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Morphological and genetic diversity in olive (*Olea europaea* subsp. e*uropaea* L.) clones and varieties

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Abstract

'Picholine marocaine' is a predominant olive cultivar in Morocco, widely spread in all the olive growing regions. Clonal selection is one of the breeding methods used for developing varieties, and the clones that showed improved traits in field were selected from 'Picholine marocaine'. In this study, we compare the potential of microsatellite markers with morphological traits to differentiate the varieties and genotypes developed through clonal selections, and to estimate the relationships among the clonal selections, Moroccan local and Mediterranean varieties. For this purpose, we evaluated 7 clonal selections from 'Picholine marocaine', five Moroccan local and seven Mediterranean varieties for variation at 15 morphological traits and 20 microsatellite loci. The results clearly showed that microsatellite markers were more efficient compared to morphological traits to differentiate closely related varieties and genotypes developed through clonal selections, in addition to distantly related varieties. Nine out of the 20 microsatellites markers tested were polymorphic, revealing a total of 48 alleles. Average number of alleles per locus was 5.3, ranged from 3 to 9 alleles. Observed heterozygosity ranged from 0.42 to 1 with a mean of 0.79. Upon comparison, the dendrograms, constructed based on morphological traits and microsatellite markers, showed a positive and highly significant relationship. The combination of microsatellite marker profiles and the morphological characters serve as reliable tools for detailed description of olive varieties.

Keywords: Olive tree; Clonal variety; Varietal identification; Microsatellites; Morphological traits.
Abbreviations: NJ- Neighbor-Joining; PCR –Polymerase chain reaction; PCOA- principal coordinates analysis; PCOs principal components; PM- 'Picholine marocaine'; SSR- Simple sequence repeats;

Introduction

In Morocco, the olive (*Olea europaea* subsp. e*uropaea* L.) plantations account for about 64 millions trees, covering 784,000 hectares (MAPM, 2011). It has major socio-economical role since it contributes to the maintenance of the rural populations. The 'Picholine marocaine' is the most predominant variety; more than 98% of the olive growing orchards are planted by this variety (Boulouha et al., 1992; Bamouh, 1998). The name 'Picholine marocaine' alludes to the French variety 'Picholine de Languedoc', which is, however, genetically distinct. This denomination was most likely originating in the marketing of Moroccan olives in southern France during 1960s and 1970s (Boulouha, 1995). Only a few other varieties ('Bouchouk', 'Bouchouika', 'Fakhfoukha', 'Hamrani' and 'Meslala') are known, and they are cultivated in restricted areas (Maestratti, 1922; Tornézy, 1922). Clonal selection and cross breeding are the two major breeding methods used for developing olive varieties. In clonal selection method, the clones that showed improved traits in the field are selected (Gregoriou, 1996). Clonal selection programs are based on different selective criteria such as high productivity, reduced alternate bearing, and suitability to vegetative propagation. Subsequently, the selected clones are multiplied and distributed for cultivation (Gregoriou 1996). Two varieties namely 'Haouzia' and 'Menara', registered for cultivation in Morocco, were developed through clonal selection (Boulouha, 1995). Other promising clones ('S7', 'S8', 'M14' and 'M16') were selected for productivity and alternate bearing. These selected clones are being tested in the Tassaout Experimental Station of INRA, Morocco (INRA, 2008). Occurrence of large number of varietal homonymy (varieties having the same name but that are genetically different) and synonymy (varieties having different names but that are genetically the same) and clonal selections have complicated varietal identification and characterization in olive tree (Bandelj et al., 2002). Morphological descriptions of the International Olive Council (COI, 1997) are usually applied for description and identification of olive varieties. Molecular techniques are also

