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MOLECULAR CHARACTERIZATION OF OLIVE (*Olea europaea L.*) CULTIVARS IN DUHOK GOVERNORATE

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ABSTRACT:

The olive tree (Olea europaea L.) was considered one of the most cultivated crops in the Mediterranean region, and it had an economically important value due to it began consumed as a table olive and oil production. In this study, AFLP and SRAP techniques were used to identify 15 cultivars of olive plants that were collected from 12 different locations in the Duhok governorate. Eleven primer combinations of sequence-related amplified polymorphism (SRAP) and ten primer combinations of amplified fragment length polymorphism (AFLP) were used to fingerprinting these cultivars. The results show that the SRAP marker had the least genetic distance 0.241between (Shtla falay olive and Khodeiri olive) and the highest value (0.946) was found between Zawita olive and Sorani olive. While AFLP marker shows the lowest value of 0.107 between Nzarky olive and Dger olive, and the highest value of (0.916) between Amedi olive and Shtla falay olive. Cluster analysis based on AFLP and SRAP combined data clearly differentiated the genotype and it showed it was a powerful tool to evaluate the genetic distance and fingerprinting of these tested cultivars. Establishing the genetic relationships between cultivars and species, and the construction of genetic maps, had the main aims for the researchers in plant breeding programs and to understand the plant evolution lingues. As a conclusion, this study demonstrates that AFLP and SRAP markers are both powerful tools in plants tree genetics.

KEYWORDS: Olive, DNA-based marker, SRAP, AFLP, Genetic distance.

1. INTRODUCTION

Olives were first planted in Crete for about 2500year BC. According to archeological data, and from there they spread to Syria, Greece, Rome, and other regions in the Mediterranean (Bazakos *et al.*, 2012). Wild olives may be found in the Saharan highlands, Macaronesia, and Mediterranean areas, from South Africa to South Asia (Therios, 2009). The species *O. europaea*, of which commercial olives are a subspecies, includes two or more subspecies: *O. sativa* (Hoffm. and Link) and *O. oleaster* (Hoffm. and Link) (Bartolucci and Dhakal, 1999). Olive is a vital crop in several countries, including Spain, Italy, Greece, Turkey, and Tunisia (Sibbett *et al.*, 2005; Sesli and Yeğenoğlu, 2010; Klonaris and Agiangkatzoglou, 2018). The Mediterranean area is the most significant olive-growing region in the world for several countries. The production of olive trees is comprised of two principal products: olive oil and table olives, both of which are cultivated in numerous places throughout the central and northern regions of Iraq. Olives are not only planted in Nineveh but also in Kirkuk, Baghdad, Erbil, and Duhok. Nineveh is the governorate that produces most of the olive fruits in Iraq (Ibrahim, 2013).

The importance of olive fruit derives from both its high weight and its great nutritional content. Olive fruit is particularly abundant in the vitamins A, B, C, D, E, and K as well as in a variety of minerals (K, Ca, Mg, and P). In addition to this, olive oil is abundant in monounsaturated fatty acids and phenolic acids, both of which possess a variety of anti-oxidant properties (Ibrahim, 2013). The traditional identification of *Olea sp.* Varieties and cultivars had been based on morphological traits, such as leaf and fruit shape, color, and size, and most of the morphological traits are influenced by environmental factors, and it was led to a significant delay in time, constraints to breeders in the surveillance of germplasm and risk for fruit growers and exporters. The DNA-based Molecular markers assessment of plant varieties and cultivars are a rapid tool to allow direct comparison of different genetic material independent of environmental influences. In the past three decades, molecular markers have become a fundamental tool for plant scientists that is useful for fingerprinting varieties, establishing phylogenies trees, and mapping plant genomes (Dez *et al.*, 2011).

