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MOLECULAR CHARACTERIZATION OF ANCIENT OLIVE TREES IN BSHAALEH LEBANON

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ABSTRACT: Amplified fragment length polymorphism (AFLP) was used to study diversity among Lebanese olive trees varieties in Bshaaleh. 15 olive trees coming from Bshaaleh were genotyped using 5 AFLP primer combinations. A total of 332 amplification products and153 polymorphic fragments were scored, with a percentage of polymorphism ranging from 33% (E-CCA / M-ACC) to 51% (E-CAC / M-ACA) depending on primer combination. Low correlation ($r<0.7$) between the primer combination E-CCA/M-ACC and all other pairswas noted. Results were analyzed for similarity among Bshaaleh accessions via unweighted pair group means cluster analysis, resulting in 2 clusters corresponding to named variety designations. Similarity coefficients of 0.965 to 1.000 among accessions were observed indicating the possible presence of varieties. Intravarietal similarity was more than 0.99 for 93% of the accessions. Only the accession tree B3 showed a relatively low genetic similarity to the cluster defined and could be considered as outlier. The cophenetic correlation between the dendrogram and the similarity matrix revealed a very good degree of fit ($r=0.994$).According to the Principal Coordinate Analysis, discrimination analysis revealed similar findings and grouped trees into one main group and other unrelated accessions and discriminated at 83% by the first three axes, suggesting that all the ancient olive trees of Bshaalehare related to the same variety. The findings of this study pointed for the need to plan for a sampling strategy that takes into consideration geographic provenances.
Key words: Olea Europea L., Olive, Lebanon, Molecular analysis, AFLP.

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INTRODUCTION

The olive tree was primary domesticated in the North-East Levant at least six millennia ago. Nowadays monumental trees are surviving across the Mediterranean Bassin. Moreover, the first olive plantation in Bshaaleh-Lebanon, that goes back to 1400 years, is still productive [1].Most studies agreed that after an ancestral spreading of few olive varieties along the Mediterranean basin, a majority of modern cultivars were derived from the crossing of these ancient cultivars among themselves, or by their breeding with wild plants, followed by local selection [2, 3,4, 5].

As one of the most important and ancient fruit crops in the Mediterranean Bassin, olive is characterized by a huge genetic patrimony, represented by cultivated and wild germplasm, ancient trees and related forms[6].

The problem of olive germplasm classification is not only complicated by the richness of its genetic patrimony, but also by the absence of reference standards and by the confusion on the cultivar names, with numerous cases of homonymy and synonymy [7].

Morphological characters such as leaves, flowers or seeds have previously been used as markers but most morphological traits are influenced by environmental factors, plant age and phenology [1]. Since objectivity is crucial for accurate morphological typing, the above factors render the use of such traits in plant identification and discernment of genetic relationships difficult [8,9].

The large amount of cultivars causes a huge problem in the management of germplasm collections and genomic traceability of olive oils, as there is a considerable uncertainty about the names of many cultivars and as olive cultivars are morphologically very similar [10].

Moreover, it was shown that genetic variability could occur within the same cultivated populations [11], which could justify heterogeneity, in production and quality traits [12].

The use of molecular markers is efficient for olive germplasm management, including the characterization of accessions and the establishment of genetic relationships between cultivars [13].

The development of various molecular marker techniques and their application in genetic diversity studies have resulted in improved discrimination among or within several olive cultivars and facilitated the estimation of their genetic inter-relationships. The ability to discriminate olive cultivars and to estimate genetic variability are important factors for a better management of genetic resources and successful breeding programs [14].

Amplified Fragment Length Polymorphism (AFLP) markers are highly polymorphic and reproducible and thus represent a powerful technique for DNA analysis that has revolutionized fingerprinting and diversity studies. AFLP analysis detects genetic variation throughout the genome by using a pair of specific restriction enzymes and their corresponding adapters combined with 2 selective rounds of PCR. The AFLPs technique may allow the simultaneous screening of a large number of loci, without any need of preliminary sequence knowledge [15]. For these advantages, and for their high reliability, many studies show that AFLP is a reliable method for generating many highly polymorphic DNA markers and inferring phylogenetic relationships among closely related *taxa*[16, 17]. Moreover, AFLPs have been widely used for genotyping olive populations [18,14].

