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Genetic Relationships between Selected Wild Olive Types and two Cultivars Grown in Moseif Region / Syria using ISSR Technique

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Abstract : This study was carried out in 2015 in the laboratory of biotechnology at the Atomic Energy Commission in Syria in order to study the genetic relationships between four wild olive types selected from Hazour and Shkarah Al Khouri in Mosief region/ Syria and two native olive cultivars Douebli and Safrawi grown in the orchards that are close to the natural forests, these selected wild types have showed a slow growth and a small size when they were evaluated according to the growth characteristics in the collection that is established for evaluating the vigor of some selected wild olives.
The fingerprinting experiment was conducted by using 16 primer of ISSR technique, so the matrix of Percent Disagreement Value (PDVs) and the dendrogram of genetic relationship were created according to Unweighted Pair Group Method with Arithmetic Mean for determining the genetic distance between the studied accessions.
ISSRs technique demonstrated the effectiveness in discrimination the studied wild olive types and cultivars, sixteen ISSR primers produced 438 amplified DNA fragmentsranging in size from 150 -1300 bp. 347 fragments were polymorphic (79.22%) with average of 21.69 polymorphism per primer. All the studied samples could be identified by positive and negative unique bands generated by twelve primers.
The percentage of Percent Disagreement Values PDVs among accessions reached in average 0.36, It was 0.31 between two studied cultivars whereas the lowest value (0.26) among the four selected wild types was between types: D1 and D2 which were selected from the same region, they also displayed a close genetic relationship with the cultivar Doebli PDVs were0.23, 0.26 respectively, the highest value of this percentage (0.47) was between the wild types D3 and D1
The studied accessions were grouped in two main clusters at the value of PVD0.46, the first cluster included two sub clusters the first one contained only the cultivar Al Safrawi and the other contained the cultivar Doebli with the two wild types D1 and D2, while the wild types D3 and D5 were settled in the second main cluster. These results agreed with the data that have been obtained from the evaluation program of growth characteristics which have showed that the two types D3, D5 were distinguished because of having short trunks whereas the other wild type D1 and D2 were faster relatively in growth and moderate in size, therefore this study added a new evidence of the genetic richness in the wild olive *oleaster* in Syria and suggested close relationships between some of them and the native cultivars.
Key words: olive, wild type, cultivar, ISSR, genetic relationship.

1. Introduction

The olive (*Oleaeuropaea* L.) is one of the most ancient cultivated fruit trees, and its cultivation has a very long history which started from the Third Millennium BC [1] in the Eastern region of the Mediterranean Sea and spread later around the basin following land and maritime routes to Italy, Spain, North Africa and France. Nowadays there are about 805 million of olive trees, 98% of which are grown in the Mediterranean countries [2].

Olive tree is of dicotyledonous perennial evergreen plants. It belongs to *Oleacea* family which comprises of 25 genera and 500 species of *Oleaeuropaea*. There are two sub species, the most important of them is Euro-Mediterranean sativa known as cultivated olive tree which has large number of improved cultivars reproduce vegetatively or by grafting. A well-known species Sylvestrisoroleaster is also called Oleaster or wild olive which grows spontaneously in form of thorny shrubs with small size fruits such these shrubs are wide spread in Spain, Portugal, Moroccan countries, Sicily, Caucasian region and in Syria [3,4].The Wild olive germplasm contains more variability than the cultivated one [5,6,7], it is distributed throughout all the Mediterranean including areas where thepresence of cultivated olive is absent or symbolic [7,8,9].

The foremost consuming countries are also the main olive oil producers. According to Food and

Agriculture Organization of the United Nations, Mediterranean countries produce more than 90% of world olives and the biggest olive producers are Spain, Italy, Greece, Turkey, Tunisia, Morocco, Syria, and Portugal[10].

