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DNA fingerprinting of millennium olive varieties in Tunisia by AFLP markers

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Abstract

Tunisia is one of the oldest agricultural settlements in history. Evidences revealed by archeological excavations indicated that olives were cultivated before about 3000 years in Tunisia. Although the importance of millennium olives, studies about molecular biodiversity and evaluation are scarce. In order to investigate intra cultivar variability on the molecular level, olive samples from nine different archeological sites were screened for amplified fragment length polymorphisms (AFLP). DNA was extracted from leaf tissue and 6 EcoRI–MseI AFLP primer combinations were used. Auto radiographs revealed 84 polymorphic markers in a total of 237 detected fragments. A set of redundant marker patterns was identified and deleted from the binary data matrix; data analysis demonstrated a high degree of polymorphism with an average of 32.7%. 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 and the Principal coordinate analysis (PCO) revealed that the genetic diversity was predominantly structured according to the morphological parameters of the fruit and the endocarp. The data obtained can be used for the varietal survey and construction of a database of millennium olive varieties in Tunisia and providing also additional information that could form the basis for the national design of breeding programs.

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Introduction

Tunisia is one of the oldest agricultural settlements in history. Evidences revealed by archeological excavations indicated that olives were cultivated before about 3000 years in Tunisia (Loussert and Brousse, 1978). The civilizations of the eastern and western Mediterranean such as the Phoenicians, Greeks and Romans, have spread this culture throughout the Mediterranean Basin (Brown, 2004). By 1200 BC, the population growth in the Mediterranean basin led to the establishment of numerous colonies in North Africa (Carthage). The distribution of *Olea* varieties in the Mediterranean basin 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 (Bartolini *et al.,* 2005). The morphological and biochemical analyses applied to millennium olive cultivars from various archeological sites in Tunisia realized by (Mnasri *et al.,* 2013 b) give a basis for comparing specimens in order to reduce the loss of genetic authenticity of Tunisian varieties and to preserve the local genetic resources of olive (*Olea europea* L.). Other than, molecular characterization of these ancient trees will be essential to the description and the protection of this patrimony. Specially, that the morphological and phenological markers have the disadvantage of the small number of polymorphism detected and of being environmentally dependent (Trigui and Msallem, 2002).

DNA-based markers are particularly useful for the correct identification of varieties, due to their independence of environmental conditions and several of them have been successfully applied for olive for example, random amplified polymorphic DNA (RAPDs) (Bogani *et al.,* 1994), amplified fragment length polymorphism (AFLPs) (Angiolillo *et al.,* 1999), sequence characterized amplified regions (SCARs) (Busconi *et al.,* 2006), inter simple sequence repeats (ISSRs) (Hess *et al.,* 2000), single nucleotide polymorphism (SNPs) (Reale *et al.,* 2006) and simple sequence repeats (SSRs) (Poljuha *et al.,* 2008). AFLP marker technology was confirmed to be a powerful tool not only for studying variation between populations of the genus *Olea* as shown by Angiolillo *et al.* (1999), but also for characterizing intraspecific variation among cultivated accessions of *Olea europaea* L. subsp. europaea. In Tunisia Kammoun *et al.* (2006) assessed genetic diversity among 29 different olive varieties using nine AFLP primer combinations and Taamalli *et al.* (2006) revealed the deference between 25 Tunisian olive cultivars by the use of five AFLP primer combinations and ten SSR loci .

In Tunisia, little is known about the millennium olive germplasm, and even though there is an important olive biodiversity (Mnasri *et al.,* 2013 b) studies about characterization and evaluation are scarce. The aim of this work was to make a molecular characterization by the use of six AFLP primer combinations. This study will explore for the first time in Tunisia the genetic diversity of millennium olive varieties. The use of molecular markers AFLP will be essential to verify the denomination of each cultivar and increase the knowledge about the diversity of this species as well as to allow participation in international programmers aiming at olive improvement and conservation.

Material and methods

Plant material

Samples were collected from nine archeological sites localized in the North, the Center and the South of Tunisia (Table 1). The results of (Mnasri *et al.*, 2013 b) have proved the wealth and the importance of the millennium olive germplasm in these sites. The study has been carried out on a sample of 30 cultivars. Three trees were sampled at random in a representative field and analyzed for each cultivar.

