as the number of heterozygotes per locus divided by the number of individuals typed); expected heterozygosity (H_e) or gene diversity (Nei, 1987), and the polymorphism information content (PIC) calculated for each locus (Botstein *et al.,* 1980). Pair wise genetic similarities were calculated using Dice similarity coefficient (Dice, 1945; Neil and Li, 1979). A dendrogram was constructed from the resultant matrix via the unweighted pair group method with the arithmetic averages algorithm (UPGMA) methods. All calculations were performed with the use of NTSYS-pc version 2.1 (Rohlf, 1998).

Results and discussion

Morphological polymorphism

The morphological characteristic of the seven olive cultivars, including mean value, variability range, variation coefficient, and minimum significant difference among accessions are shown on table 1. The morphological traits showed considerable variability among the seven cultivars, especially the fruit parameters V4, V5 and V7, as well those that were measured in the endocarp V8, V9 and V11. The fruit weight varied from 0.58 to 4.48 g, the endocarp weight ranged from 0.17 to 0.68g and the flush percentage from 70.68 to 84.82%. The variety ‘’Chemlali” which is classified as oil olive cultivar present the lowest fruit and endocarp weight, while the table olive variety “Meski” present the highest values. Previous studies explained that the description of the morphological characteristics is the usual methodology accepted from a legal point of view for patenting and registration of varieties (Badanes, 1998), especially the importance of fruit and endocarp parameters to discriminate between the olive varieties (Zaher *et al.*, 2011; Paula *et al.*, 2005, Mnasri *et al.*, 2013 a and Mnasri *et al.*, 2014).

Table 1. Descriptive statistic analysis of the morphorphological parameters.

Trait	Minimum	Maximum	Average	CV%
V1	47,33	64,76	56,64	11***
V2	9,04	15,05	11,84	16,27***
V3	4,04	6,79	4,97	19,83***
V4	13,32	24,11	19,83	21,72***
V5	8,38	18,97	14,78	26,41***
V6	1,18	1,58	1,36	10,63***
V7	0,58	4,48	2,81	55,3***
V8	10,41	16,63	14,55	18,42***
V9	5,16	9,37	7,15	22,72***
V10	1,70	2,49	2,06	15,52***
V11	0,17	0,68	0,43	47,81***

P-value: ** significant ($P < 0.05$); *** Highly significant ($p < 0.01$).

CV% Variation coefficient expressed in percentage.

The principal component analysis performed on the morphological descriptors of the fruit, endocarp, and leaf (ACP) is presented in Fig 1. The eigenvalues of the first, second and third axis of the principal components, accounted the 62.14%, 69.15%, 5.84% of the total variance, respectively. The relative magnitude of the first PC eigenvectors showed that weight, length, and maximum diameter of fruit and endocarp, as well the qualitative parameters of the fruit (symmetry in position A and nipple presence) and the endocarp (number and distribution of grooves, surface in position B and base in position A) were important attributes for the classification of cultivars in cluster.

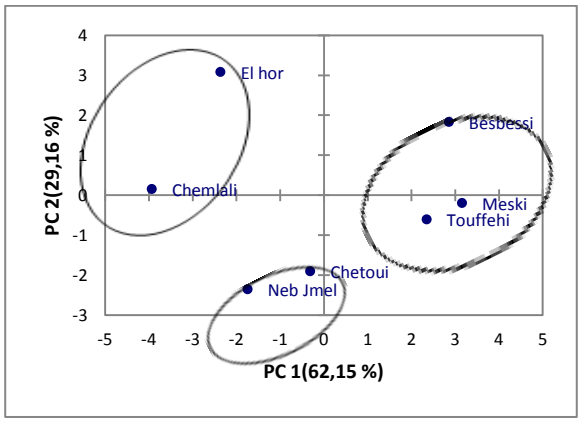

Fig. 1. Projection of the twenty two accessions in the plane generated by the first two principal components based on leaf, fruit and endocarp traits.

The inertia accounted for the second PC was due to the contribution of the fruit (shape, position of maximum transversal diameter, apex in position A and dimension of small lens) , as well with the endocarp (shape, symmetry in position A, symmetry in position B and apex in position A). The leaf traits (Length, shape and the longitudinal curvature of the blade) had relatively high eigenvectors in the third PC.

The projection of individuals in the plane generated by the axis 1, 2 and 3 showed the distribution of the seven varieties in three main groups. The cluster 1 grouped the varieties ('Meski', 'Touffehi' and 'Besbessi') characterized by the highest fruit and endocarp weight. These cultivars were classified in the olive categories of high to very high weight fruit and they can be used for canning (Barranco *et al.,* 2000). In turn, the cluster 2 which grouped the cultivars ('Neb Jmel' and 'Chetoui') is characterized by medium weight fruit and a sharp-pointed apex, as well by elliptic and mean weight endocarp, these cultivars can be used with a double aptitude. The oil varieties ('Chemlali' and 'El Hor') were grouped in cluster 3 and characterized by low weight fruit with an around apex in position A and an oval and low weight endocarp with rounding apex and base.