Phylogeography and Bayesian molecular dating analyses based on plastid genome profiling of 1263 *oleasters* and 534 cultivated genotypes reveal three main lineages of pre-Quaternary origin,Comparison of the geographical pattern of plastid diversity between wild and cultivated olives indicates the cradle of first domestication in the northern Levant followed by dispersals across the Mediterranean basin in parallel with the expansion of civilizations and human exchanges in this part of the world[11].

Syria has a very rich germplasm,there are more than seventy varieties of olive cultivated in the different areas of the country. However, only a few varieties have been extensively cultivated; in fact, five cultivars, Zaity, Sourani, Doebli, Khoderi and Kaissy, represent about 90% of the total olive trees cultivated in Syria. The others are local varieties that have a limited distribution [12].

Molecular markers revealing polymorphisms at the DNA level are very useful tools in genetics studies and in the improvement of crop plants[13,14], including ISSR markers which are helpful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of plant species [15,16,17,18,19,20].

ISSRs were applied both in phylogenetic analysis within the *Olea europaea* spices and in olive cultivar identification[13]. [9] confirmedthe wild status of some olive trees populations in the Iberian Peninsula by means ISSRs analysis. These markers were also used with success to distinguish 10 Italian varieties, by analyzing genomic DNA extracted from the olive fruit[21], and for the study of intracultivar variability of 201 accessions belonging to 11 Portuguese cultivars[22].

Our wild olive types are a result of the research programmefor five yearswhichincluded**e**valuating the vigour of a community of selected wild types in its original location in the forest (In situ) and out of the original site in the collection (Ex situ) established for this purpose, according to this programme we selected four wild olive types which showed a slow growth and smallnessvolume in compare with the other types, they are apparently suitable types for high density cultivation,so that this study aims to determine the diversity among these selected wild types and to study the genetic relationships between them and two native cultivars Al Safrawi and Doebli which are spread in the orchards close to the natural forest of wild olive in Mosief region in Syria.

2. Materials and Methods

Plant material :

This study was carried out in the laboratory of biotechnology at the Atomic Energy Commission during 2015 on four typesof wild olive selected from Mosief region / Syria and two cultivars Al Sfrawi and Doebli grown in the orchards in the same region.

The young leaves (25-30 leaves) were collected from young shoot of the wild olive trees, and they were saved in aluminum sheets and were transported to the laboratory of biotechnology at the Atomic Energy Commission.

The leaves were washed three times in distilled water, put in liquid nitrogen and stored at – 70 °C until use.

DNA extraction:

The total genomic DNA was extracted by using modified quick method of CTAB [23].A 0.5 grams of young leaves were ground to powder in liquid nitrogen using a mortar and pestle. The powder was transformed into 2 ml Eppendorf tubes and mixed with 800 µl of extraction buffer: 200 mMTris- HCl(pH: 7.5), 250 mM NaCl, 25 mM EDTA (Ethylenediamine tetra acetic acid), and 0.5% SDS. The tubes were homogenized and vortexed for 10 sec then incubated at 65 ° C for 15 min.

A 400 µl of cold 7.5 M Ammonium acetate were added to each tube and put on ice for 10 min. The tubes were centrifuged at 12000 rpm for 15 min. the upper aqueous phase (about 500 µl) was recovered to a new tube and mixed with the same volume of isopropanol and left for 5 min. The tubes were then centrifuged at 12000 rpm for 10 min, the nucleic acid precipitate was washed twice with cold 65% ethanol, then centrifuged at 12000 rpm for 10 min, dried under the laminar flow and resolvedin150µl of double-distilled and sterilized water, DNA sample concentrations were determined usingaGene Quantspectrophotometer (Amersham Biociences), and were diluted to 10 ng/µL prior to ISSR PCR amplifications.

