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AFLP and SSR markers for characterization and identification of Tunisian millennium olive varieties

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Abstract

Olive (*Olea europaea* L.*)* is one of the oldest agricultural tree crops in Tunisia, where its cultivation started before about 3000 years. 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, millennium olive samples from nine different archeological sites were studied using AFLP and SSR techniques.6 AFLP primers amplified 237 reproducible bands of which 84 were polymorphic and 10 SSR loci revealed 37 alleles with a mean number of 3.7 alleles per locus and an average heterozygosity rate ranged from 40% to 100% with a mean percentage of 74%. The principal coordinate analysis (PCO) based on AFLP and SSR similarity matrix 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

The evergreen olive cultivar (*Olea europea* L., Oleaceae) is an important Mediterranean tree (Kiple and Ornelas 2000; Kamoun *et al.* 2006). Tunisian oleiculture constitutes one of the principal economical and agricultural strategic sectors that are known for their richness of varieties (Abaza *et al.* 2001).The archeological excavations revealed that olives were cultivated in Tunisia before about 3000 years. The civilizations of the eastern and western Mediterranean such as the Phoenicians, Greeks and Romans, have spread this culture throughout the Mediterranean Basin and by 1200 BC, the population growth in the Mediterranean basin led to the establishment of numerous colonies in North Africa (Carthage) and to create a great number of millennium olive varieties (Loussert and Brousse, 1978).

The patrimony of millennium olive varieties was analyzed for the first time in Tunisia by (Mnasri *et al.,* 2013 b) with the use of morphological parameters and gives a basis for comparing specimens in order to reduce the loss of genetic authenticity of millennium varieties and to preserve the local genetic resources of this germoplasm. Although these methods are efficient, they present practical drawbacks because of the effect of environmental fluctuations on the expression of most morphological traits. To overcome these problems, different authors (Pontikis *et al.* 1980 and Kamoun *et al.* 2002) used biochemical markers such as isozymes, which have been shown very useful for varietal identification of olive. However, not all genotypes could be differentiated by this method. Furthermore, the expression of some isoenzymes may be influenced by both environmental and developmental factors, which also limit the widespread use of this technique for routine genotypic identification (Zhang *et al.,* 1999). With the advent of molecular techniques, several types of DNA markers have been used for the correct identification of varieties and numerous 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). SSR and 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.

Our research had the purpose to examine for the first time in Tunisia the potential of the AFLP and SSR markers to differentiate a number of millennium Tunisian olive cultivars and to explore the genetic relationships, among these genotypes. The use of SSR and AFLP markers will be essential to verify the denomination of each cultivar and to increase the knowledge about the diversity of this species as well as to allow participation in international programmers aiming at olive improvement and conservation. Specially, that this resource might represent an interesting reserve for breeding, with reference to biotic and abiotic factors of the olive environment, as well as an enrichment of the olive historical and cultural heritage (Mnasri *et al.,* 2014).

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.,* 2013a and Mnasri *et al.,* 2014) 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.

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.

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).

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

AFLP results were scored for presence (1) and absence (0) of amplified fragments. 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). Principal coordinate analysis has been used to highlight the pair wise relationship between millennium cultivars based on AFLP and SSR matrix. All calculations were performed with the use of NTSYS-pc version 2.1 (Rohlf, 1998).

Results and discussion

AFLP and SSR polymorphisms

Genomic DNA from 30 millennium olive cultivars was used to generate AFLP and SSR patterns, in order to study for the first time in Tunisia the genetic diversity of this patrimony. AFLP profiles were produced from sex primer combinations of EcoRI and MseI primers (Table 2). The result revealed a highly significant correlation within AFLP and SSR matrix (Mantel correlation R = 0.68, P << 0.05). The AFLP fingerprinting revealed a total number of 237 amplified DNA fragments of different size; among which 84 were polymorphic (32.7%). 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) consistent with that found in other studies (Taamaali *et al.,* 2006 , Kamoun *et al.,* 2006; Mnasri *et al.,* 2013b and Mnasri *et al.,*2014). The ten SSR primer combinations yielded a total number of 37 alleles with an average number of 3.7 alleles per locus. GAPU 59, UDO 12, DCA 09 and DCA 18 showed the lowest number of alleles (3) whereas UDO 39 presented the highest number of alleles (5). Alleles sizes vary among the ten loci, differences between the longest and shortest allele ranged from 124 to 228 bp (Table 3). Genetic variability was wide as indicated by the very high values of observed heterozygosity that ranged between 1.00 at locus (GAPU 71B, UDO 12, UDO 28) and 0.4 at DCA 18, with a mean value of 0.74. The mean PIC values were high (0.61) ranging from 0.74 at UDO 39 to 0.37 at DCA 18. This result is consistent with earlier findings

indicating the wide genetic basis of olive germplasm in Tunisia based on SSR markers (Rekik, 2008 and Abdelhamid *et al.*,2012).