AFLP markers have been widely used for the evaluation of hundreds of olive cultivars [19, 20]. This technique seems to be the most reliable and informative fingerprinting procedure for classifying and identifying olive cultivars [2]

AFLP technology was confirmed to be a powerful tool not only for studying variation between populations of the genus *Olea* as shown by [2], but also for characterizing intra specific variation among cultivated accessions of *O. europaea* L. subsp. *Europaea* [21].

Efforts to characterize eastern Mediterranean olive germplasm has been recently reported, and findings suggest high levels of genetic diversity and reveal differences between olive varieties in East and West Mediterranean countries. Sampling of the Eastern populations has been modest to date, and so the pattern of olive diversity in the Eastern Mediterranean is yet unclear [14].

In this study, we propose to assess the genetic relationships among Bshaaleh olive accessions using AFLPs analysis. Although the AFLPs method has been used to identify genetic relationships among olive cultivars throughout the world [14, 22, 20], the use of this method to determine genetic relationships between cultivars natively grown in Lebanon has been limited.

MATERIALS AND METHODS

Plant material

Fifteen old olive trees of Bshaaleh were used for molecular analysis. Fresh young leaves have been collected and stored in sterile plastic tube for 72 hrs.at a temperature below 5^{o}Cuntil DNA extraction [23].The laboratory analyses were performed at the Faculty of Agriculture – University of Perugia-Italy.

Genomic DNA isolation

Healthy leaf tissues were collected *in situ* and immediately transferred into liquid nitrogen. DNA was extracted using the Genelute Plant genomic DNA miniprep kit (Sigma®). Tissue samples were crushed with mortar and pestle; the powder immediately transferred to a 2 ml Eppendorf tube and kept on ice.

DNA release from the tissue was performed according to [24].

The concentration of the plant genomic DNA was determined by spectrophotometric analysis (DU650 spectrophotometer, Beckman) at 260 nm (1O.D. = 50 ng/µl) and the purity was calculated by $O.D._{260}/O.D._{280}$ ratio..

The size and quality of the DNA was determined by electrophoresis on 1% agarose (Gibco-BRL) gels. Dissolved aliquots were stored for longer period at -80^{o}C [24].

AFLP markers

AFLP marker analysis was performed according to [15] with some minor modifications (Fig.1).

Structure of adapters and primers

A limited set of AFLP primers can be combined to yield a large set of primer combinations, each producing a unique set of amplified fragments [23].

Adapters and primer sequences for ligation and the first round of PCR amplification were the same as those published in standard AFLP procedures and included a core adapter sequence followed by the restriction enzyme sequence [15, 2].

Primers for the second round of PCR included the same core and enzyme sequences followed by a single selective base (A or C) for one of the primers, together with 2 or 3 bases for the other.

Controls with the standard 2 selective bases on the *Eco*R I primer and 3 selective bases on the *Mse*I primer were also included. *Eco*RI primers were 5'GACTGCGTACCAATTC + x (where x = CAC or CCA). *Mse*I primers were 5'GATGAGTCCTGAGTAA + y (where y = ACA, ACC or AAG) (Table 1).