In genetics research and crop plant development, molecular markers that identify polymorphisms at the DNA level are extremely important. The extraction of proteins or DNA (for molecular markers) from plant tissues (i.e., seeds, foliage, pollen, and sometimes woody tissues) is the first step in the usage of genetic markers. Then, laboratory techniques (which are usually well-developed but may need to be tweaked for each species) are used, usually resulting typically in visual representations (Bracci *et al.*, 2011). Different biochemical and molecular markers have been used recently to characterize olive genotypes genetically(Cantini *et al.*, 1999). Among them were isozymes (Lumaret *et al.*, 2004), restriction fragment length polymorphisms (RFLPs) (Besnard *et al.*, 2001), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLP) (Owen *et al.*, 2005) and Sequence-related amplified polymorphism (SRAP) (Li and Quiros, 2001). DNA markers studies on plant genetic variation and genotyping had a promising technology on plant germplasm. The goal of this study is to determine genetic relationships among olive cultivars that grown natively in the Duhok governorate using two different DNA markers (SRAP and AFLP), to assess genetic diversity and breeding lines of olive cultivars in future improvement breeding programs.

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2. MATERIAL AND METHODS

2.1 Sample Collection

To carry out this phase of the experiment, fresh leaf samples were gathered from 15 cultivars of Olives *(O. europaea)* from the Duhok governorate named as (1) Khodeiri Olive, (2) Sorani Olive, (3) Spanish Olive or (Oil Olive),(4) Baashiqi Olive, (5) Dger Olive, (6) Kestawy Olive, (7) Nzarky Olive, (8) Kestawy Olive (Akre), (9) Shtla falay Olive (Akre), (10) Amedi Olive, (11) Batass Olive, (12) Mangeshk Olive, (13) Butiya Olive, (14) Birjini Olive and (15) Zawita Olive.

2.2 DNA extraction of the olive plant

To extract DNA from fresh, healthy tissue, a protocol published by Sesli and Yeğenoğlu (2010) was followed with some modification. The fresh leaves samples from the plant were frozen in liquid nitrogen to facilitate the grinding process, and three grams of tissue were ground to a fine powder. The fine powder was dissolved in a 2x Cetyltrimethylammonium bromide (CTAB, Sigma company made in Germany) extraction buffer was warmed to 67ºC (2x CTAB, 5M NaCl, 1M Tris-HCl, and 0.5 M EDTA). Next, the mixture was placed in a water bath and allowed to incubate at 67ºC for thirty minutes while it was agitated. To extract the combination, a volume-for-volume ratio of 24 chloroform to 1 isoamyl alcohol was utilized in the extraction process. After that, the mixture was centrifuged for half an hour at 4,000 revolutions per minute. The aqueous phase was transferred into a different tube, and the precipitate was made by adding 0.66 times its volume of isopropanol. After being precipitated, nucleic acids were dissolved in TE buffer, and stored at -20ºC until they used. DNA quality was examined using Nanodrop equipment.

2.3 PCR amplification for DNA markers

2.3.1 SRAP reaction mixture and amplification protocol

Li and Quiros (2001) developed a simple marker technique called Sequence-related amplified polymorphism (SRAP) that had used in this study which provided by the Scientific Research center, College of Science, University of Duhok. Table (1) is a list of the sequences and locus names for each of the 11 primer pairs that had been used in this study.

Table 1. Primer combination and sequences used in SRAP analysis (Li and Quiros, 2001).

Primer combination	Forward primer	Sequence (5' 3')
Em1-Me1	Me1	TGAGTCCAAACCGGATA
Em1-Me3	Me3	TGAGTCCAAACCGGAAT
Em1-Me4	Me4	TGAGTCCAAACCGGACC
Em2-Me1	Me1	TGAGTCCAAACCGGATA
Em2-Me2	Me2	TGAGTCCAAACCGGAGC
Em2-Me3	Me3	TGAGTCCAAACCGGAAT
Em3-Me3	Me3	TGAGTCCAAACCGGAAT
Em6-Me6	Me6	TGAGTCCAAACCGGACA
Em3-Me2	Me2	TGAGTCCAAACCGGAGC
Em6-Me1	Me1	TGAGTCCAAACCGGATA
Em3-Me5	Me5	TGAGTCCAAACCGGAAG
	Reverse primer	Sequence (5' 3')
	Em1	GACTGCGTACGAATTAAT
	Em1	GACTGCGTACGAATTAAT
	Em1	GACTGCGTACGAATTAAT
	Em2	GACTGCGTACGAATTTGC
	Em2	GACTGCGTACGAATTTGC
	Em2	GACTGCGTACGAATTTGC
	Em3	GACTGCGTACGAATTGAC
	Em6	GACTGCGTACGAATTGCA
	Em3	GACTGCGTACGAATTGAC
	Em6	GACTGCGTACGAATTGCA
	Em3	GACTGCGTACGAATTGAC

The reaction included the following components in a volume of 20µL, including: 1×PCR buffer, MgCl2 2.0 mmol·L-1, dNTPs 0.1 mmol·L-1, primer 0.5 µmol·L-1, template DNA15 ŋg, Taq DNA polymerase 1.5U.

