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Genetic Fingerprinting of Palestinian Olive (*Olea europea* L.) Cultivars Using SNP Markers

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ABSTRACT

The study of the genetic relationships among Palestinian olive cultivars is very important in order to assess their genetic diversity and structural details. A collection of five cultivars including Roomi, Souri, Improved Nabali, Barenge K18 and Wild Type were analyzed by using the sequences of two genes' fragments in which a panel of twenty-five Single Nucleotide Polymorphisms (SNPs) were identified. It is found that this group of SNPs is not efficient enough to discriminate among all of the cultivars. However, it is useful for varietal survey and construction of a database of Palestinian olive cultivars and for providing additional information that could constitute the basis for a rational scheme of breeding programs.

Keywords: Genetic fingerprinting, SNP markers, Olive (*Olea europea* L.).

INTRODUCTION

The Olive (*Olea europea* L.) is the most important crop in Palestinian agriculture in terms of area covered as well as economic returns. It covers about (100000) hectares distributed all over the West Bank and Gaza strip and it is distinguished as the major tree in the rain fed areas, covering about 45% of the total cultivated area in the West Bank and contributes to about 40% of total fruit production (PCBS, 2005).

Olive in the family of *Oleacea*e is one of the oldest known cultivated trees worldwide and the Mediterranean region is considered its center of origin. The archaeological findings demonstrate that the Syrian-Palestinian region was the center of origin of olive cultivation (Zohary and Hopf, 1994; Remesal-Rodriguez, 1996; Basheer-Salimia, 2004).

Genetically, olive, an ancient tree (Lopes *et al*., 2004), is an outcrossing diploid species (2n = 46) (Reale *et al*., 2006) cultivated throughout the Mediterranean basin (Reale *et al*., 2006; Taamalli *et al*., 2006). Nowadays, olive cultivation is significant to the rustic economy as well as to the environmental equilibrium of the producing regions (Lopes *et al.,* 2004).

Identification of olive cultivars is actually sophisticated because of the huge number of varietal synonyms and homonyms (Bandelj *et al.,* 2002). Hence, there is a crucial necessity for the development of methods that denominate olive efficiently and swiftly (Torre *et al.,* 2004). In olive, DNA markers are being developed to identify and characterize olive cultivars and to determine the varietal composition (Consolandi *et al.,* 2007) including randomly amplified polymorphic DNAs (RAPDs) (Hess *et al.,* 2000; Besnard *et al.,*

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2001; Sanz-Cortez *et al.,* 2001; Nikoloudakis *et al.*, 2003; Shu-Biao and Sedgley, 2004), Amplified Fragment Length Polymorphisms (AFLPs) *(*Belaj *et al.,* 2003; Sanz-Cortez *et al.,* 2003; Sensi *et al.,* 2003; Owen *et al*., 2005), Restriction Fragment Length Polymorphisms (RFLPs) (Besnard and Breville, 2000; Besnard *et al.,* 2001; De Caraffa *et al*., 2002), Sequence Characterized Amplified Regions (SCARs) (Hernandez *et al.,* 2001a; Hernandez *et al*., 2001b; Mekuria *et al.,* 2002; Shu-Biao and Sedgley, 2004), Simple Sequence Repeats (SSRs) or Microsatellite Markers (Rallo *et al.,* 2000; Sefc *et al.,* 2000; Bandelj *et al.,* 2002, Carriero *et al.,* 2002; Cipriani *et al.,* 2002; Shu-Biao and Sedgley, 2004), Inter-Simple Sequence Repeats (ISSRs) (Hess *et al.,* 2000; Vargas and Kadereit, 2001) and Single Nucleotide Polymorphisms (SNPs) (Palmieri *et al.,* 2004; Reale *et al.,* 2006; Consolandi *et al*., 2007).

Single Nucleotide Polymorphisms (SNPs) are the most abundant form of genetic variation in most organisms (Khlestkina and Salina, 2006). Because of their ability to distinguish among very similar cultivars (Consolandi *et al.,* 2007), they can be widely used for certification of cultivars and lines (Saghai Maroof *et al.,* 1994; Khlestkina and Salina, 2006; Shirasawa *et al*., 2006).