ISSR Technique:

ISSR-PCR DNA amplifications were performed using 16 ISRR primers(Table 1) AndPCR reactions were carried in a 25 µLreaction volume containing : 100 mM Tris-HCL (pH: 8.8 at 25 ° C), 50 mM$(NH_4)_2SO_4$, Bovine Serum Albumin 0.005% Tween 20- 0.00002%, 3.2 mM $MgSO_4$, 0.2mM for each of (dCTP, dGTP, dTTP, dATP) produced by Fermentas, 1 unit ofTaq DNA polymerase (Fermentas), 30 ng DNA, 125 Pmol of each primer (Alfa and Invitrogen). **Table 1.List of ISSR primers, their sequences and annealing temperatures (T°)**

Primer	Sequence	T° (°C)	Primer	Sequence	T° (°C)
A1	(CA)6RR	50	**B7**	(GTG)3GC	50
A4	(CA)6RY	50	**B10**	(GAG)5	50
A8	(CA)6RM	50	**B13**	(CAA)5	50
A14	(CA)6RS	50	**B16**	(GACA)4	50
A30	(AGC)4R	50	**C31**	(AG)8T	50
A35	(AGC)4Y	50	**6(479)**	(CAC)3GC	50
A41	(AGC)4K	50	**7(479)**	(CT)8TG	50
B2	(CT)8TG	50	**8(479)**	(CT)8AC	50

R= A+G Y= C+T M=A+C K= G+T S=G+C

Amplification was carried out in an Eppendorf Cycler with the following programm: 5 min of initial denaturating at 94 °C, followed by 40 cycles of three steps: 10 sec of denaturatinh at 94 °C, 10 sec of annealing at 50 °C, 10 sec of extension at 72 ° C, followed by a final extention for 7 min at 72 ° C.Amplification products were stored at 4 ° C until the visualization on agarose gel electrophoresis.

The PCR products were electrophoresed on 2% ethidium bromide (Fluka)- stained agarose (Q. BIOgene) gets in 0.5X Tris Borate EDTA (TBE). A 100 bp ladder (Vivantis) was used to estimate the approximate molecular weight of amplification products.

Statistical Analysis:

Results bands were screened and photographed under UV light. All reactions were repeated at least twice and only bands that were bright, reproducible were scored for the analysis. Bands were scored either (1) as present or (0) as absent, the unweighted pair group method with arithmetic average (UPGMA) [24], and percent

disagreement values (Jaccard'scoefficient) of the STATISTICA program (STAT-Soft, Inc. 2003)were used to construct the matrix and the genetic dendrogram.

2. Results and Discussions:

Table (2) shows that the use of 16 primers for DNA amplification on studied samples of wild and cultivated olive generated 160 lines of bands i.e.10 lines per primer in average, out of which 144 lines (89.24% of primers) were polymorphic. 9 primers generated polymorphism in all lines of bands (100%) whereas the polymorphism in the remaining primersranged from 60% in primer A8 to 93.8% in primer 6(479).

Table 2.The ISSR primers, the total and polymorphic lines per primer, the total amplified fragments, size of amplified fragments and polymorphic fragments per primer

% polymorphic fragments	No. of polymorphic fragments	Size of amplified fragments	No. of fragments amplified	% polymorphic lines	Total no of polymorphic lines	Total no. of lines	primer
66.67	20	150-500	30	88.9	8	9	**A1**
100	23	300-800	23	100	11	11	**A4**
48.78	20	180-1100	41	60	6	10	**A8**
100	18	150-900	18	100	6	6	**A14**
76	19	150-800	25	88.9	8	9	**A30**
100	34	150-800	34	100	13	13	**A35**
56.75	21	150-1000	37	72.7	8	11	**A41**
100	18	200-1000	18	100	10	10	**B2**
100	19	200-1100	19	100	9	9	**B7**
72.22	13	200-1000	18	60	3	5	**B10**
100	34	200-1100	34	100	11	11	**B13**
100	28	150-1200	28	100	10	10	**B16**
42.86	18	150-1000	42	63.6	7	11	**C31**
77.5	31	200-1100	40	93.8	15	16	**6(479)**
100	17	200-1000	17	100	10	10	**7(479)**
100	14	600-200	14	100	9	**9**	**8(479)**
1340.78	347		438	1427.9	144	160	**Sum**
79.22	21.69		27.38	89.24	9	10	**average**