The PCR amplification protocol for SRAP was as follow: initial denaturation for 5 minutes at 94ºC, denaturation for 60 seconds at 94 ºC, annealing for 60 seconds at 35ºC, and extension for 90 seconds at 72ºC for 5 cycles; denaturation for 60 seconds at 94ºC, annealing for 60 seconds at 50ºC, and extension for 90 seconds at 72ºC for 35 cycles; final extension for 10 minutes at 72ºC. Amplification reactions were prepared using SRAP, and the product was run on 1% agarose gel, stained using safe gel staining dye (syber-safe) in the preparation of gel, and photographed under ultraviolet illumination.

2.3.2 AFLP reaction and amplification protocol

The AFLP technique was carried out in the same manner as described by Vos *et al.*, (1995), with minor modification: The DNA from each sample was digested twice with 0.5 µl of each restriction enzyme, MseI (5'T↓TAA3') and PstI (5'CTGCA↓G3'). The digestion process was incubated at 37ºC for three hours. The DNA fragments were ligated to Pst I and MseI adapters by adding 5pmol of MseI-adapter and 5pmol of PstI-adapter to a reaction containing 0.4 l of T4-DNA ligase, 1mM ATP, and 1X-buffer, and incubated for 3 hours at 37ºC. The reaction mixture was diluted to a 1:5 ratio with sterile distillate water. PCR amplification was performed using the following protocol on a thermal cycler: 30 cycles of 30 seconds at 94ºC, 1 minute at 60ºC, and 1 minute at 72ºC.

Table 2. List of the adapter and pre-selective oligonucleotide primer sequences that correspond to the AFLP primer combinations used in this study.

Oligonucleotide		
Adaptor P	Forward	CTCGTAGACTGCGTACATGCA
Adaptor P	Reverse	TGTACGCAGTCTAC
Adaptor M	Forward	GACGATGAGTCCTGAG
Adaptor M	Reverse	TACTCAGGACTCAT
Pre-selective primers (5' 3')		
Primer P00		GAC TGC GTA CAT GCA G
Primer M00		GAT GAG TCC TGA GTA A
Selective primers amplification sequences (5' 3')		
Primer /Pstl	P107	GAC TGC GTA CAT GCA G AATA
	P174	GAC TGC GTA CAT GCA G CATG
	P20	GAC TGC GTA CAT GCA G GC
	P81	GAC TGC GTA CAT GCA G TAG
	P100	GAC TGC GTA CAT GCA G AACC
	P02	GAC TGC GTA CAT GCA G C
	P71	GAC TGC GTA CAT GCA G GGA
Primer /Msel	M82	GAT GAG TCC TGA GTA A TAT
	M43	GAT GAG TCC TGA GTA A ATA
	M88	GAT GAG TCC TGA GTA A TGC
	M182	GAT GAG TCC TGA GTA A CCCG
	M237	GAT GAG TCC TGA GTA A GATA
	M81	GAT GAG TCC TGA GTA A TAG
	M62	GAT GAG TCC TGA GTA A CTT

The pre-amplification products were diluted to a concentration of 1:5, and 2µl was employed as the template for selective amplification.

Combinations of selective MseI and Pst1 primers were used to undertake selective amplification. Amplification was performed using a 36-cycle selective program with the following profile: a 30-second DNA denaturation phase at 94ºC, a 30-second annealing step, and a 1-minute extension step at 72ºC. In this program, the annealing temperature changed in the first cycle, when it was set at 65°C, and would decrease by 0.7°C in each of the next 12 cycles (touchdown PCR). The following 23 cycles all had a temperature of 56ºC.