Table 1: Oligonucleotide adapters and primer combinations used for AFLP analysis

Name		**Sequence**
*Eco*RI adapter		5'-CTCGTAGACTGCGTACC-3'
		5''-AATTGGTACGCAGTC-3'
*Mse*I adapter		5'-GACGATGAGTCCTGAG-3'
		5''-TACTCAGGACTCAT-3'
Primers used in preamplification		
*Eco*RI+1-C	E-C	5'-GACTGCGTACCAATTC+C-3'
*Mse*I+1-A	M-A	5'-GATGAGTCCTGAGTAA+A-3'
Primer combinations used in selective AFLP amplification		
*Eco*RI+3-CAC	E-CAC	5'-GACTGCGTACCAATTC+CAC-3'
*Mse*I+3-ACA	M-ACA	5'-GATGAGTCCTGAGTAA+ACA-3'
*Eco*RI+3-CAC	E-CAC	5'-GACTGCGTACCAATTC+CAC-3'
*Mse*I+3-ACC	M-ACC	5'-GATGAGTCCTGAGTAA+ACC-3'
*Eco*RI+3-CCA	E-CCA	5'-GACTGCGTACCAATTC+CCA-3'
*Mse*I+3-ACC	M-ACC	5'-GATGAGTCCTGAGTAA+ACC-3'
*Eco*RI+3-CCA	E-CCA	5'-GACTGCGTACCAATTC+CCA-3'
*Mse*I+3-AAG	M-AAG	5'-GATGAGTCCTGAGTAA+AAG-3'
*Eco*RI+3-CCA	E-CCA	5'-GACTGCGTACCAATTC+CCA-3'
*Mse*I+3-ACA	M-ACA	5'-GATGAGTCCTGAGTAA+ACA-3'

Restriction enzyme digestion and ligation of adapters (RL)

Genomic DNA (500 ng) was digested and ligated at 37^{o}C for 4h following the method of [15] with some modifications, using 5 U *Eco*RI (having a 6 bp recognition site), 5 U *Mse*I(having a 4 bp recognition site), 1 x Restriction-Ligation buffer (20 mM Tris-acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 5 mM DTT and 2.5µg BSA), 50 pmol of *Mse* adapter, 5 pmol of *Eco* adapter, 10 mM ATP and 1 U T4 ligase (Invitrogen).

PCR amplification

Pre-amplifications (PREAMP)

The complexity of the genomic DNA was reduced by amplifying 0.01 of the restriction-ligation reaction in the pre-amplification step using primers with one selective base in a total volume of 20 µl. Pre-selective PCR amplification of prepared templates was performed by mixing 5 µl of the ten-fold diluted digested and ligated DNA with 75 ng *Eco*+C primer, 75 ng *Mse*+A primer, 1 x PCR buffer (50 mM MgCl, 1.5 mM $MgCl_2$,10 mM Tris-HCl), 10 mM dNTPs (Invitrogen) and 1 U Taq DNA polymerase (Invitrogen).

The following cycling conditions ensured optimal primer selectivity: after a first cycle of 45 s at 94°C, 30 s at 65°C, 1 min at 72°C a touch-down profile (13 cycles with a decrease of 0.7°C each) for the annealing step was added and continued with an annealing temperature of 55.9°C for the next 18 cycles followed by a final step of 5 min at 72˚C.

Three (3) µl of the resulting pre-amplification PCR products was run in 1% agarose gel to verify that a homogenous light smear is resulted [25].

B- Selective PCR amplification

Amplifications were performed in a 20 µl reaction mix containing 1/100 of the pre-amplified DNA as a template, 50 ng fluorescent-labeled *Eco*RI+3 oligonucleotide primer, 50 ng of unlabeled *Mse*I+3 primer, 2 µl 10x PCR buffer (Invitrogen), 0.2 mM dNTPs, 0.4 U Taq DNA polymerase (Invitrogen). The *Eco*-RI+3/*Mse*I+3 primer combinations used are reported in Table 1. The temperature profile adopted for PCR reactions was the same as for the pre-amplification step.