used for precise genetic characterization, ascertaining origin and elucidating the dispersal route, owning to their reproducibility, reliability and independence from environmental conditions (Poljuha et al., 2008). To distinguish between olive varieties, DNA molecular markers such as RAPDs (Gomes et al., 2008; Erfatpour et al., 2011) and AFLPs (Angiolillo et al., 1999; Rotondi et al., 2003) were applied initially. Subsequently, microsatellite markers or simple-sequence repeats (SSRs) had been developed for olive tree (Sefc et al., 2000; Carriero et al., 2002; Cipriani et al., 2002; Shabanimofrad et al., 2011) and have been successfully applied for characterization of olive tree varieties (Rallo et al., 2000; Gil et al., 2006; Baldoni et al., 2009). Microsatellite markers are suited to distinguish closely related genotypes, because of their high degree of variability (Udupa et al., 1999; Udupa and Baum, 2001; Kumar et al., 2009). In this study, we selected microsatellite markers for differentiating closely related olive varieties ('Menara' and 'Haouzia') and genotypes ('S8', 'S7', 'M14', 'M16' and 'M26') developed from the predominant Moroccan variety 'Picholine marocaine' through clonal selection. The objectives of the work reported here were (1) to compare the potential of microsatellite markers with morphological traits to differentiate varieties and genotypes developed through clonal selections, and (2) to study the relationships among the seven clonal selections, five Moroccan local and seven Mediterranean varieties, using morphological traits and microsatellite markers.

Results

Morphological characterization

The olive genotypes (Table 1) were evaluated for the morphological traits namely leaf, fruit and endocarp characters (see Supplementary Table S1). Mainly two types of morphological variants were recorded for each trait based on visual observation. However, three types of variants were also observed for some characters**,** namely, fruit nipple (absent, tenuous or obvious), endocarp symmetry position A (asymmetric, slightly asymmetric or symmetric) and endocarp surface (position B; rugose, smooth or scabrous). For these morphological traits, the clones showed less difference among themselves (for one to five characters only) and from 'Picholine marocaine' (PM; see Supplementary Table S2). Indeed, 'Menara' and 'Haouzia' varieties differed from the PM by a single character, namely the distribution of the grooves and the number of grooves, respectively. While, the clone 'M26' was distinguished by three characters, the number, distribution of the grooves and shape (position A). Similarly, the clones 'S8' and 'M16' were distinct from the PM by 3 other characters. However, the clones 'S7' and 'M14' differ from PM by 5 endocarp characters (Supplementary Table S2). The result of the two-dimensional principal coordinates analysis (PCOA) based on the 15 morphological traits revealed diversity among the 19 genotypes. The first and the second axis together explained 59% of the total variation in the standardized data set of the 19 genotypes. The first and second axis demonstrated 30% and 24.5% of the variation, respectively. Based on this morphological data, the PCOA separated the accessions into three main groups, each group with distinctive features. The scatter diagram of the first two principle components (PCOs; Fig. 1) shows the phenotypic variation among the 19 genotypes. The accessions are dispersed along the both PCO axes. 'Picholine marocaine' and its clones 'Menara', 'Haouzia', 'S7', 'S8', 'M14' and 'M16' were clustered with 'Sourani' at the positive side of the both PCOs. However, 'M26', 'Leccino', 'Ayvalik' and 'Bakhboukh Beldi' were grouped at the negative side of PCO2 and the positive side of PCO1. The local cultivars ('Bouchouk Rkik', 'Bouchouk Laghlid', 'Bouchouika'and 'Carolea') were distributed in a quarter representing the positive side of PCO2 and the negative side of PCO1. Whereas, 'Picholine de Languedoc', 'Arbequine' and 'Branquita' were distributed on the negative sides of PCO1 and PCO2. The analysis separated the 19 genotypes with Rogers-Tanimoto dissimilarity index ranging from 0.125 to 0.88. The cluster analysis based on morphological data using NJ method is presented in Fig. 2. The cophenetic correlation between the dendrogram and the dissimilarity matrix revealed a good degree of fit ($r= 0.9517; p<0.001$). The dendrogram identified mainly two major clusters, first cluster consisted of local varieties of the province of Sidi Kacem and Mediterranean varieties, second cluster consisted of 'Picholine marocaine', its clonal derivatives and 'Leccino'. The first cluster could be subdivided into 2 subgroups: the first includes local varieties and the Mediterranean varieties ('Ayvalik', 'Arbequine', 'Branquita' and 'Picholine de Languedoc'). The second subgroup includes two Mediterranean varieties ('Sourani' and 'Carolea').

