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ORIGINAL ARTICLE

Genetic Relatedness among Romanian Olive Trees and Varieties Using Amplified Fragment Length Polymorphism (AFLP) Markers

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ABSTRACT

Amplified fragment length polymorphism (AFLP) analysis was used to study the genetic variations among and within different individuals of Romanian olive trees from two regions Tafilah and Ajlun along with six varieties. Four primer combinations were used which produced about 538 amplified fragment. Dendrogram and matrix of similarity were obtained by the Unweighted Pair-Group Method analysis (UPGMA). Ajlun Romanian olive trees formed a separated group and most of Tafilah trees were formed with a separated group. High similarity (39%) among all tested individuals was observed between two samples of Ajlun. Among varieties Rasee and Improved Nabali were showed high similarity (25%). AFLP confirmed that their is a genetic variations within Romanian olive tress which lead to enlarge the studying those trees morphologically and biochemically to entering promise ones within breeding programs in the future. Finally, AFLP has a powerful tool for detecting genetic variability among cultivars and species help in known the pedigree of relatives and ancestors.

Key words: AFLP, Olive, Jordan, Romanian trees and Polymorphism.

Introduction

Olive (*Olea europaea* L.) is an evergreen tree grown at temperate regions with altitude ranged from (400m to 1500m) above sea level (a. s. l.). The production is based on the climatic conditions, tree cultivation as well the physiological state of trees, these lead to phenomenon known "alternate bearing". In Jordan, olive culture is one of the most important agriculture activities, the olive plantations accounted more than 10 million trees, where the Nabali Baladi is the most widespread and the dominant cultivar.

Oil production was exceeded the needs that allow for government to export the surplus into neighbor countries and Europe markets. Moreover, Jordan olive oil considered the best among other countries based on the percentage of PH. However, most framers said that the old trees (Romanian) are best than the introduced varieties taking into considerations the fruit quality and oil percentage. This saying oriented the direction to study the genetic relatedness among those and introduced varieties using the molecular markers.

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Changes in lifestyles and cooking habits towards healthy eating, environmental concerns, exotic cuisine, and convenience food have opened new market opportunities for olive oil products [11]. Olive oil is the principal source of fat in the Mediterranean diet, which has been associated with a lower incidence of coronary heart disease and certain cancers [15]. Adulteration is, the adding cheaper materials from other oil products, the biggest problem faced the consumers and the directions now toward to have organic farming to get high quality and guarantee production.

Traditionally, morphological and physiological traits were used to identify olive cultivars, more recently, molecular markers have been found to be a useful tool for cultivar identification and determining patterns of relatedness among cultivars. Recently, DNA–based techniques have been developed to detect genetic variations within and among varieties, populations and species. Random amplified polymorphic DNA (RAPD) used for detecting genetic variation in mutants of Nabali olive cultivar [13]. In addition, RAPD technique was used to characterization olive varieties [16], genetic relationships [6,2], cultivars analysis [10,3], genetic diversity and Albanian olive germplasm [4,5] and identification cultivars [8].

Amplified Fragment Length Polymorphism (AFLP) markers have been developed by Vos et al., [14] and widely spread due to its reliability and robustness. The use of AFLP has some advantages in terms of use in the identification of diagnostic or specific markers, it has several advantages, it doesn't require previous knowledge of the DNA sequence, generate reproducible fingerprinting profiles, allows the amplification of a high number of DNA fragments per reaction and enabling the detection of specific amplified fragments [14,9].

AFLP markers were used to study the relation between olive cultivars from Western Mediterranean [1] and discriminated among olive varieties [5].

The purpose of this study was to detect the genetic relatedness among the olive Romanian trees and olive cultivars were grown in Jordan by using AFLP markers.

Materials and methods

-Plant material

Leaves selected randomly from around the trees of eighteen olive Romanian trees and six varieties were collected from private farms North (Ajlun- Al-Hashimiah) and south (Tafilah) regions of Jordan, while leaf samples of olive varieties were collected from Al-Hussein nursery -Ministry of Agriculture during August 2006. Olive samples from Tafilah region were stored at refrigerator until processing for DNA isolation, but samples of varieties and those from North were used freshly.