After selective amplification, the products were put into polyacrylamide gels with a denaturation level of 8%, DNA fragments, which were visualized using silver-stained gels, and the gels were scanned after air drying to get a digital image.

2.4 Data analysis of SRAP and AFLP

Analyzing the data was done using an NTSYS pc 2.1 program, which was developed by Numerical Taxonomy System Applied Biostatistics in Setauket, New York. PCR products were obtained from SRAP, and the presence of each fragment's AFLP marker was determined using the present (1) or absent (0). The genetic distance between the accessions was computed using this data, and a dendrogram was created to display the results. When calculating the similarity coefficient using NTSYS-PC version 2.20 software, the original DICE similarity index was used as the basis on the calculation done by Nei and Takezaki (1994).

3. RESULTS AND DISCUSSION

3.1 (SRAP) marker analysis

The results of 11 primer combinations of SRAP, gave a reproducible result and had been used to estimate the genetic diversity of 15 olive cultivars of the genus *Olea*, after a run on 1.5% agarose gel electrophoresis as shown in Figure (1)

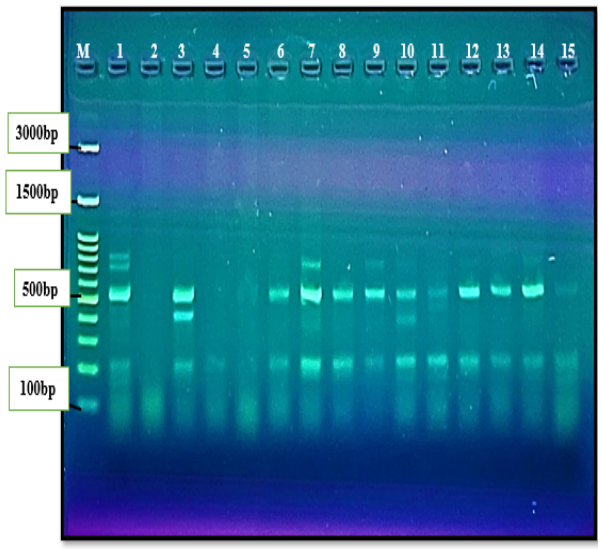

Figure 1. Amplified PCR products of 15 Olives cultivars obtained with ultraviolet light, run on agarose gel electrophoresis at 45-85v/cm, a combination between forwarding primer (2EM) and reverse primer (3ME), (M) represents DNA Marker (100bp).

On the SRAP dendrogram of the 15 cultivars of the genus *Olea*, the tree was drawn and divided into two main clusters according to their taxonomic classification as shown in Figure (2).

The first main cluster was divided into two groups:

- The first one was divided into two subgroups. Sub-group one is also divided into two sub-subgroups. The first sub-sub group was much more complex and consisted of the upper and lower part. The upper part included Khodeiri Olive, Shtla falay Olive, Nzarky Olive, and Spanish Olive. Other parts included Mangeshk Olive, Butiya Olive, and Birjini Olive. The second sub-sub group included only Dger Olive. While the other sub-group included Kestawy Olive in the upper part and Kestawy Olive (Akre) in the lower part.
- The second group was divided into two subgroups, the first sub-group included only Sorani Olive, and the second sub-group was divided into upper and lower parts, the upper part included only Baashiqi Olive, while the lower part included Amedi Olive and Batass Olive. The second main cluster included only Zawita Olive.

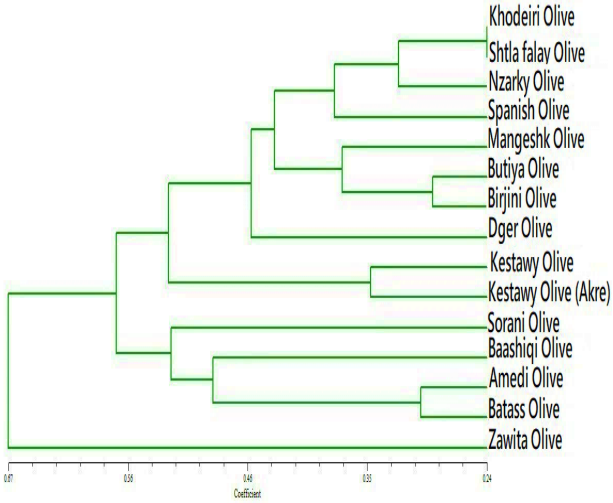

Figure 2. Dendrogram based on SRAP marker showing a relationship among Olives genotypes.

