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Fingerprinting of the main olive cultivars in Tunisia by morphological and AFLP markers.

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Abstract - A study was conducted to compare morphological and genetical data of the main local olive cultivars in Tunisia. This work was conducted in the framework of the activities of the fruit tree network in the Tunisian National Gene Bank. Samples were taken from leaves, fruits and stones for morphological characters. DNA was extracted from leaf tissue and 6 EcoRI–MseI AFLP primer combinations were used. The morphological study permitted a specific description of the characteristics for the tested varieties and their repartition into three groups according to the fruit and endocarp quantitative data. Molecular data analysis demonstrated a high degree of polymorphism with an average of 35%. The analysis of AFLP profiles found in our set of olive cultivars showed a wide genetic diversity among olive germplasm. The UPGMA cluster analyses using Jaccard's index revealed that the genetic diversity was predominantly structured according to fruit size. The data obtained was used for the varietal survey and construction of National Gene Bank fruit crops database, which will help in providing also additional information that could form the basis for the national design of olive breeding programs.

Keywords: AFLP, Gene bank, local cultivar olive, morphological data.

1. Introduction

The Tunisian National Gene bank plays a key role in the conservation, availability and use of a wide range of plant genetic diversity for crop improvement for food and nutrition security. Olive tree (*Olea europaea* L.) is among the main preserved species in the Gene bank. This species plays a strategic role in the national economy. Tunisia is the second largest producer of olive oil nation in the world after the European Union 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 2012).

The main olive varieties cultivated in Tunisia are '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).

Identification and conservation of local olive genotypes showing medium to large fruit sizes, good oil quality and considerable tree sizes are important priorities for the National gene bank to know and promote the local olive genetic resources (Mnasri et al. 2014a). Research activities aim on the one hand at further improvements in the characterization and the management of the local olive cultivars.These include the analysis of the morphological, DNA fingerprinting technology to monitor the genetic integrity of samples, the investigation of spatio-temporal patterns of genetic diversity, and the analysis of population structures.

There are many systematic identification procedures that have been developed to help identify genetic diversity in olive trees. The phenotypical characterization of the olive covered all the plant organs: leaf, fruit, inflorescence and stone. Characterization of this type was first carried out at international level by the Food and Agricultural Organization of the United Nations (FAO 1981) and Rallo and Barranco (1984). Subsequently, characterization moved further forward in studies conducted by the IOC (1997), in which as many as thirty phenotypical traits were determined on the basis of the leaf, fruit, stone, inflorescence and tree. This research served as the basis for the publication of a major catalogue reporting 134 olive varieties from 23 countries (IOC 2000).

Molecular markers, such as RAPD, AFLP, ISSR, SSR and SNP has also been used to analyze the genetic diversity in cultivated and wild type olives because morphological and biochemical traits have in general not been able to clearly differentiate between wild olive and feral, or between closely related cultivars (Kole 2011). These techniques along with their markers are currently available and are extremely useful when the morphological traits do not clearly identify the genetic diversity within related genotypes. A comparison of diversity assessments using AFLP (Grati kamoun et al. 2006; Mnasri et al. 2014 b) and RAPD (Lumaret et al. 2004) determined that AFLP illustrated greater allelic diversity than RAPD. AFLP markers have been used as an applicable tool for fingerprinting and determination of genetic similarities. (Vos et al. 1995; Angiolillo et al. 1999) pointed out that AFLP marker techniques are still being used for genetic diversity and fingerprinting due to their simplicity of use and low development cost in developing countries when the financial situation is limited.

In the present paper we focus on the characterization and the conservation of the local minor olive cultivars in Tunisia. Our work was conducted in the framework of the activities of the fruit tree network in the Tunisian National Gene Bank. The main objectives was to analysis the morphological and molecular data of the main minor local olive varieties in Tunisia and their ex-situ conservation in the germoplasm collection of the National gene Bank.

2. Materials and Methods

2.1. Plant Material

A total of 30 cultivated varieties of olive (Olea europaea L.) during the growing seasons 2012/2013 and 2013/2014 from eight different agrosystem localized in the north, the center and the south of Tunisia (Table 1). These cultivars represent the majority of named olives in Tunisia. The collection sites represent the diverse environmental conditions in which olives are grown. Three trees were representatively sampled for each cultivar; forty fruits and forty leaves were collected from each tree to evaluate qualitative and quantitative phenotypic traits.