The aim of this study is to establish an SNPs database for the Palestinian olive cultivars and to ensure that all future projects in the related field are conducted on the same cultivars.

MATERIALS AND METHODS:

Plant Materials: Fresh leaves of the five Palestinian olive cultivars (Roomi, Souri, Improved Nabali, Barenge K18 and Wild Type) were randomly collected and piled up in October 2006 from single plants on the campus of Hebron University (Hebron, Palestine) (Table 1). The first four cultivars represent some of the eminent cultivated cultivars in Palestine. Subsequently, these samples were brought and stored at 4°C at Mediterranean Agronomic Institute of Chania (Chania-Greece) where this research was carried out.

Table (1): List of olive cultivars analyzed in this study.

No.	Code	Cultivar name	Source
1	PA1	Barenge K18	Hebron University Campus, Palestine
2	PA2	Improved Nabali	Hebron University Campus, Palestine
3	PA3	Roomi	Hebron University Campus, Palestine
4	PA4	Souri	Hebron University Campus, Palestine
5	PA5	Wild Type	Hebron University Campus, Palestine

DNA Extraction: The choice of DNA extraction protocol depends on the quality and the quantity of the DNA needed, nature of samples and the presence of natural substances that may interfere with the extraction and subsequent analysis (Semagn *et al*., 2006). Therefore, CTAB method by Woolley (Woolley *et al*., 2001) with few modifications [RNase was added additionally to (Woolley *et al*., 2001) and Phenol/chloroform purification and ethanol precipitation were carried out as described in: (Sambrook *et al*., 1989)] was used for DNA extraction from the leaves of the five cultivars. The use of cycloartenol synthase and lupeol synthase genes, which are entailed in sterol biosynthesis (Haralampidis *et al*., 2001; Stiti *et al*., 2007), is based on what has been found

in the olive genome (Palmieri *et al*., In Press; Reale *et al*., 2006). The amplifications of *cycloartenol synthase* and *lupeol synthase* were carried out by Polymerase Chain Reaction (PCR) using their primer pairs *Cyclo2*, *Cyclo3* and *Lup*, respectively.

PCR: Its amplifications were carried out using 1 x AmpliTaqGold® PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl), 2.5 mM $MgCl_2$, 200 µM for each dNTP, 0.3 µM for forward and reverse primers (Table 2), 0.05 U/µL of AmpliTaqGold® *polymerase* (an ultra pure and stable polymerase with proof reading activity) and 5 µL of DNA template (25 ng/µl) per 100 µL of reaction volume. Sterile-ddH_2O was also added to the PCR reaction mix up to the appropriate volume. Polymerase chain reaction was carried out by initial denaturation of template DNA for 10 minutes at 95°C, followed by 35 cycles of denaturation (95°C for 30 s), annealing (60°C for 30 s)and extension (72°C for 60 s), with a final extension step at 72°C for 10 minutes. PCR products were then purified using (Strata Prep® DNA Gel Extraction Kit). To check the quality as well as the quantity of the DNA of these amplicons before the sequencing step of those samples, 1 µl of loading buffer and 3 µl of the purified DNA of each cultivar was loaded on a 2% (v/v) agarose gel stained with ethidium bromide and visualized with UV light.

Table (2): Primers used for targeted amplification strategy.

Locus name	Primer name	Primer sequence (5`→3`)	NCBI accession no.	T_a (°C)	Extension time (min.)	Amplicon size (bp)	Reference
Cycloartenol synthase	Cycl2-F	5`ATTCTTTTGGCTACTTGGACATCTTT3`	AY847065	60	1	901	(Reale *et* al., 2006)
	Cycl2-R	5`AACCCTCAGCTGTGCAATCTG3`					
	Cycl3-F	5`ATTTCAGATTGCACAGCTGAGG3`					
	Cycl3-R	5`ATCCTGAGGAAATCATCTCCATTT3`					
	Cyc2a-F	5`ATTCTTTTGGCTACTTGGACATCT3`	AY847065	50	0.5	257	Present study
	Cyc2a-R	5`GGAGATTGTAGCAATGTTGATATG3`					
Lupeol synthase	Lup-F	5`CTAACTCGATGGCCGTTTTCTAA3`	AY847066	60	1	501	(Reale *et* al., 2006)
	Lup-R	5`GCAACTCAAATGAATGAATCATGAT3`					
	Lup2-F	5`GCAACTCAAATGAATGAATC3`	AY847066	50	0.5	≈300	Present study
	Lup2-R	5`AACTCATGTTTTGTAGGTG3`					