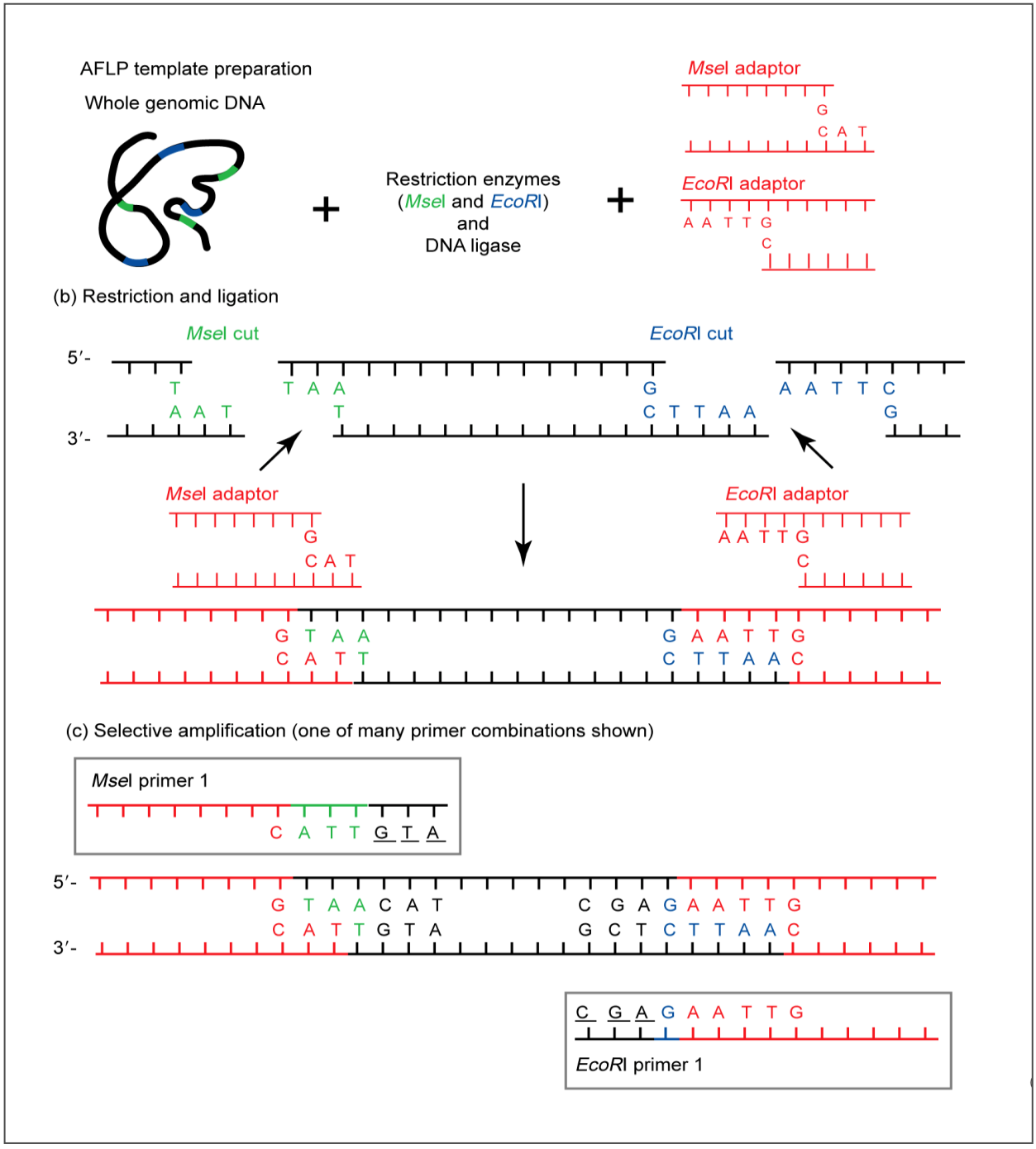

Fig. 1 Schematic AFLP technique phases

Detection of bands

One µl of each amplified sample was denatured and run on ABI 3130xl capillary sequencer (Applied biosystems).An AFLP locus was considered to be polymorphic if the amplified band was present in some samples and absent in others, and monomorphic if the band was present in all the evaluated accessions. To avoid underestimation of the genetic similarities, all loci, polymorphic or not, were considered. AFLP fragments were scored as 1 (presence of the band) and 0 (absence of the band) [26], using the Genemapper 4.0 software (Applied biosystems), and entered into a data matrix. A comparison between similarity matrices generated from individual primers combinations was conducted in order to assess the correspondence level between the primer-pairs selected for this study.