2.2. Morphological characterization

The morphological traits were systematically evaluated for the biometrical parameters of the leaves, fruits and endocarps. The methodology used in this characterization is based in the recommendations of the IOC (1997). A total of twenty nine (qualitative and quantitative) characteristics were used: 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”).

Table 1 .Descriptive statistical analysis of the morphological parameters

Variety	Code	Geographical location	Governorate
Roumi	C1	Makthar	Siliana
Chétoui	C2	Makthar	Siliana
Rajou	C3	Makthar	Siliana
Meski	C4	Makthar	Siliana
Neb Jmel	C5	Makthar	Siliana
El hor	C6	Kesra	Siliana
Esraadki	C7	Kesra	Siliana
Chetoui	C8	Zahret Medyen	Béja
Neb Jmel	C9	Zahret Medyen	Béja
Unknown	C10	Zahret Medyen	Béja
Unknown	C11	Zahret Medyen	Béja
Unknown	C12	Douamis	Bizerte
Chetoui	C13	Douamis	Bizerte
Chetoui abyeth	C14	Haouaria	Nabeul
Chemlali	C15	Haouaria	Nabeul
Unknown	C16	Sbeïtla	Kasserine
Tounsi	C17	Sbeïtla	Kasserine
Unknown	C18	Hbebsa	Siliana
Chemlali	C19	Hbebsa	Siliana
Sayali	C20	Hbebsa	Siliana
Meski	C21	Hbebsa	Siliana
El guime	C22	Hbebsa	Siliana
Souihli	C23	Hbebsa	Siliana
Neb Jmel	C24	Hbebsa	Siliana
OUESLATI	C25	La Bayed	Sidi Bouzid
Besbessi	C26	La Bayed	Sidi Bouzid
Meski	C27	La Bayed	Sidi Bouzid
Unknown	C28	La Bayed	Sidi Bouzid
Unknown	C29	La Bayed	Sidi Bouzid
Unknown	C30	La Bayed	Sidi Bouzid

2.3. Molecular Characterization

2.3.1. DNA extraction

Total genomic DNA was extracted from young leaf tissue following the method described by (Angiolillo et al. 1999) based on CTAB process. DNA was quantified on 0.8 % agarose gel by λ Hind III DNA ladder (Promega).

2.3.2. AFLP analysis

AFLP analysis was performed as previously described for olive (Angiolillo et al. 1999). Four EcoRI primers (E-AAC, E-ACC, E-ACA and E-AAG) and six MseI primers (M-CTC, M-ACG, M-ATT, M-AGG, M-GCT and M-CAA) with three selective nucleotides were used. A total of six highly polymorphic primer combinations were screened (Table 2) among those previously tested on the Tunisian olive varieties by Grati Kammoun et al. (2006). PCR amplification products were revealed by Bio-Rad Experion™ Automated Electrophoresis System (Figure 1)

2.4. 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).

AFLP results were scored for presence (1) and absence (0) of amplified fragments. Pair wise genetic similarities were calculated using Dice similarity coefficient (Dice 1945; Neil and Li 1979) and the similarities between accessions were calculated using Jaccard's coefficient for qualitative data (Jaccard 1908), according to the formula: a/ (n-d) where n is the total number of polymorphic bands, a the bands present in both accessions and d the bands absent in both accessions. The similarity matrix (calculated

with the Jaccard coefficient) was used to construct a dendrogram by means the arithmetic averages algorithm (UPGMA) methods. All calculations were performed with the use of GENEPOP 3.2 (Raymond and Rousset 1995) and NTSYS-pc version 2.1 (Rohlf 1998).

Table 2. Polymorphism rates of the six primer combinations.