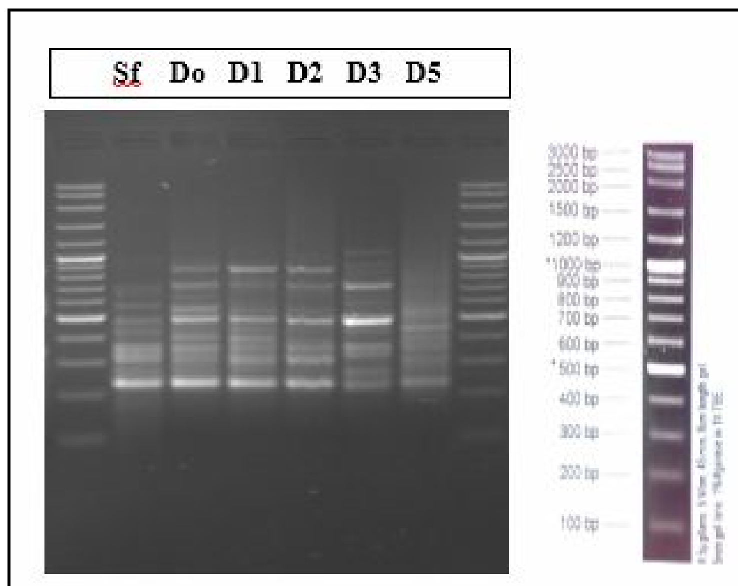

Figure1.Polymorphism generated from the use of ISSR primer 6(479) on the studied sampled (L: 100bp DNA ladder)

The table (2) shows also that The ISSR technique has a high effectiveness in discrimination the studied olive samples (*Olea europaea L.*) in both subs spices wild *(Olea europaea subsp europaeavar.sylvestris*) and cultivated olive (*Olea europaea subsp europaea var.sativa*), and the all used primers generated polymorphic fragments. The sizes of total DNA fragments shown in amplification products ranged between 150 and 1300 bp.

The used primers generated 438 fragments of which 347 were polymorphic i.e. the percentage of polymorphic fragments in average reached79.22%, a high percentage of polymorphic fragments (100%) were obtained with the primers A4, A14, A35, B2, B13, B16, 7(479), 8(479). The lowest one was 42.9% with the primer C31.Whereas most of used primers that studied the diversity among bread wheat cultivars gave 100% polymorphic bands [25].

The number of ISSR-PCR fragments generated by using the twelve primers could be used as specific markers for both wild and cultivated olive (table 3,4,) 9 primers marked all studied samples with positive unique bands,and6 primers marked the cultivar Doebli and three of studied wild types (D1, D3, D5) with negative unique bands as it shown in table 3.

Table 3.The positive unique bands and their approximate size.

primer	types	size of unique band (bp)
A4	D2	1000
A4	Sf	1100
A8	D1	500
A14	D2	1000
A41	D3	600
A30	D5	800
B2	D2	100
B5	DO	1200
B5	D5	1100
B10	D2	800
6(479)	D5	1000