DNA isolation

DNA was obtained after ground the stored leaves in liquid nitrogen (-196ºC), the quality and quantity of genomic DNA were detected on agarose gel. Total cellular DNA was extracted using anew-modified CTAB isolation protocol. Approximately (20 to 30 mg) fresh and stored leaves were ground and mixed with 750 µl of freshly and preheated of (2x CTAB solution with 0.8g PVPP) in 2ml tubes then placed at 65ºC for 30 min. the mixture was mixed with 750 µl of chlorophorm/ isoamyl alcohol (24:1) vortex a few seconds, those centrifuged at 14000g for 20 min. Upper phase (supernatant) was placed in 2ml tubes with 600ml isopropanol, tubes were shaked until the thread of DNA appeared, then centrifuged for 20 min at 14000g. The solution poured and tubes left to dry, then 600 µl of cooled 70% ethanol added and placed at refrigerator (–20ºC) overnight. Next day ethanol was poured, tubes dried and 100µl of TE added and placed at 65ºC for 30min. Four micolitter of RNAse (10mg/ml) were added per tube and left for 45min at 37ºC.

DNA quantitation was measured using machine of S2100 UV/VIS DIODE-Array-Spectrophotometer, Version 1.7.

AFLP procedure

The AFLP procedure was performed as previously described (Vose et al., 1995). Olive DNA (500ng) was double digested with *EcoRI* and *Tru91* (an isochizomer of *MseI*) and double stranded adaptors were ligated to the ends of DNA fragments, generating template DNA for subsequent PCR amplification (pre-amplification followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction tube [14].

To carry out the reaction, an enzyme master mix for 16 reactions was prepared containing 2µl *EcoRI* buffer, 2µl *Tru91* buffer, 0.25µl *EcoRI* enzyme (12u/µl), 0.25µl *Tru91* enzyme (12u/µl), 0.625µl 1mg/ml BSA, 9.875µl ddH2O and 5µl genomic DNA (500ng), the mix was centrifuged for a few seconds then stored at 37ºC for 3 hr. The restriction-ligation reactions consisted of 0.5 µl 10x T4 buffer, 0.2µl T4 DNA ligase (3u/µl), 0.5µl *Tru91* adaptor forward, 0.5µl *Tru91* adaptor backward, 0.5µl *EcoRI* adaptor forward, 0.5µl *EcoRI* adaptor backward and 2.3µl ddH2O. Those distributed 5µl for the restriction tubes, then stored at 37ºC for 3hr.The restriction-ligation reactions were diluted 1: 4, then used in pre-amplification step. The *Tru91* complementary primer had a 3'-C and the *EcoRI* complementary primer had a 3'-A.

The pre-amplification (preselective) mix was prepared by adding 2µl of 5-fold diluted DNA from the restriction-ligation reaction, 2.5µl 10xbuffer (PCR), 2.5µl dNTPs (5mM), 1µl AFLP preselective

primer (*EcoRI*+ A-3'), 1µl AFLP preselective primer (*Tru91*+ C-3'), 0.2µl *Taq* polymerase (5u/µl) and 15.8µl ddH2O. The pre-amplification (preselective) amplification was carried out in a thermal cycler programmed at 72ºC for 2min followed by 20 cycles of 94ºC for 30 s, 56ºC for 40s and 72ºC for 50s and finally incubated at 4°C. The pre-amplification DNA was diluted 5-fold with ddH2O and selective amplifications were carried out by using different *EcoRI* and *Tru91* primer combination (Alpha DNA). Primers selected for the selection amplification were from available AFLP selective primers that purchased before and stored at –20ºC at NCARTT lab. The *EcoRI* primers contained three selective nucleotides with sequence (Table 1) while the *Tru91* primers had the selective nucleotides starting with G (Table 1).