Data analysis

The similarity matrix among all accessions, calculated using the Dice coefficient [27], was utilizedto construct an unweighted pair-group method of arithmetic average cluster analysis (UPGMA), a hierarchical method of clustering (SAHN module), using NTSYSpc version 2.11Q [28]. A dendrogram was constructed from the Dice coefficient similarity data with NTSYSpc version 2.11Q by UPGMA cluster analysis.

The goodness-of-fit of the clustering was tested using the MXCOMP program, which directly compares the original similarity matrix and the cophenetic value matrix, as suggested by [28]. The best assessment of fit between dendrogram and original data was determined using the cophenetic correlation coefficient [29]. A cophenetic value matrix of the UPGMA clustering was based by computing the product-moment correlation r [30]. In order to estimate the degree of independence between AFLP profiles generated from individual primer-pairs, we computed similarity matrices and subjected these to Mantel tests [30] generated by NTSYSpc version 2.11Q [28].

To better visually depict the relationships among accessions, the matrix of the correlations based on the original AFLP data matrix was subject to Principal Coordinate Analysis (PCO). PCO analysis, which provides estimates of genetic similarity between individuals, has been used as an alternative way to represent inter individual and intergroup relationships. The analysis was performed with NTSYSpc version 2.11Q [28]. The pair wise genetic similarities among genotypes were calculated according to the Dice definition of similarity [26].

RESULTS

AFLP markers profiles for all accessions

A total of 15 olive trees were genotyped using five (5) AFLP primer combinations. A total of 332 amplification products were scored (Table 2), with 153 polymorphic fragments, with a percentage of polymorphism ranging from 33% (E-CCA / M-ACC) to 51% (E-CAC / M-ACA) depending on primer combination (Table2). The molecular weight of the amplification products ranged from 49 bp (E-CCA / M-AAG) to 467 bp (E-CAC/ M-ACC).

The average number of bands per reaction was 66.4 with a variation from 36 (E-CCA / M-ACC) to 90 (E-CAC / M-ACA) (Table 2). The number of polymorphic products per reaction ranged from 12 (E-CCA / M-ACC) to 46 (E-CAC / M-ACA).

Table 2: Primer combinations and level of polymorphism obtained by AFLP

Primer pair combination	Total number of amplified fragments	Number of polymorphic Fragments	Polymorphisms (%)
E-CAC/M-ACA	90	46	51.111
E-CAC/M-ACC	68	34	50.000
E-CCA/M-ACC	36	12	33.333
E-CCA/M-AAG	56	25	44.643
E-CCA/M-ACA	82	36	43.902
Total	**332**	**153**	
Mean	66.4	30.6	46.084

No single primer-pair was capable of identifying all of the accessions, although individual primer-pairs typically produced high numbers of different banding patterns, ranging from 36 to 90.

To be noted that 6 samples could not be assessed by AFLP markers due to the quality of the DNA extracted. These accessions belong to different regions from the North of Lebanon including: B1, O17, O19, O26, O39 and O48.

Individual primer-pairs matrices comparison

A comparison of the similarity matrices based on data from individual primer-pairs revealed a low correspondence (r<0.7) between the primer combination E-CCA/M-ACC and all other pairs, suggesting that this combination generated quite distinct banding profile (Table 3).

Table 3: Matrix correlation (= normalized Mantel statistic Z) based on data from individual primer-pairs

	E-CAC/M-ACA	E-CAC/M-ACC	E-CCA/M-ACC	E-CCA/M-AAG	E-CCA/M-ACA
E-CAC/M-ACA	-	-	-	-	-
E-CAC/M-ACC	0.916	-	-	-	-
E-CCA/M-ACC	**0.649**	**0.648**	-	-	-
E-CCA/M-AAG	0.849	0.851	**0.622**	-	-
E-CCA/M-ACA	0.905	0.923	**0.596**	0.887	-

Genetic similarities and phenetic inter-relationships for Bshaaleh accessions

Clustering analysis

The dendrogram derived from the UPGMA cluster analysis of the AFLP markers clustering Bshaaleh accession is shown in Fig.2. For a similarity coefficient higher than 0.99, it was possible to clearly differentiate one main group, whereas one accession (B3) remained unrelated to the previous group.