Table 1. Pedo-Climatic Characteristics of the different studied archeological sites

Cultivar	Site	Latitude/ longitude (grade)	Altitude (m)	Soil type	Average annual precipitation (mm)	*Average annual temperature (C°)*	Bioclimatic stage
Vm1 Vm2 Vm3 Vm4 Vm5 Vm6	Baja	3700/900	375	*Red Mediterranean Soil*	720	17.8	Sub-humid with warm winter
Vm7 Vm8 Vm9	Bizerte	3709/945	49	*Red Mediterranean Soil*	536	18	Warm temperate climate
Vm10 Vm11	Nabeul	3659/1121	350	Brown calcareous soil	435	18	Sub-humid with warm winter
Vm12 Vm13	Mehdia	3517/4685	106	*Calcimagnesic Soil*	300	23	Mediterranean climate with warm winter
Vm14 Vm15 Vm16	Sbitla	3526/906	626	*clay and sandy loam soil*	350	17	Arid with cold winter
Vm17 Vm18 Vm19 Vm20 Vm21	Kesra	3580/938	878	*Sandy brown* semi desert *soil*	411	18	Semi-arid
Vm22 Vm23 Vm24	Makthar	3586/ 915	1059	Brown calcareous soil	440-560	19	Semi-arid with cold winter
Vm25 Vm26 Vm27	Djerba	3380/1090	34	*sandy soil*	231	19.8	Arid
Vm28 Vm29 Vm30	El Ala	3566/1010	67	Brown calcareous alluvial soil	290	20	Semi-arid with cold winter

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 Kammoun *et al.,* (2006).

Data analysis

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). Principal coordinate analysis has been used to highlight the pair wise relationship between cultivars. 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).

Table 2. Polymorphism rates of the six primer combinations.

Primer combination	Total number of bands*	NPB*	PR*(%)
E-AAC/MCTC	75	28	37.3
EACC/MACG	47	17	36.1
EAAG/MATT	9	2	22.2
EACA/MAGG	25	6	24
EACC/MCAA	34	13	38.2
EACA/MGCT	47	18	38.2
Total	**237**	**84**	
Mean	**28**	**14**	**32.7**

NPB: Number of Polymorphic Bands, PR: Polymorphism Rate in Percent unit

Results and discussion

Genomic DNA from 30 millennium olive cultivars was used for the first time in Tunisia to generate AFLP patterns, in order to study the genetic diversity of this patrimony. AFLP profiles were produced from sex primer combinations of EcoRI and MseI primers (Table 2). The AFLP fingerprinting (example in Figure 1) of the 30 olive genotypes tested, revealed a total number of 237 amplified DNA fragments of different size; among which 84 were polymorphic (32.7%). The relatively large number of polymorphic markers found on the sample of olive cultivars tested suggests that the agronomical and morphological diversity, which they display, is at least partly attributable to genetic causes (Wiesman *et al.,* 1998). The number of amplified fragments varied from 9 (P-AAG/M-ATT) to 75 (P-AAC/M-CTC) with an average of 28 fragments per primer combination. The average percentage of polymorphism ranged from 22.2% for P-AAG/M-ATT to 37.3% for P-ACA/M-GCT primer combination (Table 2). This result is consistent with earlier findings indicating the wide genetic basis of olive germplasm in Tunisia (Taamaali *et al.,* 2006 , Kamoun *et al.,* 2006 and Mnasri *et al.,* 2013 a).

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). Six main groups were revealed by cutting the dendrogram at a GS value of 0.5. Cluster 1 consisted of two main oil cultivars (Vm11 and Vm22) localized in the region of Makthar.