Characterization with microsatellite markers

The 20 microsatellites primer pairs used were successfully amplified the corresponding microsatellite fragments in all the 19 genotypes. Nine out of the 20 microsatellite markers showed polymorphism when tested with the 14 varieties and 5 clones of olive tree (Supplementary Table S3). The degree of differentiation of genotypes varied by the individual microsatellite locus. The microsatellite locus ssrOeUA-DCA17 efficiently differentiated 'M16', 'Menara', 'Haouzia' and 'Picholine marocaine' (PM) variety, whereas 'S7', 'S8', and 'M14' clones showed same profiles as PM. The microsatellite marker ssrOeUA-DCA17 can be efficiently used for differentiation of PM and the varieties developed through clonal selection namely, 'Haouzia' and 'Menara', even at an early age, in the nurseries. The SSR marker GAPU 71A showed three profiles, the first profile for PM and its clones and 'Branquita', 'Ayvalik', 'Carolea', 'Sourani' and 'Arbequine', the second for all the Moroccan local varieties and the third profile for 'Leccino'. The combined profiles of all the nine microsatellite markers failed to distinguish between 'Bouchouk Rkik' and 'Bouchouk Laghlid'; and also between 'S7', 'S8', and 'M14' clones. The nine polymorphic microsatellites markers revealed a total of 48 alleles among the 19 genotypes. The number of alleles ranged from 3 (for GAPU 71A locus) to 9 (for ssrOeUA-DCA 17 locus), with an average of 5.3 alleles per locus (Supplementary Table S3). Observed heterozygosity ranged from 0.42 to 1 with a mean of 0.79 (Supplementary Table S3). The results from the principal coordinates analysis (PCOA) revealed that a considerable amount of variation (64.3%) was explained by the first three axes: axes 1, 2 and 3 explained 36.9%, 17% and 10.3% of the variation respectively. The scatter diagram of the first two PCOs (Fig. 3) shows the molecular variation among the 19 genotypes. The accessions were dispersed along the both PCO axes. 'Picholine marocaine' and its clones 'Menara', 'Haouzia', 'M26', 'S7', 'S8', 'M14' and 'M16' were clustered together at the positive side of PCO1, whereas the local cultivars ('Bouchouk Rkik', 'Bouchouk Laghlid', 'Bouchouika'and Bakhboukh Beldi') were distributed in the negative side of the PCO1 and PCO2. The

Table 1. *Olea europaea* subsp. *europaea* L. used in the study

Name of variety/clone	Nature of the variety/clone	Main area of cultivation
'Picholine de Languedoc'	Exotic variety	France
'Leccino'	Exotic variety	Italy
'Branquita'	Exotic variety	Portugal
'Ayvalik'	Exotic variety	Turkey
'Carolea'	Exotic variey	Italy
'Sourani'	Exotic variety	Syria
'Arbequine'	Exotic variety	Spain
'Picholine Marocaine'(PM)	Local variety	Morocco
'Menara'	Clone selected from PM	Morocco (from Haouz region)
'Haouzia'	Clone selected from PM	Morocco (from Haouz region)
'S8'	Clone selected from PM	Morocco (from Tadla region)
'S7'	Clone selected from PM	Morocco (from Tadla region)
'M14'	Clone selected from PM	Morocco (from Tadla region)
'M16'	Clone selected from PM	Morocco (from Tadla region)
'M26'	Clone selected from PM	Morocco (from Haouz region)
'Bakhboukh Beldi'	Local variety	Morocco (from Sidi Kacem region)
'Bouchouk Rkik'	Local variety	Morocco (from Sidi Kacem region)
'Bouchouika'	Local variety	Morocco (from Sidi Kacem region)
'Bouchouk Laghlid'	Local variety	Morocco (from Sidi Kacem region)