Similarity coefficients of 0.965 to 1.000 among accessions were observed. Intravarietal similarity was more than 0.99 for 93% of the accessions. Within this cluster, a clear sub-cluster was identified, it included11 accessions: B2, B4, B5, B6, B7, B8, B10, B12, B11, B14, and B9. Six accessions among this sub-cluster showed a 100% genetic similarity.

Only the accession tree B3 showed a relatively low genetic similarity to the cluster defined and could be considered as outlier.

Among the accessions studied, the lowest genetic similarity (0.962-0.964) was observed between B3 and B15, B13, B2, B9, B11 respectively and the highest genetic similarity (1.000) was observed between B4 and B5, B6, B7, B8, B10, B12 (Table 4).

Table 4: Dice similarity matrix of 14 olive trees of Bshaaleh determined using 332 amplified fragment length polymorphism markers

	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15
B2	1.00													
B3	.964	1.00												
B4	.996	.966	1.00											
B5	.994	.967	1.00	1.00										
B6	.996	.966	1.00	1.00	1.00									
B7	.996	.966	1.00	1.00	1.00	1.00								
B8	.996	.966	1.00	1.00	1.00	1.00	1.00							
B9	.993	.964	.998	.997	.998	.998	.998	1.00						
B10	.996	.966	1.00	1.00	1.00	1.00	1.00	.998	1.00					
B11	.993	.964	.998	.997	.998	.998	.998	.996	.998	1.00				
B12	.994	.967	1.00	1.00	1.00	1.00	1.00	.997	1.00	.997	1.00			
B13	.989	.962	.993	.991	.993	.993	.993	.991	.993	.991	.991	1.00		
B14	.993	.966	.998	.997	.998	.998	.998	.996	.998	.996	.997	.996	1.00	
B15	.989	.960	.993	.991	.993	.993	.993	.991	.993	.991	.991	.991	.991	1.00

