DNA Fingerprinting of Two Oil Olive (*Olea europaea* L) Based on Different Molecular Markers

[1]Zahraa S. Sultan, [1]Ahmed E. Abdalla, [2]Nader R. Abdelsalam
[1]Department of Food Science, Faculty of Agriculture (Saba Basha), Alexandria University,
[2]Agricultural Botany Department, Faculty of Agriculture (Saba Basha), Alexandria University, 21531 Alexandria, Egypt (nader.wheat@alexu.edu.eg)

ABSTRACT: Olive (*Olea europaea* L.) is an economically significant fruit and oil crop in the Mediterranean region where it has been cultivated since ancient times. Two virgin olive oils were obtained from two olive cultivars i.e. “Wetagen” and “Chemlali” collecting from 15 different plants localized in Marsa Matroh and Siwa Oasis. DNA was extracted and three molecular techniques i.e. RAPD, SSR and AFLP were used to made DNA fingerprinting and calculate the genetic similarity between both oil cultivars. Data showed that, genetic similarity between the both oil cultivars was 0.13% based on the eight RAPD primers, 0.07% for AFLP primers and 0.03% for SSR markers. From these data we have been able to use these markers with oil to fingerprint and differentiate olive oils in markets. The differences found in the DNA patterns can be used as a characteristic fingerprint of each oil with the certainty that these amplifications are only obtained with olive DNA and not with DNA from any other contaminant origin.
Key words: Olive oils, RAPD, SSR, AFLP, Fingerprint

INTRODUCTION

Olive tree (2n = 46) is an ancient woody crop with more than 46,000 years of cultural history, and is mainly spread around the Mediterranean basin. In recent decades, olive trees have been introduced and cultivated in the United States, Australia, South Africa, China and in other countries (Breton *et al.,* 2008). Olive has become one of the major oil crops in some countries because it can produce a high-quality oil with great economic value (Bandelj *et al*., 2004). This species is a member of the family *Oleaceae*, which contains about 30 genera and 600 species (Bracci *et al*., 2011). Olive has been one of the most diverse tree species with about 1200 olive cultivars grown worldwide (Bartolini *et al*., 1998). At present, olive cultivars are identified using biochemical and molecular markers such as isoenzymes (Lumaret *et al*., 2004), RAPDs (Martins-Lopes *et al*., 2008), AFLPs (Montemurro *et al.,* 2008), SSRs (Sefc *et al.,* 2000; Cipriani *et al*., 2002; Erre *et al*., 2010), SCARs (Pafundo *et al*., 2007), and SNPs (Belaj *et al*., 2012). Molecular markers are valuable tools that can be used to study olive genetics, and have been applied to the identification of cultivars. For instance, ISSR and SSR markers were used to analyze 41 olive cultivars and resulted in an efficient distinction of each accession (Gomes *et al*., 2009). Virgin olive oils are the oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration. International Olive Council (2013) has defined the quality of olive oil based on parameters that include free acidity, peroxide value (PV), UV absorption

(K232nm and K270nm) and sensory analysis. Fatty acids composition which is a purity parameter for olive oil is strongly affected by environmental conditions (Mousa *et al*., 1996). It is worth to mention that Arbequina cv. under Egypt conditions showed low oleic acid content (44.00%) so it considered out of the limit (55-83%) establishment by the International Olive Council (Benincasa *et al*., 2011). Matteo *et al*. (2003) reported that DNA recovery from food samples might be of great importance when the raw material used in the production process must be traced. They were interested in verifying the presence of nucleic acids in extra virgin olive oil to determine the cultivar of origin of the olives used for the production. They defined a reliable DNA extraction method for extra virgin olive oil, as far as both quantity and quality, and the possibility of using this DNA for fingerprinting. DNA extraction was tested on four mono variety oils, plus four commercial extra virgin olive oils. The DNA in the extracted solution was of chloroplast and nuclear origin since we could amplify cloned cultivar RAPD and AFLP fragments homologous to nuclear DNA of other species. It has also been shown that DNA purified from oil can be used for AFLP analysis and that the profile of the DNA purified from a mono variety oil corresponds to the profile of the DNA purified from the leaves of the same cultivar.