Note: The sequences, T_a (anneling temperature), extension time used for PCR and expected amplicon sizes in the PCR are given. *Cyc2a-F/R* and *Lup2-F/R* primer pairs were designed by using PrimerExpress® 2.0 Software (Applied Biosystems) to amplify shorter fragments than those given by *Cyclo2-F/R*, *Cyclo3-F/R* and *Lup-F/R* and their PCR products will be used for the genotyping step. To check the uniqueness of the complementary DNA region against new primers, a BLASTn search (Altschul *et al*., 1990) was carried out.

SNP Discovery: It was carried out based on the amplification of DNA fragments using locus-specific (LS) primers, Table 2, and on the sequencing of PCR products instantly from all five olive cultivars.

Sequencing was performed with one of the PCR primers using BigDye® Terminator cycle sequencing in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). Three methods; namely visual inspection, BioEdit software (Hall, 1999) and SeqDoc software (Crowe, 2005) were used to identify the polymorphisms among the sequencing traces obtained from Genetic Analyzer by aligning them against each other.

SNP Genotyping

Genotyping of the cultivars' SNPs was carried out in 10 µl reactions. Purified PCR products (5 µL) was mixed with 2 µL ExoSAP-IT® (USB Corporation) and incubated twice at 37°C for 45 minutes and at 80°C for 15 minutes, respectively. The primer extension reaction was carried out by mixing 5 µL of SNaPshot™ Reaction Mix, 3 µL of previously cleaned up PCR product, 1 µL of 2 pM SNP primer (Table 3) and 1 µL sterile-ionized-distil H_2O. The mixture was incubated at 95°C for 25 cycles for 10 s, 50°C for 5 s, 60°C for 30 s and eventually at 4°C. The whole primer extension product (10 µl) was mixed with 1 µL shrimp alkaline phosphatase (USB Corporation), incubated at 37°C for 60 minutes and ultimately at 75°C for 15 minutes . Subsequently, 0.5 µL of the sample was blended with 0.5 µl of GeneScan™ -120 LIZ™ size standard and 9 µL of Hi-Dye™ formamide before incubating at 95°C for 1 minute and placing on ice to be inescapably loaded into the ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

Table (3): SNaPshot™ extension primers used.

Locus name	Primer name	NCBI accession #	Name of SNP	SNP Probe Sequence 5`→3`
Cycloartenol synthase	Cyc2a-F/R	AY847065	Cyc2-2	GCTCTCAAAAGGGTCAAGT
Lupeol synthase	Lup2-F/R	AY847066	Lup2-1 Lup2-2	GGGTATGCTACTACCTAATCTT TGTGTTTCGATTTGCTGC

RESULTS AND DISCUSSION

Leaf DNA Extraction

The products of the successful PCR reactions of the five cultivars (PA3, PA4, PA2, PA1and PA5) (Table 1), in combination with a positive control (a Greek olive cultivar; Koroneiki), using *Cyclo2, Cyclo3*and *Lup* primer pairs were run on a 2% (v/v) agarose gel, respectively (Fig. 1). The application of both *Cyc2a* and *Lup*2 primer pairs (Table 3) designed to give shorter fragments than *Cyclo2, Cyclo3 and Lup* was also accomplished successfully as shown in Figure 2. The use of negative control was useful for excluding any false negative, which for example results from the amplification of a non-target DNA.