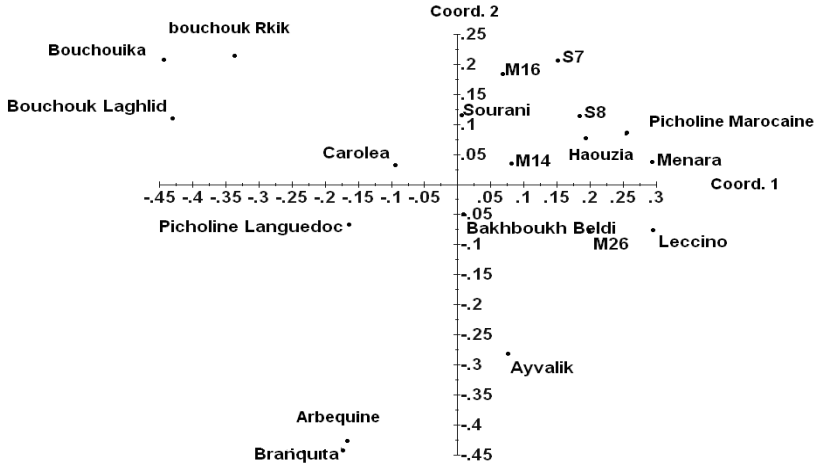

Fig 1. Principal coordinates analysis of 19 olive varieties based on morphological data. The first and the second axis together explained 59% of the total variation. 'Picholine marocaine' and its clones 'Menara', 'Haouzia', 'S7', 'S8', 'M14' and 'M16' were clustered with 'Sourani' at the positive side of the both PCOs. 'M26', 'Leccino', 'Ayvalik' and 'Bakhboukh Beldi' were grouped at the negative side of PCO2 and the positive side of PCO1. The local cultivars ('Bouchouk Rkik', 'Bouchouk Laghlid', 'Bouchouika'and 'Carolea') were distributed in a quarter representing the positive side of PCO2 and the negative side of PCO1.

Mediterranean varieties were distributed in a quarter representing the positive side of PCO2 and the negative side of PCO1, except 'Carolea' which was distributed in the negative side of the PCO1 and PCO2. The dissimilarity coefficients ranged from 0.11 to 0.92. The NJ dendrogram constructed based on the dissimilarity matrix revealed a good degrees of fit with dissimilarity coefficient (r= *0.9851; p<0.001*). The NJ dendrogram highlights the formation of three distinct clusters (Fig. 4).The first cluster consists of the Mediterranean varieties ('Leccino', 'Picholine Languedoc', 'Ayvalik, 'Arbiquine', 'Souran', 'Carolea' and 'Branquita'). The second one was composed of two branches. One of the branch comprised of 'S7', 'S8', 'M14' and 'M16' clones selected from 'Picholine marocaine' and the other branch with, 'Picholine marocaine' and its clones 'Menara', 'Haouzia', and 'M26'. The third cluster was formed by the Moroccan local varieties originating in the Sidi Kacem Province ('Bouchouk Laghlid', 'Bouchouk Rkik', 'Bakhboukh Beldi' and 'Bouchouika'). The cluster analysis clearly showed a relationship for microsatellite profiles of the local varieties ('Bouchouk Laghlid', 'Bouchouk Rkik', 'Bakhboukh Beldi' and 'Bouchouika') and the clones to their respective geographical origin. In order to study the extent of agreement between dendrograms derived from morphological traits and microsatellite markers, the respective distance matrices were compared using the Mantel (1967) matrix correspondence test. The analysis revealed a positive and highly significant correlation (r=0.538*; p*<0.001, 1000 random permutations) between the morphological and microsatellite marker-derived dissimilarity matrices.

Discussion

In Morocco, previous studies showed the predominance of only one genotype, referred to as 'Picholine marocaine' in the cultivated olive populations (Khadari et al., 2007). A survey

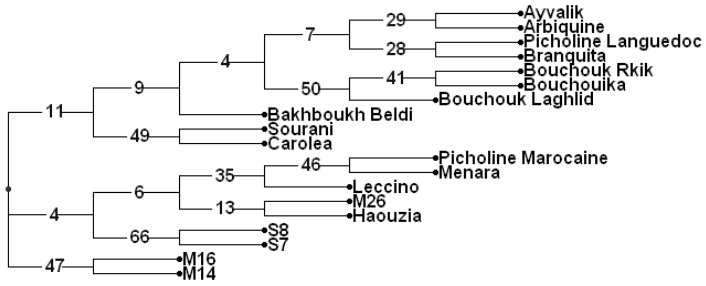

Fig 2. Dendrogram (NJ method) of 19 olive genotypes based on genetic distances calculated using morphological data. Bootstrap values supporting nodes are shown. Distances were obtained with Rogers-Tanimoto dissimilarity coefficient.