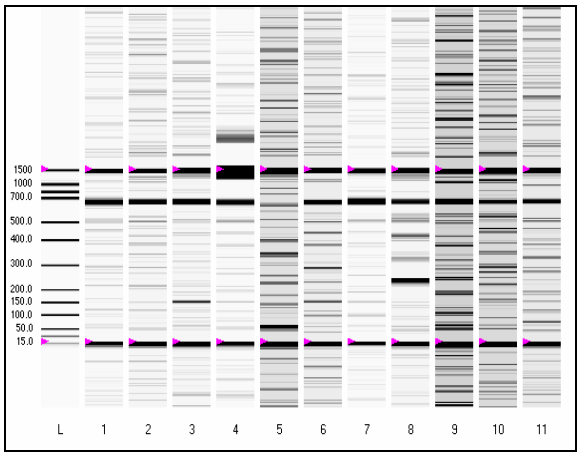

Fig.1. Example of AFLP banding patterns in olive using the prier combination EACA/MAGG.

The second cluster grouped 10 cultivars which are classified in the olive categories of medium to low weight fruit and they can be used with a double aptitude (Barranco *et al.*, 2000). The third cluster is represented by the alone table variety Vm23 localized in the region of Makthar (fruit weight > 5g), essentially used for canning. The cultivars of the fourth cluster are characterized by ovoid and asymmetric fruits with medium-size (2-4g). The fifth cluster grouped essentially the cultivars (Vm10, Vm12, Vm14, Vm16 and Vm27) typically used for oil production and characterized by their height oil quality (Mnasri *et al.,* 2013 b). The sixth cluster grouped the cultivar Vm13 witch present an important morphological similarity with the accessions of the fifth group, however on the molecular level, it is clearly distinguishable from these cultivars at a distance of similarity (0.42%).