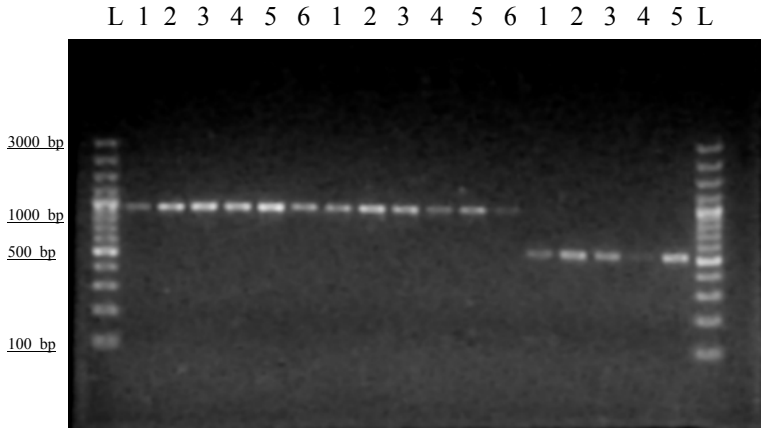

Fig. (1): Agarose gel electrophoresis stained with ethidium bromide of the PCR products extracted and purified using the StrataPrep® DNA Gel Extraction Kit (STEATAGENE®) of the five Palestinian olive cultivars (PA1, PA2, PA3, PA4 and PA5) in combination with a positive control (6). L: 100 bp ladder; (1: PA3; 2: PA4; 3: PA2; 4: PA1; 5: PA5; 6: positive control using the three primer pairs (Cyclo2, Cyclo3 and Lup), respectively).

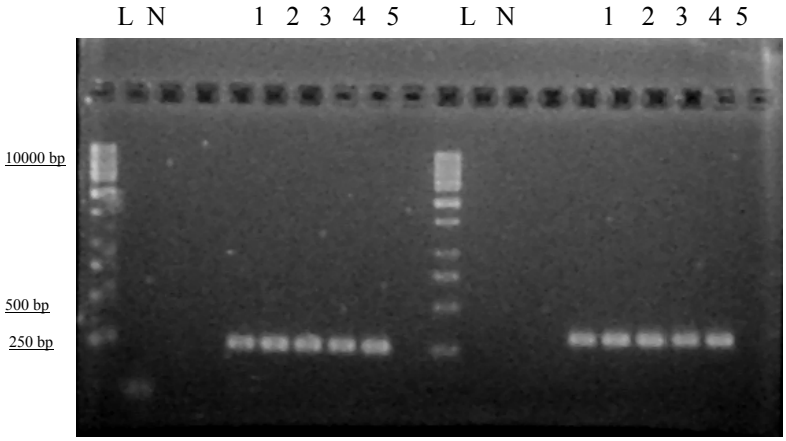

Fig. (2): Agarose gel electrophoresis stained with ethidium bromide of the PCR products of the five Palestinian olive cultivars with Cyc2a and Lup2 primer pairs, respectively. L: 1K bp ladder; N: negative control; 1: PA3; 2: PA4; 3: PA2; 4: PA1; 5: PA5.

Direct sequencing of gene fragments amplified from genomic DNA has the advantage of not requiring cloning of the PCR products and potential SNPs can be identified in the form of heterozygotes (Palmieri *et al.*, In Press) together with the homozygous ones. For example, SNP-1 (Table 4) in (PA3+PA4+PA2) is heterozygous whereas it is homozygous in (PA1+PA5)). In spite of analyzing two genes out of the proposed three genes, the number of the discovered SNPs in cycloartenol synthase and lupeol synthase was similar to what Reale *et al.* (2006) identified. When *Cyclo2-F/R*, *Cyclo3-F/R* and *Lup-F/R* primer pairs were used to give longer fragments than those of what (Reale *et al.,* 2006) used, considering that the ample sequences needed to be aligned as a first necessity to identify polymorphisms and to distinguish real genetic changes from those generated by sequencing errors (Barker *et al*., 2003), a number of twenty-five SNPs (Tables 4 and 5) were identified in a total length of 2303_{bp} sequences analyzed, corresponding to the DNA fragments of those genes in five olive cultivars; their distribution was 1 SNP per $\approx 100_{bp}$. This level of polymorphism in the olive tree compared to other plant species reported to have polymorphisms is closer to maize (an out-crossing plant species) where the frequencies of its nucleotide changes are 1 per 104_{bp} (Giancola *et al.,* 2006). Of the twenty-five SNPs, five were transversions and twenty were transitions. Transitions constitute 80 % of these identified twenty-five SNPs encountering the principle that transitions are more common than transversions (Strachan and Read, 2003; Morley *et al*., 2004). One probable explanation would be a high spontaneous rate of deamination of 5-methyl cytosine (5mC) to thymidine (C←→T) SNPs, with (G←→A) on the other strand (Strachan and Read, 2003; Morley *et al*., 2004).