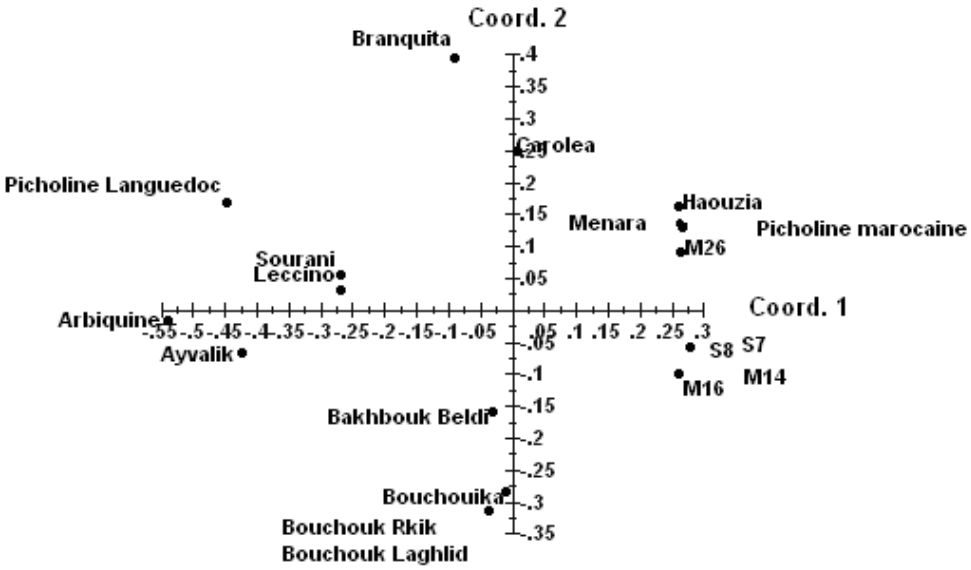

Fig 3. Principal coordinates analysis of 19 olive genotypes based on microsatellites data. The first and the second axis together explained 53.9% of the total variation. 'Picholine marocaine' and its clones 'Menara', 'Haouzia', 'M26', 'S7', 'S8', 'M14' and 'M16' were clustered together at the positive side of PCO1, whereas the local cultivars ('Bouchouk Rkik', 'Bouchouk Laghlid', 'Bouchouika'and Bakhboukh Beldi') were distributed in the negative side of the PCO1 and PCO2. The Mediterranean varieties were distributed in a quarter representing the positive side of PCO2 and the negative side of PCO1, except 'Carolea'.

of trees from 'Picholine marocaine' varieties grown in Haouz and Tadla areas of Morocco was carried out and the clones that showed unusual traits were selected from the 'Picholine marocaine' populations. Two of these clones were subsequently registered as varieties, and named as 'Menara' and 'Haouzia' (INRA, 2008). These clones resemble to 'Picholine marocaine' for most of the characters and differ for the few. In olive tree, microsatellites had been successfully applied for diversity analysis of ancient varieties (Cipriani et al., 2002), varietal identification and characterization (Rekik et al., 2008; Muzzalupo et al., 2009; 2010) and to address the issue of homonymy and synonymy (Khadari et al., 2003; Bracci et al., 2009). However, these studies did not address the potential of microsatellite markers for characterization of varieties developed through clonal selection. In a recent study by RAPD and ISSR analysis revealed the existence of a high level of genetic variability among 'Cobrancosa' clones (Martins-Lopes et al., 2009), Similar levels of polymorphism were detected in the Portuguese olive varieties 'Galega' (Gemas et al., 2004) and 'Verdeal-Transmontana' (Gomes et al., 2008). Our study clearly showed that microsatellite markers are highly useful for varietal identification and characterization of olive trees developed through clonal selection. The level of polymorphism observed in this study agrees with results of previous studies carried out in olive varieties with microsatellite markers (Fabbri et al., 1995; Angilillo et al., 1999; Baldoni et al., 2009; Rallo et al., 2000; Belaj et al., 2003; Besnard et al., 2001b; Rallo et al., 2003).