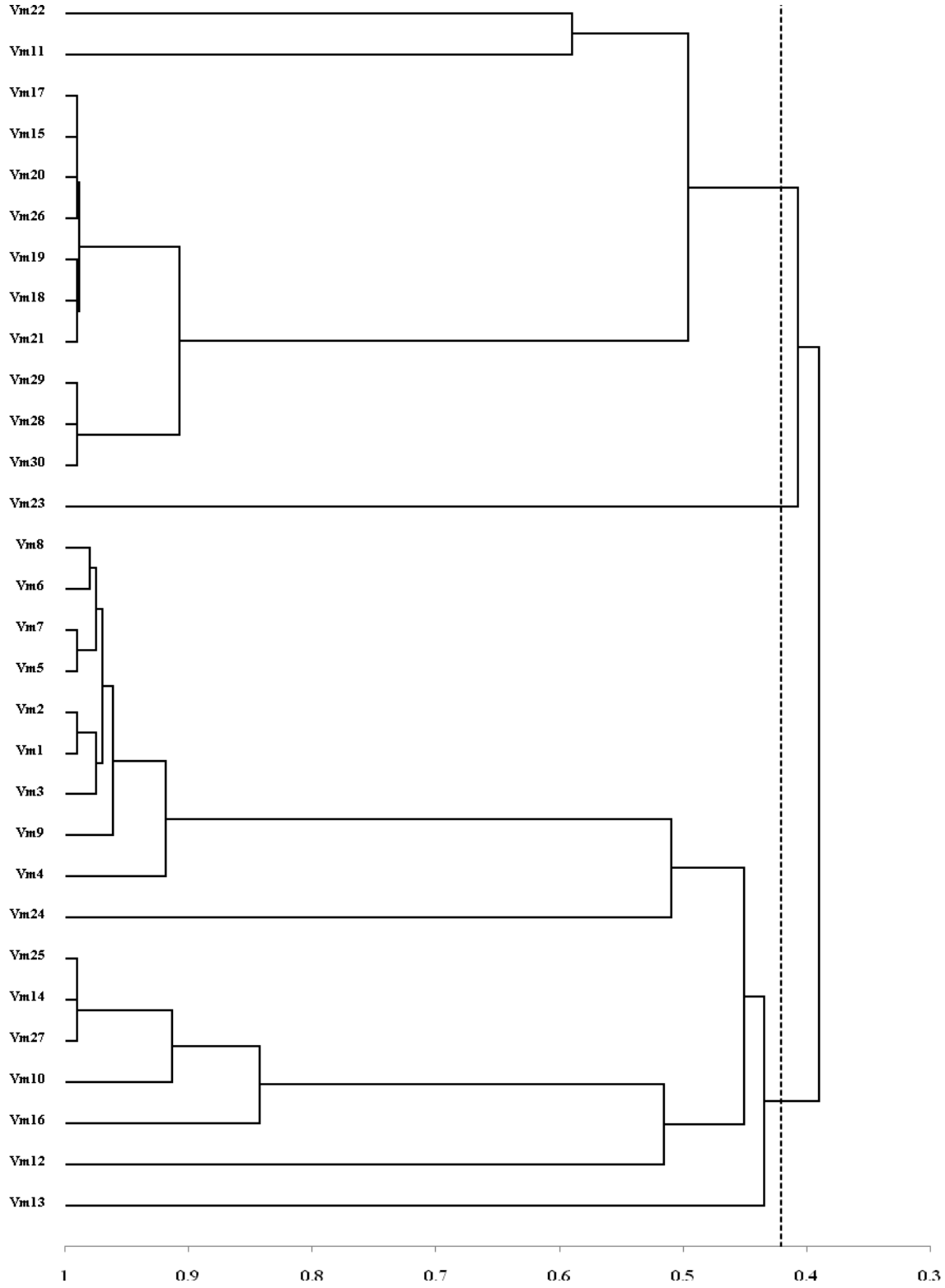

Fig. 2.UPGMA dendrogram based on dice similarity matrix between 30 samples of millennium olive cultivars .

Loukas and Krimbas (1983), in their isozyme study, Fabbri *et al.* (1995), in their analysis of olive cultivars by RAPD and (Kamoun *et al.,* 2006 ; Mnasri et *al.,* 2013 a) in their analysis of olive biodiversity in Tunisia by AFLP obtained a comparable clustering of cultivars based on fruit and endocarp size. That these similar results emerge from analysis of different olive cultivars using different approaches would seem to indicate that fruit and endocarp size is a morphological marker that can efficiently discriminate olive germplasm. Moreover, the lack of any apparent correlation between DNA polymorphism and the origin of cultivars is consistent with the hypothesis 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; Mnasri *et al.,* 2013 a).

The taxonomic structure was further investigated by Principal coordinate analysis (PCO), based on the same matrix of pair wise distances. PCO consists on a representation of the dissimilarity among several cultivars in a reduced multidimensional Q space. On the first three principal coordinates, preservation of the original pair wise distances is very good (cophenetic correlation of 0.88). The 30 tested cultivars were separated along the two-principal dimensional PCO plot (fig. 3) into three main clusters. The first two principal components accounted for 28.85% of the variance, seems to support the results obtained by cluster analysis.