Table (4): The Palestinian database of five olive cultivars of the lupeol synthase (Lup-F/R) fragment.

A	**1**	**2**	**3**	**4**	**5**
Code / **SNP No.**	**PA3**	**PA4**	**PA2**	**PA1**	**PA5**
1	**CG**	**CG**	**CG**	CC	CC
2	CC	CC	CC	CC	**TT**
3	CC	CC	CC	CC	**AA**
4	AA	AA	AA	AA	**GA**
5	AA	AA	AA	AA	**TT**
6	AA	AA	AA	AA	AA
7	GG	GG	GG	GG	GG
8	AA	AA	AA	AA	**CC**
9	TT	TT	TT	TT	**CC**
10	GG	GG	GG	GG	GG

Table (5): The Palestinian SNP database of five olive cultivars of the cycloartenol synthase fragment.

A	1	2	3	4	5
Code / SNP No.	PA3	PA4	PA1	PA2	PA5
1	**AA**	**AA**	TA	TA	**AA**
2	**AA**	**AA**	TA	TA	**AA**
3	AA	AA	AA	AA	AA
4	**AA**	**AA**	AC	AC	**AA**
5	**AA**	**AA**	AC	AC	**AA**
6	GG	GG	GG	**AG**	**AG**
7	**TT**	**TT**	TC	TC	TC
8	**CC**	**CC**	TC	TC	TC
9	**GG**	**GG**	AG	AG	AG
10	**GG**	**GG**	TG	TG	TG
11	TT	TT	TT	TT	TT
12	**CC**	**CC**	TC	TC	**TT**
13	**TT**	**TT**	TC	TC	**TT**
14	CC	CC	CC	CC	CC
15	CC	CC	**AA**	**AA**	**AA**

Note: SNP positions (1-11) were found in the cycloartenol synthase fragment using the *Cyclo2-F/R* primer pair, whereas (12-15) were discovered in the same fragment of the aforementioned gene using the *Cyclo3-F/R* primer pair.

SNP Database

SNP database of the Palestinian olive cultivars was created to initiate the first step on the way for genotyping all of what Palestine genuinely owns. Although the discovered set of SNPs in the two genes' fragments, lupeol synthase and cycloartenol synthase, using their own primer pairs in the five olive cultivars is ineffective enough to discriminate among all of them, it has thoroughly differentiated three out of five, forming a percentage of 60 %, which is actually due to less polymorphism among them.

When the minimum efficient number of the discovered SNPs is used, the five cultivars can be assembled into four groups. For instance, SNP-1 in lupeol synthase of those cultivars (Table 4) divides them into two groups: (PA2, PA3 and PA4) and (PA1 and PA5). Consequently, using SNP-2 in lupeol synthase (Table 4); (PA1 and PA5) group can be broken off into two groups; PA1 and PA5. To discriminate among the (PA2, PA3 and PA4) cultivars, SNP-2 in cycloartenol synthase (Table 5) divides them into two groups (PA2) and (PA3 and PA4) (Fig. 3).

Another efficient discrimination can be carried out among these five cultivars when two SNPs of cycloartenol SNPs are used (Table 5). For example, when SNP-1 is used, the five cultivars can be split into two groups: (PA1, PA2) and (PA3, PA4 and PA5). When SNP-6 is also used, the (PA1, PA2) group can be

broken apart into two groups: (PA1) and (PA2), and (PA3, PA4 and PA5) class can also be separated into two groups: (PA3, PA4) and (PA5).

No more SNPs were discovered in these two genes' fragments which can tell apart between the PA3 and PA4 class. The inability of these SNPs to distinguish these two olive cultivars confirms their identity and the close genetic relationship between them, making them potential cultivar authentication tools. Assaf (1994) along with many other scientists mentioned that the Souri cultivar (PA4), also called Nabali Baladi, is similar to the Roomi cultivar (PA3), also called Romani, without any evidence proving a difference between them. Therefore, the SNP database of the Palestinian cultivars proves that the Souri and Roomi cultivars are indistinguishable at all discovered SNP positions, unless it refutes by future work.