The values of allele number compared to those in the literature, using a comparable number of SSR loci, are somewhat lower than the number of alleles detected by Lopes et al. (2004). The reason for low number of alleles observed in our study could be due to use of large number of clones derived from 'Picholine marocaine' than the exotic varieties.In this study, both morphological and molecular data were used to estimate relationships among the varieties, in order to establish an evidence for reliability of microsatellite

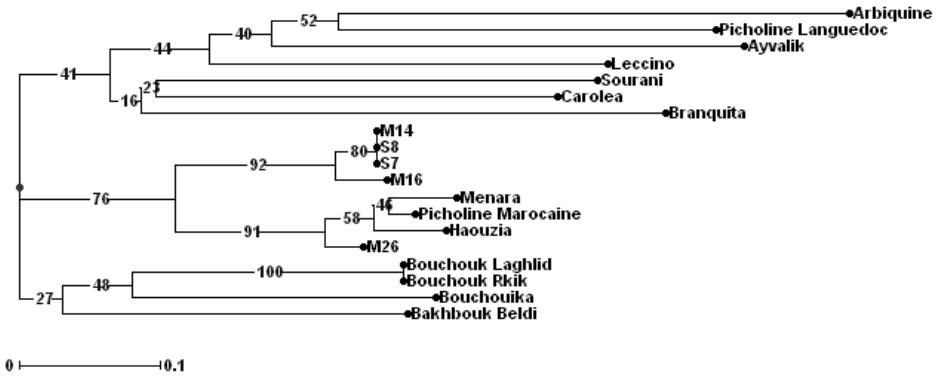

Fig 4. Dendrogram (NJ method) of 19 olive genotypes based on genetic distances calculated using microsatellites data. Bootstrap values supporting nodes are shown. Distances were obtained with the Jaccard coefficient.

markers for differentiation and to establish a combined fingerprint (of both morphological and molecular information) of each variety. The differentiation based on morphological traits and microsatellite markers showed good correlations, indicating their high reliability and usefulness in differentiation of olive varieties. A trend of clustering of varieties originating from the same or adjacent regions was also detected. 'Menara', 'Haouzia' and 'M26' selected through clonal selection in Haouz area formed a cluster including 'Picholine marocaine'. Whereas, the varieties 'S8', 'S7', 'M14' and 'M16' selected from the Tadla area formed a separate cluster. Similarly local varieties from Province of Sidi Kacem, Morocco ('Bouchouk Laghlid', 'Bouchouk Rkik', 'Bakhboukh Beldi' and 'Bouchouika') formed a different cluster, indicating a relationship between geographic origin and genetic relationships at microgeographical scale. The clustering of native or local varieties or clonal selections provides evidence that soil and climate of the geographic location have substantial influence on their differentiation over the years. The similar behaviour was observed by Sarri et al. (2006) for wider geographical scale with microsatellite analysis and by Sanz-Cortes et al. (2001) for microgeographical scale using AFLPs. The clustering of the varieties from the same or nearby region further suggests a common genetic base and an autochthonous origin for these olive tree varieties.

Materials and methods

Plant materials

The olive varieties used for this study are maintained in the olive orchard of the Regional Agronomic Research Center, INRA, Marrakech, Morocco. The list of genotypes and the main area of their cultivation are presented in Table 1. These olive genotypes were evaluated for morphological traits and microsatellite polymorphisms.

Morphological characterisation

Morphological description was carried out by using the "methodology for primary characterisation of olive varieties" proposed by the International Olive Council (COI, 1997). Most of the characters that were evaluated were also similar to UPOV guidelines for the conduct of tests for olive distinctness, homogeneity, and stability (DHS), which includes 15 characters that had a very high discriminating power for the identification of olive varieties, namely, one character of leaf, 4 characters of fruit and 10 characters of endocarp (see Supplementary Table S1).