For the selective amplification, the reactions were set up as follows: 2µl of 5-fold diluted pre-selective amplification reaction product, 1.0µl *Tru91* primers, 1.0µl *EcoRI* primers, 2µl of 10 x buffer, 2µl dNTPs (0.25mM), 0.2µl*Taq* polymerase (5u/µl), 0.25µl of MgCl2 (15mM) and 11.55ddH2O.

Selective amplification was carried out in a thermal cycler programmed at 94ºC for 2min, followed by 13 cycles of 94ºC for 20s, 68ºC (0.7ºC /cycle) for 30s and 72ºC for 1min, and 23 cycles of 94ºC for 30s, 59°C for 30s and 72ºC for 1min and final incubation at 4ºC.The samples were loaded on 6% polyacrylamide gel on Apelex gel sequencing apparatus (vertical). The selective amplification reaction product (6µl) was mixed with 4ul of loading buffer (98% deionized foramide, 10mM EDTA (pH: 8), 0.05% bromofenol, 0.05% xylene cyanol), from which 6µl was finally loaded on the gel.

Four primer pair combinations (*EcoRI- / Tru91-*) were used in analysis (Table 2), six olive varieties and eighteen samples of Romanian trees from each Ajlun and Tafilah regions were subjected to the selective amplification with these primer combinations, and treated under the same conditions by the same researcher. To determine the size of the AFLP fragments, we used an AFLP DNA marker (50bp step ladder) (Promega), ranging in length from 50 - 800bp.

Data analysis

AFLP polymorphic bands were scored as present (1) or absent (0) an estimates of similarity among all varieties wee calculated according to Nei and Li, [12] definition of similarity Sij= 2a/ 2a+b+c, where Sij is the similarity between two varieties (individuals) i and j, a is the number of bands present in both individuals, b is the number of bands present in j and absent in i. The matrix of similarity was analyzed by the Unweighted Pair-Group Method (UPGMA) and the dendrogram was obtained using spss. Version 10. Program.

Results and Discussion

Only one primer combinations out of five, AGC-CAT, was failed to produce at least one amplified fragment. The AFLP fingerprinting of the six olive varieties and eighteen samples of Romanian trees were collected from each Ajlun and Tafialh regions (Table 1), tested using four random primer pair combinations (Table 2 and 3), revealed a total number of 1119 amplified DNA fragments, 538 out of them turned to be polymorphic and were distributed across the entire lanes. The average percentage of polymorphism ranged from 25% for EAAC-MCCT (Tafialh Romanian trees) to 71% for EAGC-MCAC for (Tafialh Romanian trees) (Table 3) for olive varieties high percentage of polymorphism showed by EACT-MCAA and the lowest percentage of polymorphism is 45% for EAAC-MCCT (Table 3). Some bands wee specific to one or more of Ajlun and Tafialh also the same to olive varieties.

Variations among olive varieties, Ajlun and Tafilah Romanian olive trees

Dendrogram constructed by UPGMA derived from similarity matrix described above, showed five main distinct clusters (Figure 1). The first cluster was formed twelve olive samples of Ajlun. The second cluster formed two individuals from Ajlun. The third group formed four samples of Ajlun, four samples of Tafilah and two samples of Tafilah with one Ajlun sample. The four cluster formed two samples of Tafilah Romanian trees with one sample of Ajlun. The fifth cluster can be divided into two groups, the first included two sub–sub clusters; one formed olive variety K18 with one individual tree from Tafila, the second formed two individuals of Tafilah and Nabali Baladi showed separated group (Figure 1). The second sub-sub cluster is included two groups; the first has two Tafilah individuals which are more closely and one sample has separated group.

The second group of the fifth clusters were formed three groups the first formed Nasohee variety and one individual Tafilah sample also it was wholly separated from this group (Figure 1). The second group showed that Rasee and improved Nabali more closely but showed sharing with one Ajlun sample and all of them collected in one group. Within this group one sample of Tafilah individuals was showed separated from other. One sample of Tafilah was showed a separated one among all samples within this group.

