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Morphometric contribution and molecular markers for the characterization and identification of olive tree (*Olea europaea* L.) varieties

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Abstract

This study focused the variability of morphological types and polymorphism level in olive tree. Ten SSR microsatellite markers and 23 morphological descriptors were used to characterize 15 indigenous cultivars of this species growing in Algeria. A significant morphological variability was observed between the cultivars. The type of the endocarp exhibited a discrimination potential higher than that of the fruit, leaf and the inflorescence. Molecular markers have also shown a very high level of polymorphism and a high discriminatory power to discriminate easily the whole sample. It is possible to achieve the same results using only 3 markers (ssrOc UADCA 18, UDO 099-043 and ssrOcUA-DCA9). These results underline the importance of using ssr markers and morphological descriptors for the inventory and identification of olive varieties.

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Introduction

The olive tree occupies the 24th place of the 35 most cultivated species in the world. The phenological diversity of cultivars is remarkable and the socio-economic interest of the species is major. It is estimated that 98% of the olive-growing area is located in the Mediterranean basin. It was described 2600 different olive cultivars (Bartolini *et al.*, 2005).

Algeria has important resources of olive trees little valued until now. Over 150 local olive cultivars were counted by Chaux (1955). Recent work of varietal identification developed from the combination of morphological, agronomic and Phrenological have identified 36 varieties (Mendil and Sebai, 2006). They also helped to resolve some cases of homonyms and synonyms. Many cultivars remain unknown, despite their interest.

The name of cultivars is most often attributed according to their main agronomic and/or morphological characteristics. The Chetoui cultivar for example has fruits that ripen in winter. The Chemlal of Kabylie is so named because its presence in mass in this region of Algeria.

The richness of genetic diversity of Algerian olive cultivars and the essential criteria of their domination amply justify their characterization and identification. Studies on the genetic diversity and identification of Algerian olive cultivars have not been performed to date and very few cultivars have been included in previous studies at the Mediterranean level (Belaj *et al.*, 2002).

The difficulties that sometimes arise from the morphological characterization "variation between individuals of the same variety grown at different altitude levels have forced some researchers to undertake new varietal characterization studies based on genetic markers especially type SSR markers (simple sequence repeat) and type SPM (single point mutation). They are highly polymorphic reproducible markers.

The current study was undertaken to characterize fifteen varieties of olive tree cultivated in the East of Algeria.

Material and methods

Plant Material

The plant material studied consists of 15 indigenous cultivars (Table 1). They come from three different sites (differences base on ecologic, climatic and edaphic point of view). Five Traditional varieties (Chemlal, Bouricha, Lahmar, Zeradj, Balbal obtained from *Said Bousbaa's* farm located in south where the olive tree dates back to the Constantine region by the mountains of Toumiates and El Milia. Nine local varieties (Kerdoussi, Braouhi, Lohmiri, Lokchiri, Bliltii, EL-kharfi, Chetoui, Laaninbi and Rouihni) obtained from different places of the Skikda region. These trees are not irrigated and depend on the winter rains. The soil fertilization, plant protection and size, are never practiced.

Morphological Characterization

The characterization of studied varieties concerned the morphological description of the main tree organs (fruit, endocarp, leaf and inflorescence). Twenty three morphological characters (Table 2), currently used for primary characterization of olive varieties (Barranco and Rallo 1984; COI, 1997) were selected for this study. The observations were carried by a single individual per variety. Each individual was selected and identified based primarily on its phytosanitary status. The characters related to leaf and inflorescence were determined on a sample of 40 leaves and 40 inflorescences collected respectively on the median of 8 to 10 shoots of the year and 10 fruiting branches. The description of the fruits was performed on a sample of 40 fruits per individual taken on the median of 10 fruiting branches. These fruits selected from the most representative eliminating the smallest and the largest and those with malformations. These 40 fruits served after pulping for determination of characters from the endocarp. The samples of different organs were collected in the southern part of the tree.

Morphological characters have been structured based on quantitative descriptors (gram, centimeter, units) and qualitative (Form, expressions of morphological characteristics).