Molecular polymorphism

Microsatellites were successfully amplified in the seven analyzed varieties with the ten primer pairs. A total of 41 alleles were observed across the used markers, the number of alleles per locus ranging from 5 (GAPU103A) to 3 (DCA09 and DCA18) with a mean value of 4.1 alleles per locus (Table 2). Allele sizes vary among the ten loci, differences between the longest and shortest allele ranged from 121 to 228 bp. The observed heterozygosity ranged between 1.00 at locus (GAPU71B, UDO12) and 0.42 at DCA18, with a mean value of 0.75 which proved the important variability of the analyzed cultivars. The mean PIC values were high (0.65) and ranging from 0.78 at locus (UDO28) to 0.57 at locus (DCA18). In fact, this diversity may be associated with the variation in the loci. An important number of reports have indicated the high variability in the average number of alleles per locus in olive cultivars (Carriero *et al.,* 2002; De

La Rosa *et al.,* 2002; Diaz *et al.,* 2006; Sarri *et al.,* 2006 and Abdelhamid *et al.,* 2012). Moreover, these findings are in good agreement with those of other authors working on the molecular variability of Tunisian olive cultivars based on SSR markers (Rekik, 2008 and Tamalli *et al.,* 2006).

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

SSR locus	N° alleles	Observed Heterozygosity	Expected heterozygosity	PIC	Range size (pb)
GAPU59	4	0,71	0,62	0,58	208-218
GAPU71A	4	0,57	0,65	0,61	210-228
GAPU71B	4	1	0,69	0,64	121-144
GAPU103A	5	0,85	0,82	0,76	136-184
UDO03	4	0,85	0,64	0,6	135-202
UDO12	4	1	0,69	0,64	166-193
UDO28	5	0,71	0,84	0,78	143-210
UDO39	5	0,71	0,81	0,75	108-220
DCA09	3	0,71	0,69	0,64	182-206
DCA18	3	0,42	0,61	0,57	174-190
Total	41				
Mean	4.1	0,75	0,7	0,65	0,75

The diversity of the studied sample was approached by calculating a dendrogram of genetic similarity (fig 2) based on Jaccard index (1901) with NTSYS-PC (Rohlf, 1998). Two main groups were revealed by cutting the dendrogram at a GS value of 0.7. The first cluster grouped the cultivars (Besbessi, El Hor, Chetoui and Chemlali) characterized by oval fruit symmetric in position A and presented an around base. The second cluster grouped the cultivars (Touffehi, Meski and Neb Jmel) characterized by elliptic fruit and endocarp asymmetric in position A. In fact, there is no clear structuration of the seven varieties relative to their fruit weight and their end-use. Nevertheless, the seven cultivars clustered according to the qualitative parameters of their fruits and endocarps and proved our previous analyses of the molecular biodiversity of the autochthon Tunisian olive cultivars based on AFLP markers (Mnasri *et al.,* 2013b and Mnasri *et al.,* 2014). Further, Kamoun *et al.,* (2006), Taamaalli *et al.,* (2006) and Abdelhamid *et al.,* (2012), in their analysis of Tunisian olive cultivars by AFLP and SSR obtained a comparable clustering of cultivars based on the qualitative morphological parameters of their fruits and endocarps. These similar results emerge from analysis of different olive cultivars using different approaches would seem to indicate the efficiently of the qualitative morphological marker to discriminate olive germplasm.

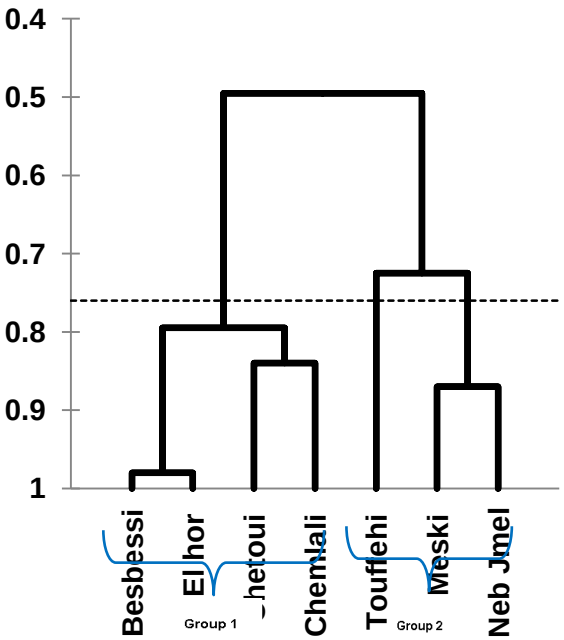

Fig. 2. Dendrogram of the seven olive cultivars based on SSR data using Jaccard's GS matrix and the UPGMA clustering method.

Conclusion

The morphological and molecular analyses of the seven predominant olive varieties in the region of Hbebsa proved the importance of this germoplasm. The studied cultivars featured phenotypic variability for all the analyzed traits, especially for the fruit and endocarp parameters. The principal components analysis based on morphological markers revealed the distribution of the seven varieties in three main groups according to their

fruit and endocarp weight. Whereas, the molecular analyses based on SSR markers didn't present any clear segregation of the seven olive varieties relative to their fruit weight and their end-use. These results proved the insufficiency of the morphological markers, especially the quantitative traits to discriminate the olive varieties. For that reason, the use of SSR markers is essential to verify the denomination of each cultivar and increase the knowledge about the olive germoplasm in the region of Hbebsa which is in despite of its arid climate characterized by an important olive biodiversity. These biotechnological tools can provide significant insights for research in crop breeding and germplasm conservation.

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