3.2 AFLP marker analysis

Fourteen AFLP primers were used to characterize the genetic diversity of 15 Olive cultivars gathered from 12 different geographic locations in the Duhok governorate, ten primers gave a reproducible result and were used to screen the genetic distance among these tested cultivars. Figure (3) showed the result of the AFLP primer combination between (P20, and M88) that gave a successful result.

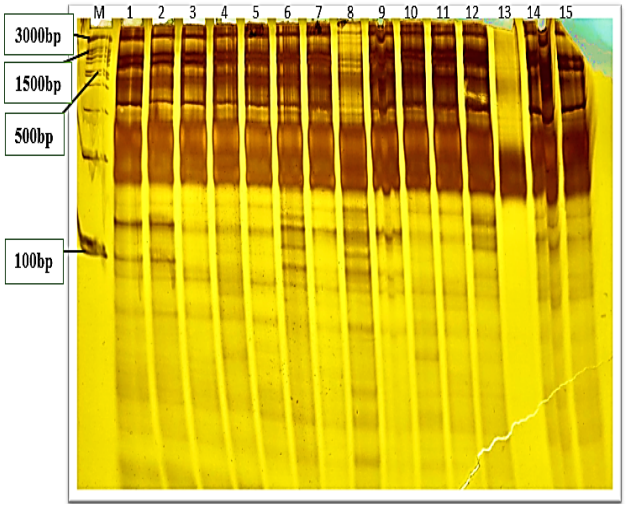

Figure 3. Amplified PCR products of 15 Olives cultivars obtained with ultraviolet light, run on Polyacrylamide gel electrophoresis at 150-250v/cm, a combination between (P20) primer and (M88) primer, (M) represents DNA Marker (100bp).

The obtained results from the dendrogram revealed that there were two main clusters, as shown in Figure (4).

- The first main cluster was much more complex and consisted of approximately all tested varieties, which contain two groups. The first one included only Khodeiri Olive, and the second group was divided into two subgroups. The first one is also divided into two sub-subgroups, sub-subgroup one was divided into upper and lower parts, and the upper part of sub-subgroup one included two groups, Sorani Olive, Spanish Olive, Dger Olive, Birjini Olive, and Zawita Olive in group one.
  The second group in the upper part of sub-subgroup one included Nzarky Olive, Shtla falay Olive, Amedi Olive, Batass Olive, and Mangeshk Olive. While the lower part in sub-subgroup one included only Baashiqi Olive. The second sub-subgroup included only Kestawy Olive. The other subgroup included only Butiya Olive.
- While the second main cluster included only Kestawy Olive (Akre).

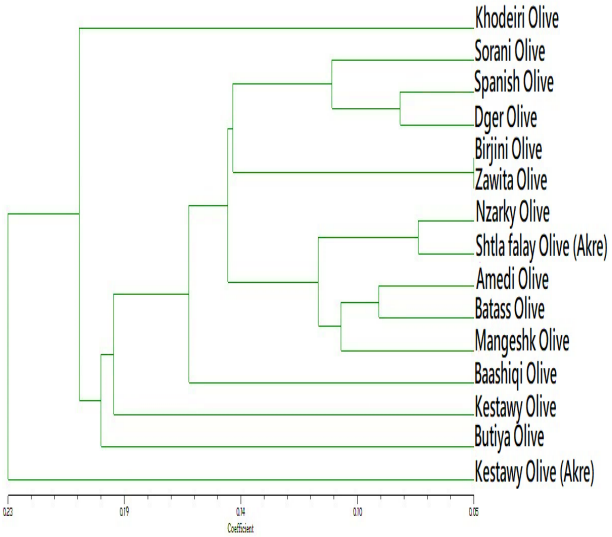

Figure 4. Dendrogram based on AFLP marker showing a phylogenetic relationship among Olives genotypes.