Primer combination	Total number of bands*	NPB*	PR*(%)
E-AAC/MCTC	75	30	40
EACC/MACG	47	19	40
EAAG/MATT	9	2	22
EACA/MAGG	25	7	28
EACC/MCAA	34	13	38
EACA/MGCT	47	21	44
Total	**237**	**92**	
Mean	**28**	**15**	**35**

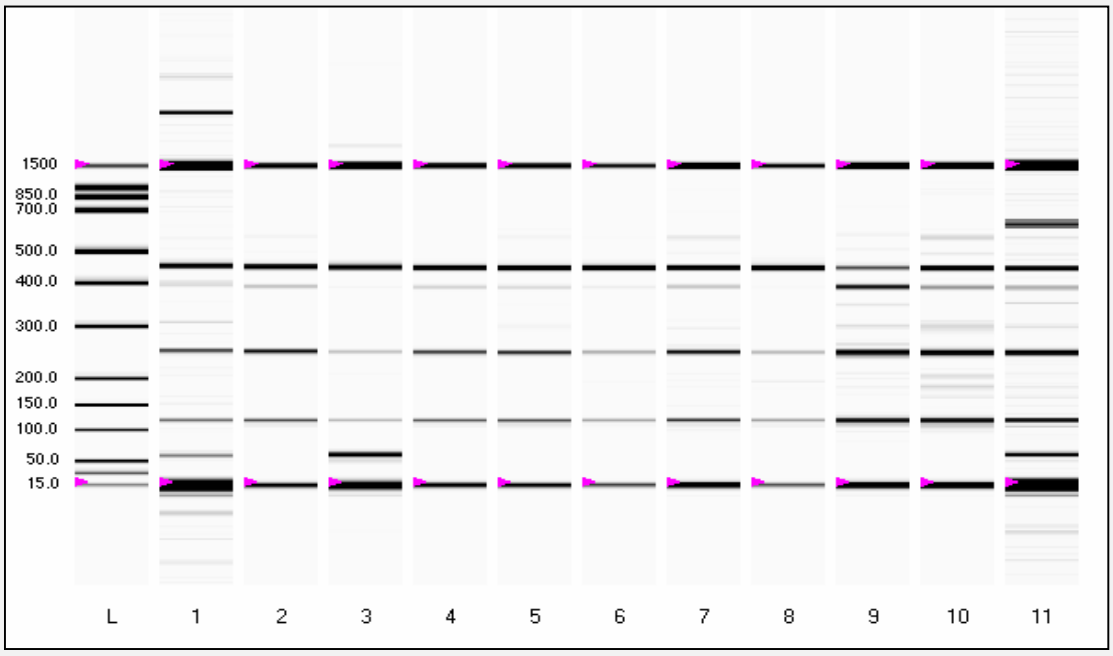

Figure 1. Electrophoretic profile of the amplification products of some Tunisian autochthon olive cultivars using the AFLP primers (EAAG/MATT)

3. Results and discussion

3.1. Morphological polymorphism

The morphological analyses of the leaf, fruit and stone data revealed an important variability of the tested olive cultivars (Table 3). A high significant difference between the cultivars was observed for the most considered characteristics ($P < 0.05$). Especially the length (V4), maximum diameter (V5) and the weight (V7) of the fruit, as well the length (V8), the maximum diameter (V9) and the weight of the endocarp. The fruit weight ranged from 0.25 to 5.14g, the endocarp weight varied from 0.1 to 0.72g. The local olive oil cultivar ‘’Souihli’’ localized in the region of Hbebsa present the lowest fruit and endocarp weight, while the table olive variety “Meski” localized in the region of Makthar present the

highest values. These results are in good agreement with previous works by (Lousser and Brousse 1978 ; Trigui and Msallem 2002; Hannachi et al. 2008 a; Hannachi et al. 2008 b; Mnasri et al. 2013a ; Mnasri et al. 2014a).

The results of the geometric method of multivariate statistical analysis (PCA) performed on eleven quantitative and eighteen qualitative morphological data (Figure 2) revealed that axis1 and axis 2 of the PCA accounted respectively 43.5% and 21,14% of the total variance. The first axis is highly correlated with the fruit traits (length "V4", maximum diameter "V5", weight "V7" and the qualitative data nipple presence "V17") also with the endocarp data (length "V8", maximum diameter "V9", weight "V11", symmetry in position B "22", base "V25", surface "V26" and distribution of grooves "V28"). The second axis is correlated with the leaf traits (length "V1", width "V2", and Longitudinal curvature of the blade "V12") as well as some fruit and stone qualitative data (shape, symmetry in position A, apex form, presence and dimension of small lens).These results proved the importance of the endocarp and fruit morphological parameters to discriminate between olive cultivars as demonstrated by (Paula et al. 2005; Zaher et al. 2011; Mnasri et al. 2013a ; Mnasri et al. 2014a). Badanes in 1998 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 (Caldo et al.1996).