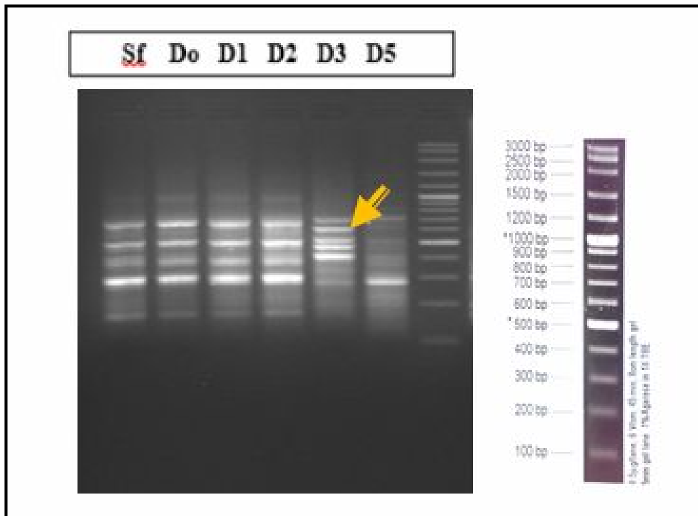

Figure2. The positive unique band (600 bp) produced by primer A41 identified the wild type D3

Table 4.The negative unique bands and their Approximate size

primer	types	Size of unique band (bp)
A8	D5	500
A35	D3	400
A41	D5	500
B2	D5	700
8(479)	D1	500
B16	Do	600

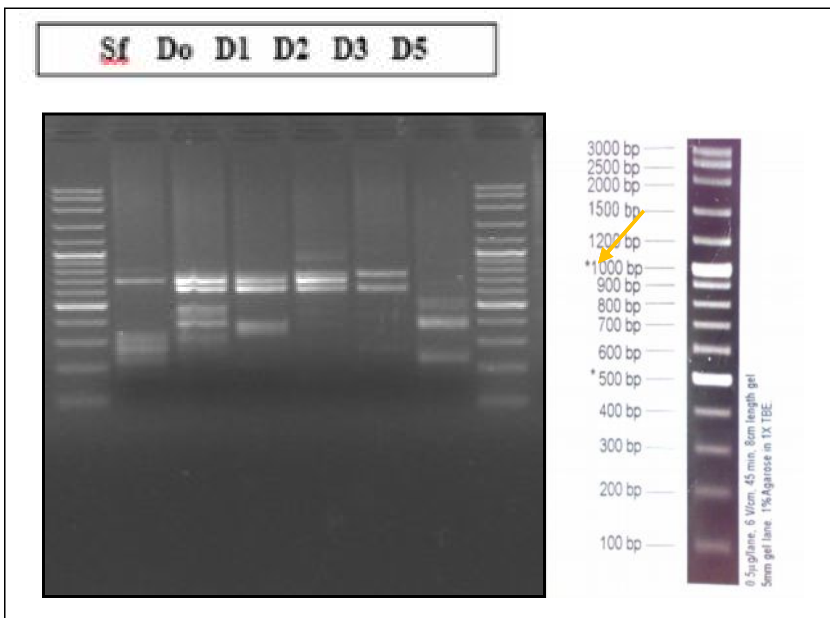

Figure3. The negative unique band(700 bp) produced by primer B2 identified the wild type D5

Notably some samples were characterized by more than one primer e. g. the cultivar Doebli characterized by unique fragments which generated by primer B5 (positive unique band) and primer B16 (negative unique band), the capability of ISSR primers to give unique bands stated by many research such as [26].

The sizes of unique positive bands ranged from 100 to 1200 bp, whereas the negative ones were between 400 to 700 bp,

The high level of polymorphism observed (79.22%) with the primers used in this study, indicate a high level of genetic variation among the studied accessions including wild olive types, in agreement to[27] confirmed high efficiency of ISSR technique indetermination the genetic similarity among some genotypes of Durum Wheat (*Triticumsp*) and wild species, and also in agreement to [28]reportedthat the disagreement values among seven wild olive types from Turkey based on RAPD-PCR assay varied from 0.1391 to 0.4957.