The description of the fruit is realized upon or is terminated at veraison. In a variety, if an organ sample studied has two categories for the same character in different percentages, use is made in the calculation of averages and takes into consideration the category that dominates. In case where the two categories have similar percentages, the number of samples studied was increased thereby allowing the definition of the dominant class characteristic of the variety studied.

To authentify the varieties, we have conducted a verification tests comparing the authenticity of varieties studied with the characterization established in the global catalog of olive varieties and varietal sheets available in the literature.

Varietal identification

All 15 studied varieties were characterized by applying 23 morphological descriptors for the samples of fruits, endocarp, leaves and inflorescences. Within 15 varieties analyzed, the character presence of mucro in the endocarp is homogeneous. This character was placed in inactive variable. The DNA was extracted from young leaves (0.25 g) according to the protocol described by De la Rosa *et al.* (2002). Then, 10 molecular markers type microsatellites (SSR) were used to characterize the different studied varieties of olive tree.

Statistical analysis

For each quantitative character, we have conducted a comparison of means by analysis of variance (ANOVA). When a significant difference was found between varieties for a given character, ANOVA was completed by the *LSD test* that identifies the varieties that are significantly different. Given that most of the characters are qualitative, multivariate analysis was more appropriate to identify all 15 varieties studied and identify morphological characteristics that contribute most to this identification in order to group the varieties according to their morphological resemblance degrees and view existing phylogenetic relationships between them (Table 3). The main factor axes are maintained for the hierarchical classification. This is based on the aggregation criterion of Ward (1963) and provided a tree that is interpreted and subjected to partition. The tree cut level is determined based on the number of classes and the interclass inertia ratio of the total inertia (which must be high so that the classes reflect the diversity of morphological varieties) (Benzecri, 1982). These analyzes were performed using SPAD software programm).

The program obtained containing fluorescent SSR data was assesses and verified by Genscan Genotype version 3.7 soft ware from applied biosystems. Relations between olives genotypes are determined on a binary matrix established based on the Jaccard similarity coefficient. We used the aggregation method called "Neighboor joinning (NJ Three) developed by Nei (1977), or UPGMA algorithm to build phonograms from distance matrices calculated.

Results

Morphological characters

The results showed that characters exhibiting highly significant position on the first factorial axis are: the endocarp category, the shape from the endocarp, the symmetry of the fruit and the top of the fruit. Indeed axis 1, characterized mainly the appearance of the endocarp and fruit. This axis, opposes varieties with a truncated base of the fruit and a rounded top of the endocarp. In addition, varieties having a rounded base of the fruit and a pointed tip of the endocarp. The high total inertia obtained for the character form of the endocarp and fruit shape demonstrated the significant discriminating power of these characters.

The second axis also provides information on the appearance of the fruit. It opposes varieties with few lenticels with a large dimension as having a slightly asymmetric symmetry of the fruit, will generally have

a position towards the base of the maximum transverse diameter of the fruit. It opposes also varieties with many lenticels with a small dimension as having asymmetric symmetry of the fruit, will generally have a position towards the top of the maximum transverse diameter of the fruit.

The assessment of the similarity of varieties allowed us to discern four main groups of varieties studied of *Olea europa* L. (Table 4):

Group 1: varieties with few lenticels with a large dimension. This group consists of four varieties representing 26.67% of the sample analyzed. We find the varieties of "Lohmiri", "Chetoui", "EL-kharfi" and "Zeradj".

Table 1. Studied cultivars and their origins.

Cultivar	Source	Cultivar	Source
Laaninbi	Ecole Felfla	Chetoui	Chrea, Tamalous
Rouihni	Ecole Felfla	Chamlal	El Harrouch
Kerdoussi	Mechta Bniguendouz	Bouricha	El_Harrouch
Braouki	Bniguendouz	Laha	El_Harrouch
Lohmiri	Bniguendouz	Mar	El_Harrouch
Lokchiri	Bniguendouz	Zeraj	El_Harrouch
Leblilti	Bniguendouz	Balbal	El_Harrouch
El-kharfi	Bniguendouz		El_Harrouch

Group 2: varieties with a wide range of morphological type. Characterized by a low representation of characteristics and the absence of specific morphological characters to this second group which consists of 6 varieties (40% of the sample analyzed). Where there are individuals of the variety "Balbal", "Bouricha", "Braouki", "Lahmar", "Lokchiri" and "Kerdoussi". Group 1 and 2 are in the same axis and can be combined into one group.