Table 3: List of the studied olive cultivars and their geographical locations

Variable	Minimum	Maximum	Average	CV%	LSD
V1	37,25	66,27	55,73	11,72	0.021^{*}
V2	9,05	16,60	11,99	16,53	0.167^{NS}
V3	3,53	6,79	4,82	19,31	0.013^{**}
V4	7,73	25,85	16,71	27,56	0.001^{***}
V5	2,72	20,18	11,88	35,99	0.006^{***}
V6	1,18	2,85	1,49	21,56	0.002^{***}
V7	0,26	5,61	1,85	73,55	0.002^{***}
V8	8,75	17,16	13,09	19,43	0.001^{***}
V9	4,78	9,37	6,41	19,04	0.0001^{***}
V10	1,45	2,55	2,05	13,37	0.01^{**}
V11	0,10	0,72	0,30	54,72	0.0001^{***}

P-value: NS: no significant ** significant ($P < 0.05$); *** highly significant ($p < 0.01$) CV% Variation coefficient expressed in percentage

The projection of the thirty olive cultivars in the plane generated by the first and second PCA axis permitted the separation of the accessions in two main groups according to the fruit and the endocarp form and weight. The first cluster encloses table olives as "Meski" (C4, C20 and C27) and "Besbessi" (C26) and also dual-purpose cultivars as "Neb Jmel" (C5, C9 and C24), "Tounsi" (C17), "Rajou" (C3), Chétoui (C2), "Sayali" (C21) and Sradki (C7). The second group includes only oil olive cultivars which are characterized by low weight fruits, as the varieties "Chemlali" (C15 and C19), "Oueslati" (C25), "Souihli" (C23) and "El Hor" (C6). In fact, several researchers have demonstrated the richness of the Tunisian olive patrimony by oil olives, table olives and also dual-purpose cultivars (Loussert and Brousse 1978; Trigui and Msallem 2002 ; Grati Kammoun et al. 2006).

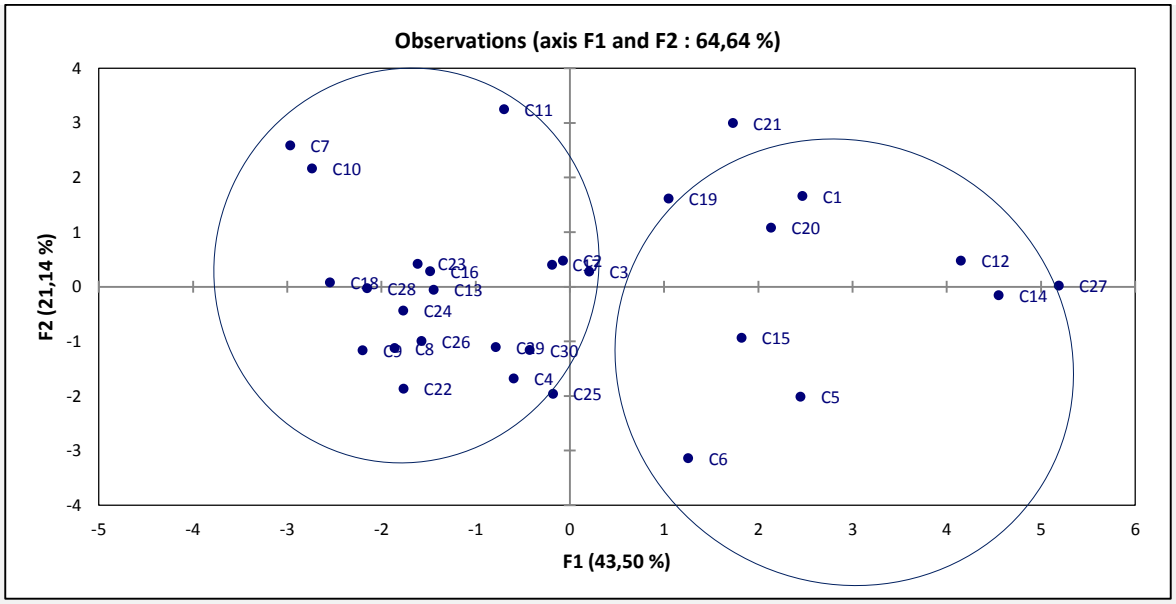

Figure 2. Projection of the thirty studied olive varieties in the plane generated by the first two principal components based on leaf, fruit and endocarp traits.