3.3. Compound (AFLP + SRAP) markers analysis

According to this program, a phylogenetic tree was then constructed to determine the relationship between these varieties as shown in Figure (5). The analysis was based on compound (AFLP+SRAP) markers, the dendrogram generated a dendrogram based on Dice genetic similarity coefficients using UPGMA cluster analysis. The obtained results from the dendrogram revealed that there were two main clusters.

- The first main cluster was much more complex and consisted of approximately all tested varieties, which contain two groups. The first one included only Khodeiri Olive, and the second group was divided into two subgroups. The first subgroup was divided into two sub-subgroups, sub-subgroup one consisted of upper and lower parts. The upper part of sub-subgroup one included two groups, group one included Sorani Olive, Spanish Olive, and Dgery Olive. The second group included Nzarky Olive, Shtla falay Olive, Amedi Olive, Batass Olive, and Mangeshk Olive.
  The lower part in sub-subgroup one included two groups. Group one in the lower part of sub-subgroup one included only Butiya Olive and the second group in the lower part of sub-subgroup one included Birjini Olive and Zawita Olive. The other sub-sub group included only Baashiqi Olive. The second subgroup included only Kestawy Olive.
- While the second main cluster included only Kestawy Olive (Akre).

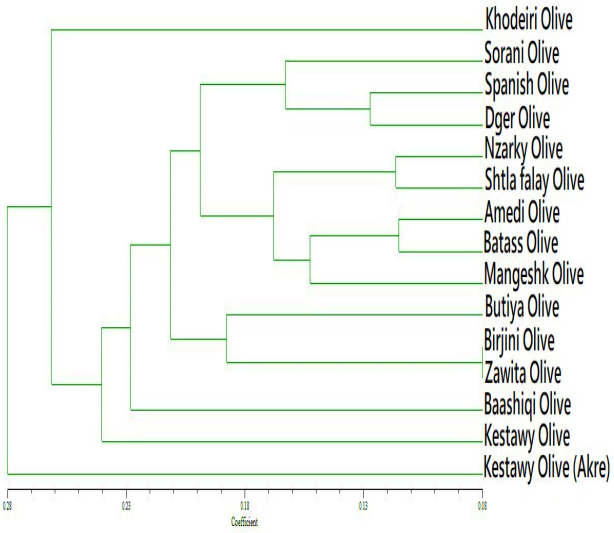

Figure 5. Dendrogram based on compound (AFLP + SRAP) markers showing a phylogenetic relationship among Olives genotypes.

Studying plant genetic diversity patterns and descriptions of plant genotypes can help determine breeding strategies and facilitate the introgression of diverse germplasm into the current plant genetic base (Tsivelikas *et al.*, 2009). One of the most important and accurate technologies is DNA markers, which assess genome sequence composition and genetic make-up information genetic variability for breeding.

The variation may have been contributed by gene flow, natural hybridization, propagation by seed, and human selection (Choudhary *et al*., 2018; Gbadamosi *et al.*, 2020)

AFLP and SRAP techniques are both considered powerful tools for fingerprinting and estimating genetic distance. In the present study, both techniques were used to verify the fingerprint of fifteen varieties of olive plants from different locations in the Duhok governorate. Ten AFLP primers combinations were used and successfully discriminate the selected olive varieties. In SRAP marker (number of primers that showed monomorphic) showed a monomorphic pattern, while the eleven primers combination exhibited polymorphic as well as monomorphic. The combined phylogenetic tree results showed that the results incline to the AFLP marker more than the SRAP, and this may due to the principles of AFLP (using two different sets of enzymes). The data obtained in this study confirmed the efficiency of both AFLP and SRAP techniques for verification and discrimination of the genetic distance of olive varieties, and illustrate the importance of molecular markers.

The results that were obtained from this study were the first step to a more extensive analysis that will include more cultivars to cover all the olive genetic diversity and elucidate its geographic distribution. As a sequel to the present work, new surveys should be made to cover all the areas of Kurdistan Regional to sample more cultivars and to draw a clearer picture of the diversity of the olive germplasm, trying to apply the methodology to a wider range of samples from this region.

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