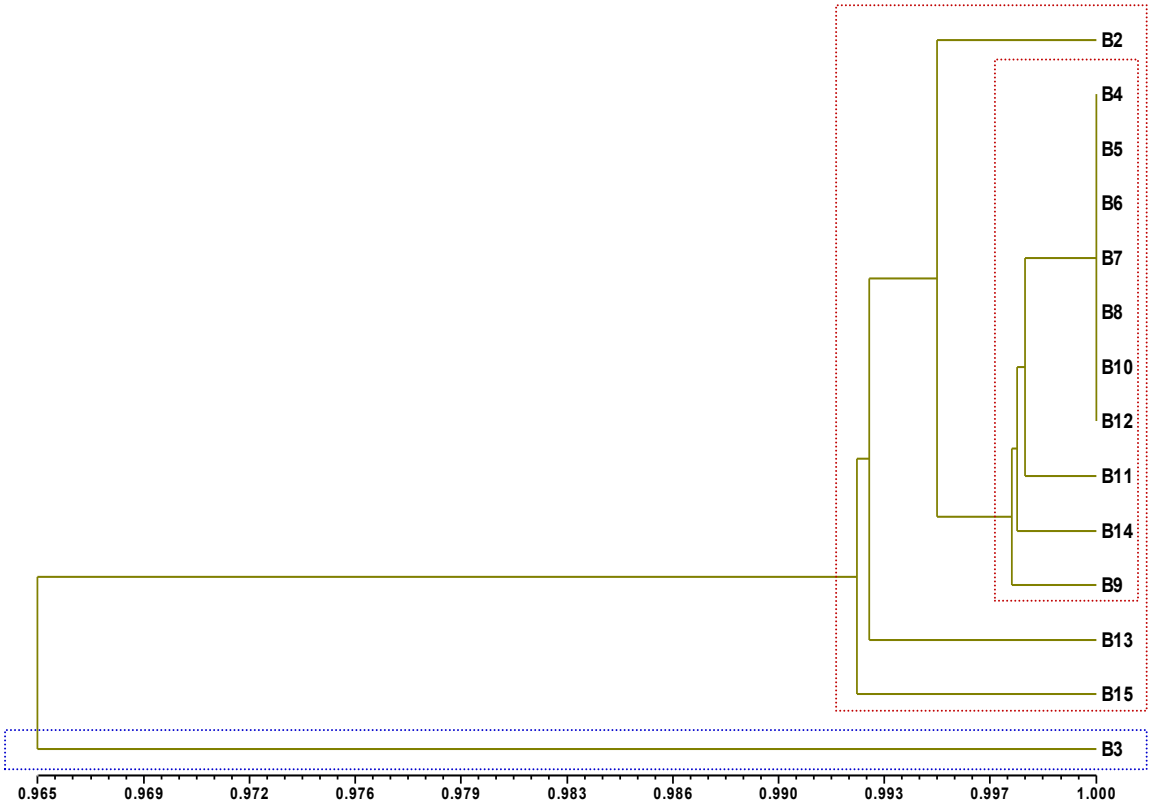

Fig. 2 UPGMA Dendogram based on Dice similarity matrix and illustrating the relative similarity among 14 old olive trees of Bshaaleh

Cophenetic correlation and Mantel test

The cophenetic correlation between the dendrogram and the similarity matrix revealed a very good degree of fit (r=0.994). This high value of the cophenetic correlation indicated that the presence of a cluster was more than a random event.

Principal Coordinate Analysis (PCO)

According to PCO, discrimination analysis revealed similar findings and grouped trees into one main group and other unrelated accessions (Fig.3) and discriminated at 83% by the first three axes (Table 5). B3 remains the far unrelated accession to the others. B13, B14 and B15 were plotted separately from the rest of the group. This genetic variation could be explained by probable somatic mutations as mentioned by[31].

Table 5: Principal Coordinate Analysis of Bshaaleh olive trees

I	Eigen value	Percent	Cumulative
1	**0.06148**	58.0027	58.0027
2	**0.01650**	15.5755	73.5782
3	**0.00989**	09.3272	82.9055

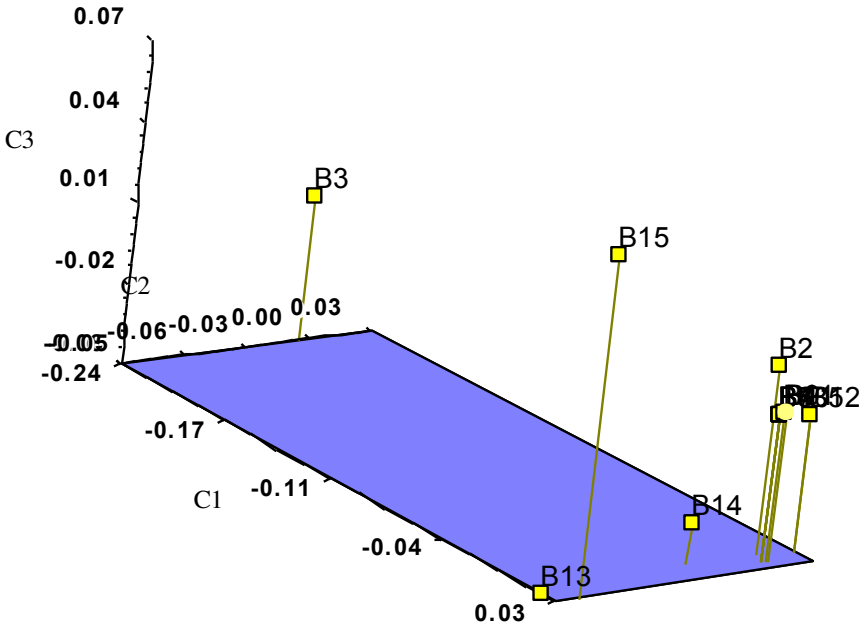

Fig.3 Principal coordinate plot of Bshaaleh accessions (14) for the first three principal coordinates estimated with AFLP markers using the genetic similarity matrix

DISCUSSION

The purpose of this study was to characterize an ancient group of olive trees present in the Bshaaleh area, in the North of Lebanon, by performing molecular analysis. Molecular markers were used to estimate genetic relationships among olive accessions. The high level of genetic similarity we detected among the studied accessions is consistent with results obtained by [20] using AFLP and SSR markers, where the molecular characterization of Lebanese olive germplasm was able to separate, by cluster analysis, the trees according to their geographic provenance.