The ISSR matrix (table 5) shows that the general mean of disagreement values (PDVs) was 0.36, and it reached 0.31 between the two cultivars, the lowest value of disagreement among wild types was between the types D1 and D2, these mentioned types showed relatedness with the cultivar Doebli with the PDV 0.26, 0.23 respectively, whereas the highest value of disagreement was between the types D1 and D3 (0.47). Regarding the two wild types D3 and D5 which were distinguished as a dwarf type (the result of research program for 5 years) The PDVs between them and the studied cultivars were moderates. It was 0.46, 0.48 between the type D3 and the cultivars Al Safrawi, Doebli respectively, and 0.41, 0.49 between the type D5 and the two cultivars.

Table 5. The matrix of percent disagreement values (PDVs) between the studied accessins (cultivated and wild olive) resulted from ISSR technique

	Sf	Do	D1	D2	D3	D5
Sf	0.00					
D0	0.31	0.00				
D1	0.33	0.26	0.00			
D2	0.33	0.23	0.26	0.00		
D3	0.46	0.48	0.47	0.46	0.00	
D5	0.41	0.49	0.43	0.43	0.44	0.00

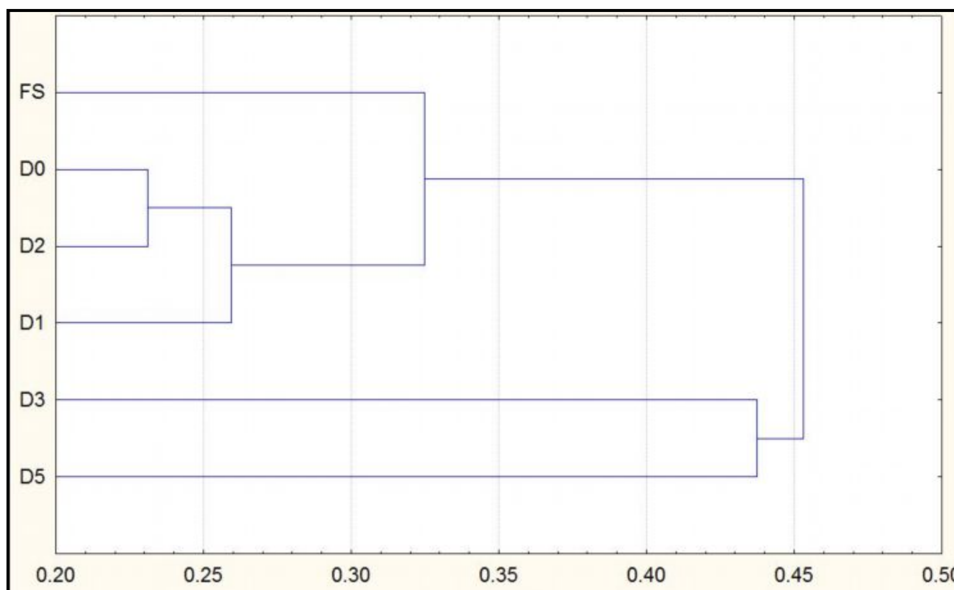

Figure 4. Genetic dendrogram showing the degree of relationships among the studied samples (cultivated and wild olive) based on disagreement values

The UPGMA dendrogram constructed using the disagreement values (Figure 4) clustered olive samples into two major groups at disagreement value 0.46. The olive samples in the first major group further clustered into two subgroups, one of them included only the cultivar Al Safrawi, the other subgroup included the wild types D1, D2 and the cultivar Doebli. The wild types D3 and D5 were settled together in the second main group.

This result agrees with the result published by [29] in Tunisia suggesting close relationship between some cultivar and some oleaster trees, and also interpreted by [8] assumed that a hybridization occur between true oleasters and cultivated varieties in areas of close contact between the two forms. Many studies assumed that a majority of modern cultivars were derived either from inter–crossing of ancient cultivars, or from interbreeding with wild plants followed by local selection [30,31,32,33].

Conclusion:

This study confirmed thatISSR markers were polymorphic markers suitable to detect the genetic diversity of olive cultivated and wild oliveat the DNA level, and added a new evidence of the genetic richness in the wild olive *oleaster* in Syria.

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