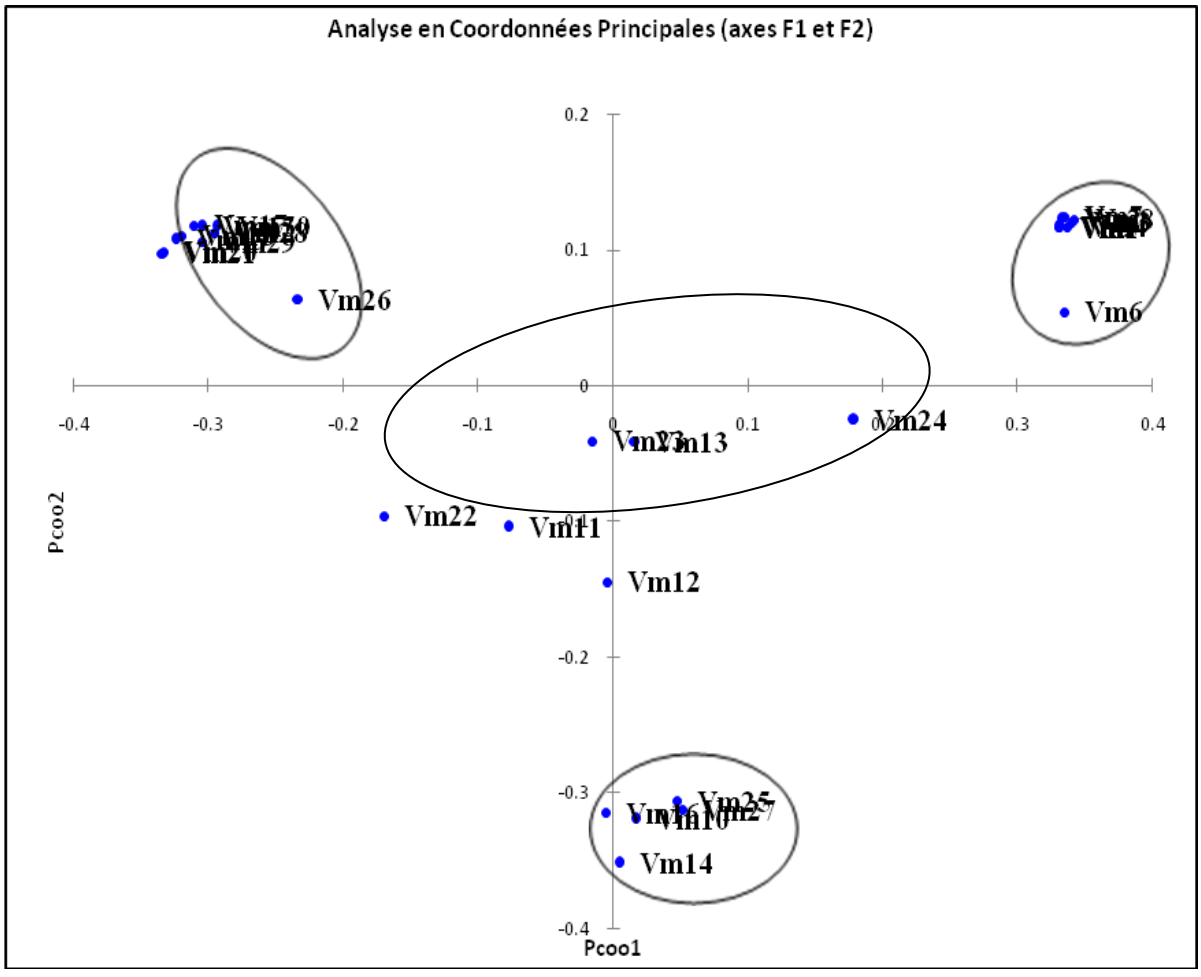

Fig. 3. Principal coordinate plot of olive genotypes for the first and second principal coordinates estimated with 84 AFLP markers using the GS matrix.

The projection of cultivars in the 1-2 plot exhibits the aggregation of the 30 millennium accessions in four main groups. The PCO separated the oil producing cultivars characterized by small seize fruits from the table varieties essentially used for canning and the medium fruit seize cultivars typically utilized for oil and canning. Further the accessions of the first, second and the third groups are totally superposed which proved that they present different clones of three principal varieties localized in the North, the Center and the South of Tunisia, whereas the fourth cluster grouped cultivars with different DNA fingerprinting and proved the importance diversity of the germoplasm of millennium olive varieties in the regions of Makthar, Haouria and El Jem. The ancient manuscripts revealed that the civilizations of the eastern and western Mediterranean such as the Phoenicians led to the establishment of numerous olive varieties in the North and the Center of Tunisia, and then this culture has been spread from the north to the south of Tunisia with the Roman and the Arabic civilizations (Camps –Fabrer, 1997; Loussert and Brousse, 1978).

Conclusion

The results obtained in this work, aimed at testing the reliability of the AFLP markers for cultivar discrimination and clarifying the local millennium olive cultivars‟ identity” in Tunisia. The AFLP markers showed a wide genetic diversity among the millennium cultivars, especially in the north and the center of our country, approved the phenotypic and biochemical diversity observed among the olive accessions (Mnasri *et al.,* 2013 b) and suggests a high genetic potential, which could be used from the agronomic point of view to substantially improve the olive production in Tunisia.

These results also, were the first step to a more extensive analysis that will include more cultivars to cover all the millennium olive diversity in Tunisia. As a sequel to the present work, new surveys should be made in the archeological sites localized in North and the Center of Tunisia (Haouaria, Makthar, Baja, Bizerte and Sbeitla) to sample more cultivars and to draw a clearer picture of the diversity of the Tunisian millennium olive germplasm. Specially, that these cultivars are living archives, and although we were not successful in extracting dendroclimatological information from them, it is likely that in future we can extract valuable information on the history of local weather, or on the history of the cultivation of olive trees in Tunisia.

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