DNA isolation and amplification of microsatellites

Total genomic DNA was extracted from young growing leaves of the tree collected from each variety using the Wizard Genomic DNA purification Kit (Promega, USA). We initially analyzed the varieties by using twenty microsatellite loci selected among three sets of microsatellite markers: three markers (GAPU59, GAPU71A and GAPU71B) from the primer set designed by Carriero et al. (2002), eight markers (UDO6, UDO12, UDO14, UDO17, UDO27, UDO34, UDO36 and UDO44) from Cipriani et al. (2002) and nine markers (ssrOeUA-DCA01, ssrOeUA-DCA03, ssrOeUA-DCA04, ssrOeUA-DCA05, ssrOeUA-DCA07, ssrOeUA-DCA11, ssrOeUA-DCA13, ssrOeUA-DCA15 and ssrOeUA-DCA17) from Sefc et al. (2000) (Supplementary Table S3). PCR amplification of microsatellites was carried out in a reaction volume of 10 µl containing 20 ng genomic DNA, 1x PCR green buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 unit of *Taq* DNA polymerase (Promega) and 10 pmol of each primer. Polymerase chain reaction (PCR) was carried out using Eppendorf Master Cycler gradient thermocycler (Eppendorf, Germany). PCR cycles consists of 2 min initial denaturation at 95°C, followed by 35 cycles, each cycle with 30 s denaturation step at 95°C; a 30 s annealing step at either 50 or 55°C, depending on the optimum annealing temperature for a primer pair (see Supplementary Table S3) and 30 s elongation at 72°C. The PCR concluded with a 5 min final extension at 72°C. PCR products were separated on 6% denaturing polyacrylamide gels (Sequi-Gen GT System, gel size 38 x 30 cm; Bio-Rad, USA), containing 1x TBE and 7.5 M urea. The gel was run in 0.5x TBE at constant power of 35 W for 1.5-2 hrs. Gels were stained with silver using the protocol described by Bassam et al. (1993) with minor modifications. Digital images of gels were taken using a scanner. Microsatellite allele sizes were determined with a 100 bp DNA ladder (Promega, USA).

Data analysis

The morphological data were used in multivariate analysis with the major goals to distinguish between olive varieties and to determine the main characters that allow differentiation between the varieties. Each of morphological traits namely, leaf, fruit and endocarp characters had 2 to 5 variants and were denoted as numbers from 1 to 5, respectively. The principal coordinates analysis (PCOA) was used for analysis of the morphological traits. The Rogers-Tanimoto dissimilarity coefficient's between genotypes that summarised variation and discriminated the weight of qualitative characters, was computed. The two-dimensional PCOA was performed based on the dissimilarity matrix. The genotypes were then clustered by the Weighted Neighbor-Joining (NJ) method. A cophenetic value matrix (Sneath and Sokal, 1973) of the (NJ) clustering was used to test for the goodness-of-fit of the clustering to the dissimilarity matrix on which it was based, by computing the product-moment correlation (*r*) with 1000 permutations (Mantel, 1967).
Microsatellite polymorphisms were scored for the presence (1) or absence (0) of amplified bands and were used for estimation of the dissimilarity coefficients between cultivars using Jaccard's coefficient method (Jaccard, 1908). The individual microsatellite polymorphism for the varieties were plotted in a bidimensional space using principal coordinates analysis. The dissimilarty matrix was used to construct a dendrogram using the NJ method. The reliability of the inferred tree was tested by a bootstraping with 1,000 resamplings. These analyses were carried out using the DARwin 5.0.148 software program (available at http://darwin.cirad.fr/darwin/Home.php). The relationship between the dissimilarity matrix based on morphological traits and the genetic dissimilarity matrix based on microsatellite polymorphism was analyzed according to Mantel (1967) using the NTSYSpc ver. 2.01 program (Sneath and Sokal, 1973).

Conclusion

The results from the present study clearly showed that microsatellite markers are able to differentiate closely related varieties such as clonal selections, in addition to distantly related varieties. The combination of microsatellite marker profiles and the morphological characters serve as reliable tools for detailed description of varieties. The improved varietal identification and sanitary process could result in improvement in oil quality and enhance opportunity for marketing via product labeling by variety.

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