Table 2. List of morphological characters studied of olive tree.

Studied characters			
Leaf (2 characters)		Endocarp (10 characters)	
Form (FOL)	-Elliptic (1)	Form (FOE)	-Spherical (1)
	- Elliptic-lanceolate (2)		-Ovoid (2)
	-Lanceolate (3)		-Elliptic (3)
Longitudinal curve of the lamina (LCL)	-Epinastic (1)		-Lying (4)
	-Plane (2)	A Symmetry (position A) (SAE)	-Symmetrical (1)
	-Hyponastic (3)		-Slightly asymmetrical (2)
	-Helical (4)		-Asymmetric (3)
Inflorescence (2 characters)			
Length of the bunch (LOB)	-Short (1)	A Symmetry (position B) (SBE)	-Symmetrical (1)
	-Medium (2)		- Slightly asymmetrical (2)
	-Long (3)		
Number of flowers /inflorescence (NFL)	-Feeble (1)	Position of maximum transverse diameter (DIE)	- Towards the base (1)
	-Medium (2)		-Central (2)
	-High (3)		-To the summit (3)
Fruit (9 characters)			
Form (FOF)	-Spherical (1)	Summit (SOE)	-Sharp (1)
	-Ovoid (2)		-Rounded (2)
	-Lying (3)		
Symmetry (SYF)	-Symmetrical (1)	Base (BOE)	-Truncated (1)
	-Slightly asymmetric (2)		-Sharp (2)
	-Asymmetrical (3)		-Rounded (3)
Position of maximum transverse diameter (DIF)	- Towards the base (1)	Surface (SUE)	-Smooth (1)
	-Central (2)		-Rough (2)
	- Towards the top (3)		-Planer (3)

Table 2. (continued).

Studied characters			
Summit (STF)	-Sharp (1) -Rounded (2)	Number of fibrovascular grooves (NGE)	-Reduces (1) -Middle (2) -High (3)
Base (BOF)	-Truncated (1) -Rounded (2)		
Nipple (NOF)	-Absent (1) - Draft (2) -Evident (3)	Distribution of fibrovascular grooves (DFG)	-Uniform (1) - Near the suture (2)
Presence of lenticels (POL)	-Few (1) - Many (2)	End of the peak (EPE)	- Without mucro (1) - With mucro (2)
Dimension of lenticels (DOL)	- Small (1) - Large (2)		
Initial localization of veraison (VRF)	- From the base (1) -Uniform (2) -From the summit (3)		

Group 3: varieties with asymmetric symmetry of the fruit. This group consists of three varieties with 20% of the sample analyzed. Where found individuals of the variety "Rouihni", "Derdouri" and "Chemlal".

Group 4: varieties with low weight of the endocarp. This group consists of two varieties with 13.34% of the sample analyzed. Where there are individuals of the variety "Laaninbi" and "Blilti". These two varieties have enregistred the lowest weight of the endocarp according to ANNOVA ($p<0.05$).

Varietal identification

The 10 SSRS markers used in this study (Table 5) showed a significant polymorphism and very high discrimination capability allowing distinguishing different cultivars (Algerians, Tunisians and Moroccans). A total of 76 alleles were revealed using 10 SSR loci. The number of alleles per locus ranges from 5 (locus GAPU 71B) to 10 (loci ssr OeUA-DCA 5; DCA9 and SSr UADCA18 Oe) with an average of 7.6 alleles per locus. The expected heterozygosity was spread between 0.729 (GAPU59) and 0.901 (ssr Oe AU. DCA18), with a mean value of 0.81.

Table 3. Selected variables for analysis in multiple components of morphological descriptors of studied olive varieties.