3.2. AFLP analyses

Six AFLP primer combinations (Table 2) were used to analysis the molecular polymorphism of the thirty autochthon olive cultivars. The PCR amplification products were revealed by Bio-Rad Experion™ Automated Electrophoresis System (figure 3). The results revealed a total number of 237 amplified DNA fragments of different size (50pb to 1300pb); among which 92 were polymorphic. The number of polymorphic bands varied from 2 (EAAG/MATT) to 30 (EAAC/MCTC) with an average of 15 bands per primer combination. These results suggest that the morphological diversity of the Tunisian olive cultivars is at least partly attributable to genetic causes and proved the observations of Wiesman et al. (1998). Moreover, AFLP markers have been used as an applicable tool for fingerprinting and determination of olive genetic similarities in Tunisia and proved the wide genetic basis of olive germplasm in our country (Grati-Kamoun et al. 2006 ; Tamaali et al. 2006 ; Mnasri et al. 2013 b ; Mnasri et al. 2014a).

The diversity of the studied sample was approached by calculating a dendrogram of genetic similarity (figure 3) based on Jaccard index (1901) with NTSYS-PC (Rohlf 1998). Four main clusters were revealed by cutting the dendrogram at a GS value of 0.48. The first group consisted of three table olive accessions with high genetic similarity (Gs=0.99), in fact these cultivars present different clones (C4, C21 and C27) of the same variety "Meski" localized in the regions of Makthar, Hbebsa and La bayed. The second group include essentially the dual-purpose olive cultivars (C1, C2, C3, C5, C8, C9, C10, C11, C12, C13, C14, C18, C23 and C24) characterized by medium fruit weight, ovoid shape and the presence of the nipple. The third group encloses four oil cultivars (C6, C22, C25 and C28) and the table olive (C26), 127 polymorphic AFLP bands assembled the cultivars of the third cluster, which are distinguished by their spherical fruits. The last group consisted of six oil olive cultivars (C7, C29, C30, C16, C15 and C19) and three dual-purpose olives (C1, C17 and C20) grouped by 118 polymorphic bands and characterized by their asymmetric and ovoid fruits and endocarp. As a consequence, there isn't any apparent correlation between DNA polymorphism, the fruit weight and the origin of the olive cultivars, these results consist with the hypothesis proved that early after domestication, olive cultivars of horticultural value were moved widely from region to region by human migration which have favored the dispersal of olive, cultivated in the whole Mediterranean basin along many centuries (Chevalier 1948; Cifferi 1950 ; Fabbri et al. 1995; Ouazzani et al. 1995). Further, the UPGMA cluster analyses revealed that the genetic diversity of the tested autochthon olive cultivars was predominantly structured according to the morphological data (shape and symmetry in position A) of the fruit and the endocarp, which proved the importance of these parameters in the classification of the olive varieties. Grati

Kamoun et al. (2006) and Mnasri et al. (2013 b) in their analysis of olive biodiversity in Tunisia by AFLP obtained a comparable clustering of cultivars based on fruit and endocarp parameters.