The SNP-12 of cycloartenol synthase in the PA5 variety is unique (Table 5), which can discriminate it from the (PA1, PA2, PA3 and PA4) cultivars. Furthermore, in the lupeol synthase gene, the PA5 variety is highly distinct among all the studied cultivars at almost all its SNPs (Table 4), it is considered unique in its zygosity, in which one SNP is adequate enough to discriminate among all the other cultivars.

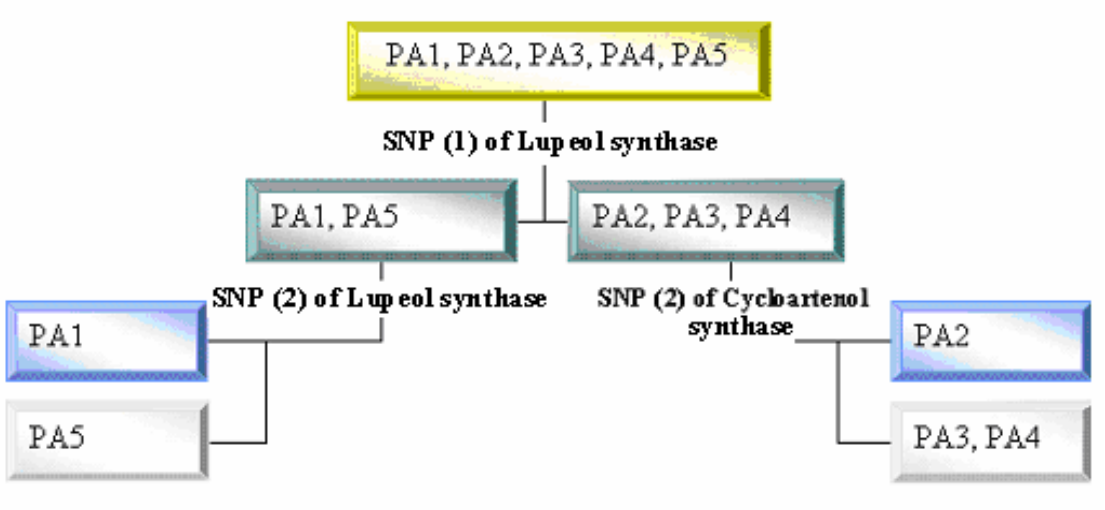

Fig. (3): Using the minimum efficient number of SNPs (SNP-1, 2) of *lupeol synthase* gene and (SNP-2) of *cycloartenol synthase* gene, the five Palestinian cultivars can be grouped into four classes.

SNaPshot™ Analysis

The amplified fragment of lupeol synthase using its primer pair, *Lup2-F/R,* contains all its discovered SNPs, whereas that of cycloartenol synthase contains the first 3 SNPs when its primer pair *Cyc2a-F/R* was used. This set of SNPs is effective enough to discriminate among those cultivars, which can be differentiated using the discovered set of twenty-five SNPs. The result of the genotyping was in agreement with that of the sequencing, (Table 6, Fig. 4).