Var.	FOL	LCL	LOB	NFL	FOF	SYF	DIF	STF	BOF	NOF	POL	DOL	VRF	FOE	SAE	SBE	DIE	SOE	BAE	SUE	NGE	DFG	EPE
Elhamri	2	2	2	2	2	2	2	2	2	1	1	2	2	3	2	1	3	1	3	3	2	1	2
Rouihni	1	2	2	2	3	3	3	2	1	1	2	1	1	3	3	2	3	1	3	1	2	1	2
Lokchiri	2	2	3	3	3	2	2	2	2	1	2	1	3	4	2	1	3	1	3	2	2	1	2
Chetoui	2	3	2	2	3	2	2	1	2	3	1	2	2	4	2	1	3	1	2	1	2	1	2
Chamlal	2	2	1	2	3	3	2	1	1	2	2	1	3	4	2	2	3	1	2	2	2	1	2
Brouaki	2	3	2	3	3	2	2	1	1	3	2	1	1	3	2	2	3	1	3	2	2	1	2
Elkharfi	3	3	2	2	2	2	1	1	2	1	1	2	1	3	2	1	2	1	3	1	2	1	2
Kerdoussi	2	3	3	3	2	1	1	2	2	1	2	1	3	2	2	1	3	1	3	2	2	1	2
Blilti	2	2	2	2	3	1	2	2	2	1	2	1	2	2	1	1	2	1	3	1	1	1	2
Laaninbi	1	2	2	2	1	1	2	1	2	1	2	1	2	2	2	1	1	2	3	1	2	2	2
Zeradj	2	2	2	1	3	2	1	1	2	1	1	2	3	3	2	2	2	1	3	1	2	1	2
Balbal	2	2	2	2	2	2	1	2	1	2	2	1	3	3	2	1	2	1	3	3	3	1	2
Bouricha	2	3	3	2	3	2	2	1	1	1	2	1	1	3	2	1	2	1	2	1	2	1	2
Lahmar	2	3	3	3	2	2	2	2	1	1	2	1	3	3	3	1	3	1	3	1	1	1	2
Derdouri	1	2	2	2	3	3	3	1	1	1	2	1	1	4	2	2	2	1	3	2	2	2	2

FOL: Form of leaves, LCL: Longitudinal curve of the lamina, LOB: Length of the bunch, NFL: Number of flowers, FOF: Form of fruits, SYF: Symmetry of fruits, DIF: Position of maximum transverse diameter of fruit, STF: Summit of fruit, BOF: Base of fruit, NOF: Nipple of fruit, POL: Presence of lenticels, DOL: Dimension of lenticels, VRF: Initial localization of veraison of fruits, FOE: Form of endocarp, SAE: A Symmetry of endocarp, SBE: B Symmetry of endocarp, DIE: Position of maximum transverse diameter of endocarp, SOE: Summit of endocarp, BAE: Base of endocarp, SUE: Surface of endocarp, NSE: Number of fibrovascular grooves of endocarp, DFG: Distribution of fibrovascular grooves, EPE: End of the peak of the endocarp.

The observed heterozygosity ranged from 0,636 to 1 with an average value of 0.84. Null alleles were found in 4 of SSR primers (DCA3, UDO 99 043, GAPU103A and EMOO3). They ranged between 0.0086 and 0.0806. The peak values have ranged from 0.860 (De A18) to 0.663 (GAPU59) with an average of 0.7477, and allowed to classify all cultivars, they are informative markers (Pic> 0.5).

Variables identification

The combination of allenic profiles obtained with the ten loci SSRs or allelic models obtained could identify all the plant material studied. Anyway, morphometry is very useful in the field of phylogeny and perhaps complement the contribution of genetic markers, recently developed for reliable identification of varieties.

Genetic relationship between the olive cultivars

The average value of similarity between the different genotypes is 0.47 (data not shown here). The similarity between genotypes extends between 0.36 and 0.90.

Table 4. Olive groups obtained according to similarity of varieties.

Olive groups	Varieties	Percentage of sample analyzed (%)
Group 1	Lohmiri, Chetoui, EL-kharfi, Zeradj.	26.67
Group 2	Balbal, Bouricha, Braouki, Lahmar, Lokchiri, Kerdoussi.* Rouihni, Derdouri, Chemlal.	40
Group 3	Laaninbi, Blilti.	20
Group 4		13.34

* Group 1 and 2 are in the same axis and can be combined into one group.

The correlation coefficient between the cophenetic dendrogram (not shown here) and the original distance matrix is not very high but significant through this dendrogram, we see that cultivars are classified into three main groups whose first group contains five Algerian cultivars. The second group consists of three beams of which the first is composed of 4 cultivars: Chemlal, El-Kharfi, Derdouri and Chetoui.