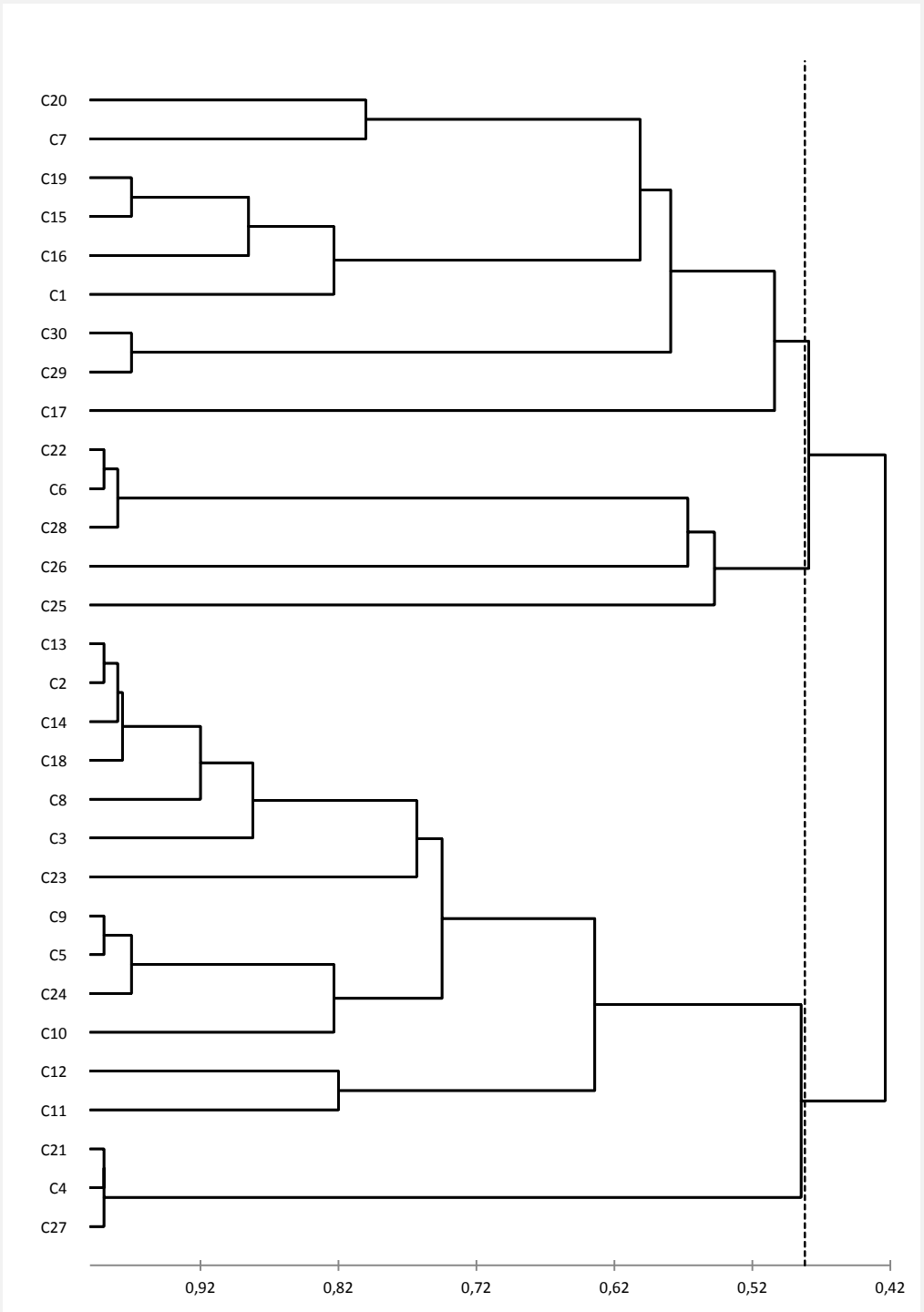

Figure 3. UPGMA dendrogram based on dice similarity matrix between 30 samples of autochthon Tunisian olive cultivars.

4. Conclusion

Tunisia, having been the crossroads of many civilizations, the basis for trade exchange between the East, Africa and Europe, inherited from these flows a rich olive-growing genetic inheritance. Besides the two principal varieties Chemlali and Chetoui, several minor local varieties widespread in different Tunisian olive growing areas. The inventory and characterization of thirty different olive cultivars localized in

eight different restricted areas localized in the north, the center and the south of Tunisia by the National Gene Bank discovered the richness of the Tunisian olive patrimony. The morphological data revealed an important variability of the fruit and the endocarp parameters and gives a basis for comparing the different studied olive cultivars. Although this method is efficient, it presents practical drawbacks because of the effect of environmental fluctuations on the expression of most morphological traits. The use of the AFLP marker technology confirmed the performance of this method not only for studying difference between varieties, but also among the clones of the same variety. The comparison of the AFLP and the morphological tools proved the absence of correlation between DNA polymorphism, the fruit weight and the origin of the olive cultivars. However, we have noticed an important correlation between the DNA polymorphism and the qualitative morphological data of the fruit and the endocarp. These results proved the importance of the morphological data and the AFLP markers to establish a fingerprint of each cultivar and to more analysis their intra-clone diversity. The data obtained was used for the first time in Tunisia to construct the National Gene Bank olive crops database, which will help in providing also additional information that could form the basis for the national design of olive breeding programs.

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