Jaccard´s genetic similarity (Table 4) estimated within and among all tested individuals showed the highest similarity values in Rasee variety and Ajlun sample (0.182) and the lowest value (0.000) between improved Nabali and Ajlun. High similarity was registered between K18 and onTafilah sample (0.205) and the lowest value (0.017).

Table 1: Olive varieties were collected from Al-Hussein nursery / Ministry of Agriculture in Jordan.

Variety name	Source of collection
1-K18	AL-Hussein nursery / Ministry of Agriculture
2-Nabali baladi	AL-Hussein nursery / Ministry of Agriculture
3- Rasee	AL-Hussein nursery / Ministry of Agriculture
4- Grosadee	AL-Hussein nursery / Ministry of Agriculture
5- Nasohee	AL-Hussein nursery / Ministry of Agriculture
6-Improved Nabali	AL-Hussein nursery / Ministry of Agriculture

Table 2: Oligonucleotides adaptors and primer combinations used for AFLP analysis.

Name	Sequence
EcoRI adaptor	5'- CTCGTAGACTGCGTACC-3'
	3'- AATTGGTACGCAGTC-5'
Tru91 adaptor	5'-GACGATAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
Primers used in pre-amplification	
EcoRI+1-A	*5'-GACTGCGTACCAATTCA-3'*
Tru91+1-C	5'-GATGAGTCCTGAGTAAC-3'
Primers used in selective amplification	
EcoRI+3-AGC	5'-GACTGCGTACCAATTC+AGC-3'
EcoRI+3-ACT	5'-GACTGCGTACCAATTC+ACT-3'
EcoRI+3-AAC	5'-GACTGCGTACCAATTC+AAC-3'
MseI (an isochizomer of *Tru91*)	
MseI+3-CAC	5'-GATGAGTCCTGAGTAAC+CAC-3'
MseI+3-CAA	5'-GATGAGTCCTGAGTAAC+CAA-3'
MseI+3-CCT	5'-GATGAGTCCTGAGTAAC+CCT-3'
Primer pair combinations (*EcoRI/ MseI).*	
1-AGC-CAC	
2-ACT-CAC	
3-AAC-CCT	
4-ACT-CAA	

Table 3: Polymorphism rates related to the four AFLP primer combinations among eighteen Romanian olive trees from each Tafilah and Ajlun governorate and six varieties.

Primer combination	Source of genome	Total number of fragments	Polymorphic fragments	Polymorphism (%)
AGC-CAC	-Tafilah-Romanian olive trees	70	50	71
	-Ajlun-Romanian olive trees.	86	49	57
	-Six olive varieties	67	37	55
ACT-CAA	-Tafilah-Romanian olive trees	49	24	49
	-Ajlun-Romanian olive trees.	129	44	34
	-Six olive varieties	62	45	72
AAC-CCT	-Tafilah-Romanian olive trees	154	38	25
	-Ajlun-Romanian olive trees.	104	46	44
	-Six olive varieties	60	27	45
ACT-CAA	-Tafilah-Romanian olive trees	113	59	52
	-Ajlun-Romanian olive trees.	172	85	49
	-Six olive varieties	53	34	64
		Total: 1119	Total: 538	Mean: 51

Variations among olive varieties

Dendrogram (Figure 1) was showed that the two following varieties Rasee and Improved Nabali were more closely but formed with Ajlun sample. Nabali baladi and K18 were shared with Tafilah Romanian olive trees. High similarity was showed between improved Nabali and Rasee (0.254) and the lowest was showed between Nabali Baladi and improved Nabali (0.074).

Variations within Ajlun Romanian olive trees

Out of 18 individuals of Romanian olive trees, twelve individuals were formed a separate group and isolated from the whole dendrogram. Within this group, the dendrogram can be divided and formed two sub-groups, the first includes six samples, but the second formed five samples, further the first group have two sub groups, while the second formed two sub groups, also four individual trees were shared with four samples from Tafilah. High similarity (0.39) was showed between individuals numbers (5 and 6) while the lowest (0.000) was recorded for individuals numbers (10 and 14).