Table 1. List of the studied millennium olive cultivars and the 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

Vm: millennium cultivar.

PCO SSR and AFLP analyses

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. The PCO analyses based on AFLP and SSR rendered similar results, but SSR gave greater resolution than AFLP, probably due to the higher number of SSR loci and their high reproducibility as co-dominant markers. Axis 1 and Axis 2 accounted for a high percentage of variance (23.73% and 17.4% in SSR and 19.75% and 9.1% in AFLP analyses; Fig. 1 and fig.2) and clearly separated the 30 millennium accessions in four main groups. Along the first axis, most of the millennium oil cultivars characterized by small seize fruits plot separately from the table millennium varieties essentially used for canning and the medium fruit seize cultivars and typically utilized for oil and canning. Slight differentiation between oil and table millennium cultivars was observed along the second PCO axis and based essentially on the form of the fruit and the endocarp. Further, the AFLP PCO analysis revealed a perfect superposition of the individuals of the first, second and the third groups 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 (Mnasri *et al.,* 2014). At the same time SSR PCO analysis permitted the separation of the individuals of the first three groups and proved an important intra-clone genetic diversity of the Tunisian millennium olive varieties. These results are confirmed by the ancient manuscripts which demonstrate 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 improved and spread from the north to the south of Tunisia with the Roman and the Arabic civilizations (Camps–Fabrer, 1997; Loussert and Brousse, 1978).

Table 2. Polymorphism rates of the six AFLP 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

Table 3. 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	3	0.7	0.55	0.54	212-218
GAPU71A	4	0.76	0.71	0.7	210-228
GAPU71B	4	1	0.65	0.63	121-144
GAPU103A	4	0.76	0.74	0.72	136-159
UDO03	4	0.56	0.58	0.57	135-202
UDO12	4	1	0.52	0.51	166-193
UDO28	5	1	0.7	0.69	143-210
UDO39	5	0.53	0.76	0.74	108-232
DCA09	3	0.73	0.65	0.63	182-206
DCA18	3	0.4	0.37	0.37	174-190
Total	**37**				
Mean	3.7	0.74	0.62	0.61	

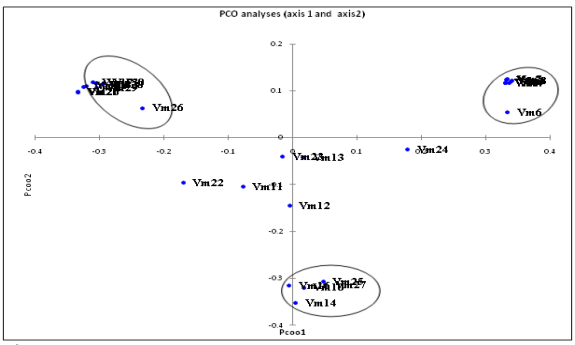

Fig. 1. Principal coordinate plot of olive genotypes for the first and second principal coordinates estimated with 6 AFLP markers using the GS matrix.

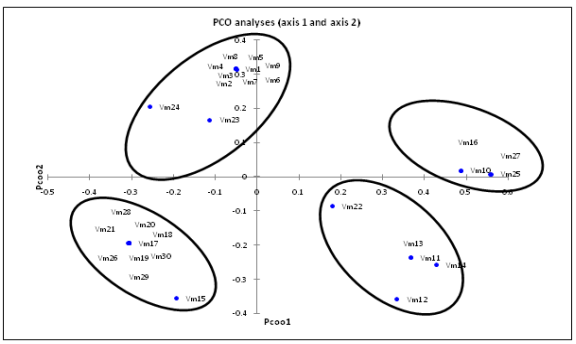

Fig. 2. Principal Coordinate plot of olive genotypes for the first and second principal coordinates estimated with 10 SSR markers using the GS matrix.

In fact, 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 b and Mnasri *et al.*,2014) in their analysis of olive biodiversity in Tunisia by AFLP and (Rekik, 2008 and Abdelhamid *et al.,*2012) by SSR markers demonstrated 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 the whole Mediterranean basin along many centuries (Chevalier, 1948; Fabbri *et al.,* 1995; Ouazzani *et al.,*1995; Mnasri *et al.,* 2013 and Mnasri *et al.,* 2014).

Conclusion

Dominant (AFLP) and co-dominant (SSR) markers have been highly consistent in the estimation of genetic diversity of the Tunisian millennium cultivars in Tunisia. PCO analyses based on the AFLP and SSR matrix proved the important biodiversity of this patrimony, especially in the North and the Center of Tunisia, were the ancient civilizations such as the Phoenicians and Romans have develop the olive culture for many centuries. However, with this work, we have proven that for the management of the millennium olive germopalsm in our country, it is necessary to use the morphological and biochemical information in addition to the fingerprint, especially when dealing with accessions presenting a microsatellite and AFLP profiles with a high similarity index.

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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