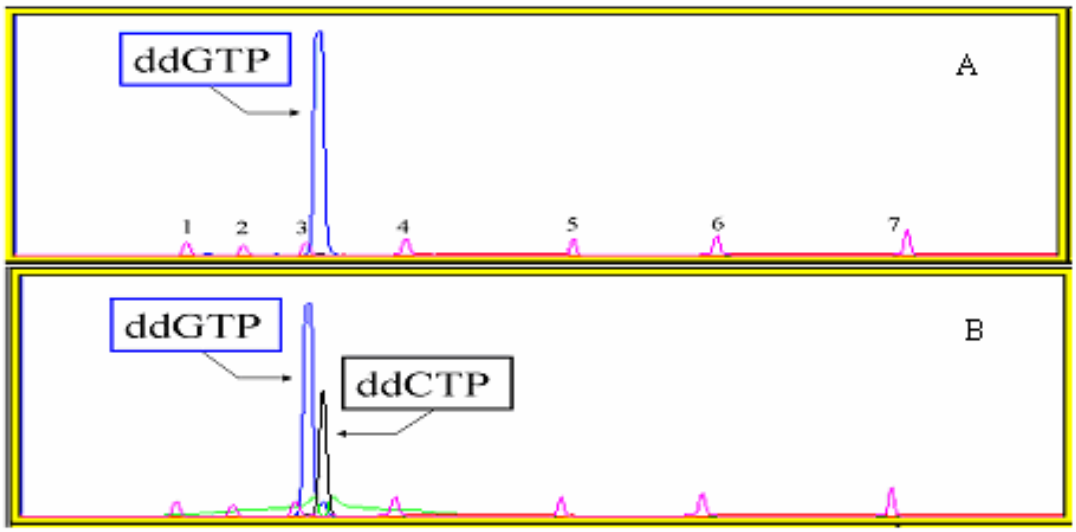

Fig. (4): SNaPshot™ results of some Palestinian cultivars when applied on their leaf DNA. A*) Lup2-1* SNP position on PA5; B) *Lup2-1* SNP position on PA3. Small serial peaks refer to the GeneScanTM-120 LIZTM size standard with the following size: 1) 15 nt; 2) 20 nt; 3) 25 nt; 4) 35 nt; 5) 50 nt; 6) 62 nt; 7) 80 nt; 8) 110 nt long.

Note: The ABI SNaPshot™ result files were viewed with the GeneScanView 1.2_4. A software developed by Davide Campagna at the University of Padova, Italy (http://www.bmr-genomics.it/), which is freely available from the internet.

Table (6): Genotyping summary of the Palestinian cultivars using a combination of three SNPs.

Variety Code	Lup2-1	Lup2-2	Cyc2-2
PA1	GG	CC	AT
PA2	GC	CC	AT
PA3	GC	CC	AA
PA4	GC	CC	AA
PA5	GG	TT	AA

CONCLUSIONS AND RECOMMENDATIONS

In the current work, the discovered set of twenty-five single nucleotide polymorphisms is not effective enough to discriminate among the five olive cultivars indigenous to Palestine. For example, among them both Souri and Roomi are indistinguishable. As a result, more SNPs need to be discovered to prove if those two cultivars are alike or different and to genotype the rest of the Palestinian cultivars, which means that more fragments of the same genes or other genes should be involved in future studies. In addition, the allelic variants which were not recognized in some cultivars as a result of inappropriate sequencing signals should be carried out for the completion of the database.

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البصمة الوراثية لبعض أصناف الزيتون الفلسطينية عن طريق معرفة الاختلافات بينها على مستوى النيوكليوتيدة الواحدة (SNP)

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ملخـص

تعتبر دراسة العلاقات الوراثية بين أصناف الزيتون الفلسطينية ذات أهمية عالية في تحديد التنوع الجيني والتركيبي في أشجار الزيتون. ولتحقيق ذلك، تم في هذه الدراسة جمع عينات من أوراق الزيتون من الأصناف: رومي، صوري، نبالي محسن، برنجي K18، بالإضافة إلى صنف الزيتون البري (داعي استخدامه هو معرفة بعد مادته الوراثية عن الأصناف المزروعة ومعرفة ما إذا كان منها ما نتج من تهجينه مع صنف آخر)، حيث تم تحليل العينات باستخدام المتتاليات لقطعتين من الجينات التي احتوت على مجموعة من الاختلافات على مستوى النيوكليوتيدة الواحدة.

أظهرت النتائج أن هذه المجموعة من الاختلافات غير كافية للتمييز بين الأصناف الخمسة المدروسة، لكنها ذات قيمة عالية في التمييز الوراثي بين هذه الأصناف من ناحية وفي تشكيل قاعدة بيانية حقيقية من ناحية أخرى، وكذلك توفير معلومات إضافية قد تفيد في وضع الأسس العلمية الصحيحة لبرامج تحسين أشجار الزيتون.

الكلمات الدالة: البصمة الوراثية، SNP، الزيتون.

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