Variations within Tafilah Romanian olive trees

Out of 18 individuals of Romanian olive trees, six trees were showed separated group, while two individuals formed alone, two individuals formed alone and shared with K18. Four individuals formed alone but shared with same samples of Ajlun. Olive variety "Nabali Baladi" was formed with the group of Tafilah, in addition Nashoe variety shared with one Tafilah sample. Not only Tafilah but also one

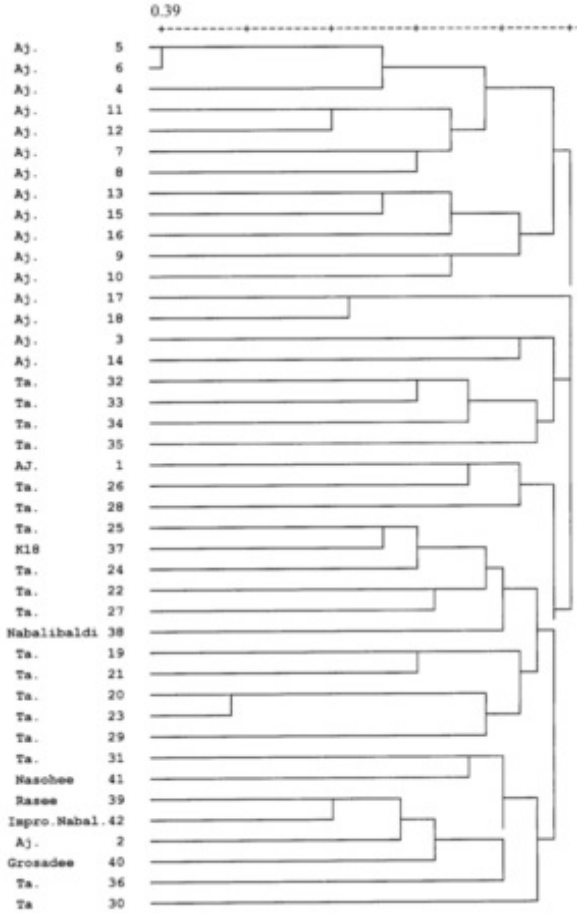

Fig. 1: Dendrogram generated among Romanian olive Trees and six varieties by using four primer pair combinations based on AFLP markers. Aj.: Ajlun souree for Romanian trees, Ta.: Tafailah source for Romanian tree. Impro. Nabal: improved nabali.

sample from Ajlun shared with the same group of Rasee, Grosadee and improved Nabali. Tafilah. High similarity was showed between (0.20) between number of individuals (22 and 25) but the lowest value is (0.000) between individual numbers (25 and 35).

Discussion

The minimum using of primer combinations and samples are better to have highly polymorphic amplified fragments and easily to discriminate among tested genotypes. Among tested primers, ACT-CAA and AGC-CAA were very important primers that can be used for either screening wild, landraces accessions and remaining olive varieties were unknown or untested.

AFLP analysis demonstrated that Romanian olive trees either fromTafilah or Ajlun had high genetic variability within and among tested individuals this was showed through dendrogram. Moreover, there is some individuals from Ajlun or Tafilah also showed broad genetic variability when they are formed a separated group. This variability is related to the genetic changes over all past years were exposed to through spontaneous mutations of naturally selection or sometimes human capacities.

Local farmers and some researchers said that their is a relationship between Rasee and improved nabali, this study was confirmed this saying through a dendrogram that showed both were grouped together. More over, Nasohee variety which commonly known its origin from Tafilah so, in our results this variety was grouped with some individuals of this region. Moreover, in addition to genetic make up there is also geographical features, isolation and climatic conditions also play a great role for creating variability within or among individuals.

AFLP has a powerful for detecting genetic variability among cultivars and species also help in known the pedigree relatives and ancestors.

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