The third group consists of Laaninbi, Lahmar and Blilti. The number of alleles and expected heterozygosity found in all samples were similar as that reported by several authors in studies about the genetic material to other branch of olive cultivation.

Discussion

In this study, we presented the first results on the characterization of olive cultivars using molecular markers type microsatellites (SSR) and using morphological descriptors.

Simultaneous use of specific markers locus and morphometry allows no doubt, to overcome the disadvantages of each other, and get a safe varietal identification in order to build a reference database of genotypes.

These markers, most of which were used in varietal characterization, are considered reliable molecular markers. Our results confirm the high efficiency of these markers as to the genotyping of olive cultivars and detection of the genetic richness offered by this genetic material of olive.

This richness has previously been reported by some authors (Barranco *et al.*, 2000; Mendil and Sebai, 2006) using morphological descriptors.

Indeed, the results obtained in this study showed significant morphological polymorphism in the sample studied cultivars with a discriminating power of the order of 100%. It goes beyond the discriminatory power obtained in identification studies using enzyme labels (Trujillo *et al.*, 1995), "DNA" such as RAPD (Random Amplified

Polymorphism DNA) and AFLP (Amplified Franment Lenght Polymorphism) (Belaj *et al.*, 2001; Khadari *et al.*, 2008). The SSR markers used in this study were highly polymorphic. The number of alleles obtained is similar to that reported by Bracci *et al.* (2009), by against; it is potentially less than that released by Alba *et al.* (2009).

Table 5. Genetic parameters and discriminatory power.

SSR	N° of alleles	H_o	H_e	pic	F (null)
ssrOeUA-DCA3	7	0,647	0,777	0,725	0,0673
ssrOeUA-DCA5	6	0,882	0,768	0,712	-0,0871
ssrOeUA-DCA9	10	0,939	0,883	0,84	-0,0516
ssrOeUA-DCA18	10	1	0,901	0,86	-0,0704
Udo99-43	10	0,8	0,851	0,801	0,0086
GAPU103 A	7	0,769	0,862	0,806	0,0363
ssrOeUA-DCA11	6	1	0,779	0,703	-0,1703
GAPU71B	5	0,846	0,738	0,664	-0,0859
EMO03	7	0,636	0,766	0,703	0,0806
GAPU59	8	0,846	0,729	0,663	-0,1014
Media	7,6	0,8365	0,8054	0,7477	-0,03739
Max	10	1	0,901	0,86	0,0806
Min	5	0,636	0,729	0,663	-0,1703

This probably results in the use in this study of a few samples of cultivars and by the use of a range of different microsatellites. In addition, the expected heterozygosity in all cultivars was similar to that reported by several authors in studies on olive genetic material from other growing areas (Bandelj *et al.*, 2002; Belaj *et al.*, 2004).

Our results are also consistent with those reported in several studies using SSR markers and genetic olive hardware companies in other North African countries (Charafi *et al.*, 2008; Rekik *et al.*, 2008; Taamalli *et al.*, 2008).

A high level of polymorphism was also detected between Chemlal of Skikda and Chemlal of Kabylie. The difference is so important and should be considered as different genotypes.

Previous studies using morphometry and molecular markers showed that the cultivars with the same name may be included in different genotypes (Besnard *et al.*, 2001; Belaj *et al.*, 2004; Rekik *et al.*, 2008). Differences between these Chemlal were also revealed by studies using chloroplast and mitochondrial markers (Besard *et al.*, 2000), and by AFLP (Grati Kamoun *et al.*, 2006).

Conclusion

It can be concluded that SSR markers are a very suitable tool for exploring the genetic variability of olive cultivars. These morphological descriptors complement undoubtedly the contribution of these microsatellite markers that allow a very reliable identification of cultivars. They may have utility for the characterization of genetic resources of the olive tree. The existence of a homonymous assumes systematic surveys and characterization of both molecular and morphological to sort the genetic material so rich in Algeria, which has been barely explored.

This diversity should be evaluated and preserved *in situ*, in gene banks. In this way the farmers can rely in the future on their indigenous plant material to begin a breeding program for the renovation of the olive oil sector.

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