AFLP coefficient of similarity ranged between 0.96 and 1.00 among 90% of the trees sampled in this study, further supporting the suggestion that all the ancient olive trees of Bshaalehare related to the same variety, using the set of 5 primers combinations selected.

In fact, in our study, we were able to cluster 90% of the accessions into the same genetic pool, showing high level of similarity in the North of Lebanon confirming the usefulness of AFLP in discriminating between cultivars [32] and in addressing genetic diversity within the *Olea* complex [2].

The only published study assessing the existing *in situ* diversity of old Lebanese olive groves, was conducted by [20]. The genetic diversity among old trees from 14 groves located in the different regions of Lebanon has been assessed using AFLP and SSR techniques. The coefficient of similarity resulted from AFLP indicates the possible presence of different varieties, but the findings of this study point for the need to plan for a sampling strategy that takes into consideration geographic provenances.

The morphological characterization conducted in a previous study showed that the accessions of Bshaaleh were clustered into three main groups, whereas the molecular characterization showed that all accessions were clustered in one main group. Differences between the two methods of characterization has also been reported in several other studies, where discrepancies were explained by taking into account the possible interferences of environmental factors [32, 33, 9].

Classification by AFLP markers is more reliable than morphological classification, where characters are influenced by environmental effects [34]. Historical and/ or geographic information, which has been the basis of much variety classification, is limited by the reliability of the persons being interviewed. Therefore, materials developed before modern record-keeping methods are likely to be inaccurately described.

The results obtained in this work, aiming to test the reliability of the morphological parameters for cultivar discrimination and clarifying the local cultivars' identity and their relationships within the local population, by the use of the AFLP markers, has led to very interesting findings.

The estimates of genetic relationship can be helpful for organizing germplasm for conservation of genetic resources for the identification of cultivars for selection of parents for hybridization, for predicting favorable heterotic combinations. Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles, which are likely to uncover the largest number of unique and potentially agronomically useful alleles [35].

[11] have noticed that phenotypic variations observed in field, despite of genetic similarities or differences, are most probably associated with genetic expression varying with changing environmental conditions and growing applications.

The proximity of the North of Lebanon with Syria and Turkey has, likely, favored close regional and multiple selection of cultivars in the area as showed by [2] and [14], who found that the Lebanese Souri accession showed very high genetic similarity with an Ayrouni accession, both accessions being closed to a Syrian Saurani accession and the Buyuk Topak Ulak Turkish accession.

The apparent unique nature of the North of Lebanon olive germplasm coming from Bshaaleh revealed by our results supports the case for the implementation of more intense characterization and conservation strategies. Studies like the one presented here are useful to provide new insights that can help to recognize the different genotypes, to perform programs for the development of new varieties and for a better choice of the variety to use in relation to the successful productive objective and environmental conditions.

The set of primers combinations used revealed high level of similarity among the accessions. To confirm this result, the usage of other combinations will be interesting; especially those reported in the literature and that have been used for Lebanese olive germplasm characterization [20].

The characterization of olive germplasm is an essential task in the modern olive culture. Genetic identification is important to protect and to preserve the genetic resources, to safeguard the typical oils [36] and to certificate the propagation material.

CONCLUSION

Lebanese olive trees were locally selected during the beginning stages of olive growing and served as basic plant material for the current traditional varieties derived by both sexual and clonal propagation.AFLP was evaluated as a tool to identify the intraspecific and intravarietal diversity of olive tree. The molecular characterization of the ancient olive trees of Bshaaleh resulted in clustering all the trees into the same genetic pool. Further interdisciplinary studies on diversity, biogeography and phylogeny of this genus seem promising.

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