MATERIALS AND METHODS

A set of two olive cultivars were collecting from 15 different plants localized in Marsa Matroh and Siwa oasis (Siwa: Ain Ragwa, Dakrour, Kadwsa, Mishandid and Maraqi; Marsa Matroh: Elnegala and Marsa Matroh city) used to obtain the virgin oil. DNA was extracted according to Fernando *et al.,* (2004) Eight RAPD-PCR has been developed, in which DNA is amplified by the polymerase chain reaction (PCR) using arbitrary short (10 nucleotides) primers (Williames *et al*., 1990). RAPD were used in the current study recorded in Table (1).

Table (1). Primers name, and nucleotide sequences of primers used for RAPD analysis

Code	Primer	Sequence (5`-3`)
1	OPA-05	AGG GGT CTT G
2	OPA-10	GTG ATC GCA G
3	OPA-15	GTC GTA GCG G
4	OPB-07	GGT GAC GCA G
5	OPB-17	AGG GAA CGA G
6	OPC-12	TGT CAT CCC C
7	OPD-04	TCT GGT GAG G
8	OPD-11	AGC GCC ATT G

Decamers with a GC content of 60% were obtained from Operon Technologies (Table 1). The eight primers were selected from the Operon Kit (Operon Technologies Inc., Alabameda, CA). Reactions were carried out in 25µl containing 67mM Tris-HCl pH 8.8; 16.6mM $(NH_4)_2SO_4$; 0.01% Tween-20; 2mM $MgCl_2$; 0.1mM each of dNTP; 0.4mM primer; 0.5µ of Eco *Taq* and 20ng genomic DNA. Amplification reactions were performed in a Biometra *T1* gradient thermalcycler programmed for 1 cycle of 1min at 94°C followed by 35 cycles of 1min at 94°C; 1min at 36°C and 1.5min at 72°C for denaturing; annealing and primer extension phases, respectively. The last cycle was followed by 7 minutes at 72°C (Bautista *et al.,* 2003). Amplification products were separated on 1% agarose gels at 100 volts for 1.30 hrs., with 1x TBE buffer. Agarose gels were examined on ultraviolet transilluminator (302nm wavelength) to detect the ethidium bromide/DNA complex and photographed. DNA fragment lengths were determined by comparisons with 100pb DNA ladders (V-gene Biotechnology Limited, shiqao, P.R. China) run on each gel. The reproducible DNA bands from two runs were scored for their presence or absence in each genome. AFLP procedure was applied according to Angiolillo *et al.* (1999). Genomic DNA (500ng) was digested with two restriction enzymes (*Eco*RI and *Mse*I) for 14–16h at room temperature and ligated to double-stranded *Eco*RI and *Mse*I adapters. The ligates were pre-amplified with pre-selective primers using 1 cycle of 2 minutes at 72°C and 30 cycles, each consisting of 30sec at 94°C; 60sec at 56°C and 1min at 72°C. A final cycle was performed for 1min minutes at 60°C. For the selectively amplifications, three sets of AFLP *Eco*RI and *Mse*I primers (Table 2) were used. For each reaction, 15µl super-hot start Master Mix; 1µl *Eco*RI primer and 1µl *Mse*I primer were mixed. The PCR program consisted of an initial warm at 94ºC for 1 min then one cycle at 94ºC for 30 sec; 66ºC for 1 min and 72ºC for 1 min, followed by 10 subsequent cycles each at 1ºC and finally 25 cycles at 94ºC for 30sec; 56ºC for 1min and 72ºC for 1min. A final cycle was performed at 60ºC for 30min. The PCR products were separated on 6% polyacrylamide gels; stained with silver staining and photographed. DNA fragment lengths were determined by comparisons with 100pb DNA ladders run on each gel.

Table (2). The nucleotide sequences of primers used for AFLP analysis

Primer code	Sequence (5`-3`)
*Eco*RI- ACA & *Mse*I-CAT (A)	5'-GACTGCGTACCAATTC ACA-3' 5'-GATGAGTCCTGAGTAA CAT-3'
*Eco*RI-AAG & *Mse*I-CTG (B)	5'-GACTGCGTACCAATTC AAG-3' 5'-GATGAGTCCTGAGTAA CTG-3'
*Eco*RI-ACT & *Mse*I-CAT (C)	5'-GACTGCGTACCAATTC ACT-3' 5'-GATGAGTCCTGAGTAA CAT-3'

For Simple Sequence Repeat (SSR) analysis Genomic DNA was extracted from 80 mg of oil with the DNeasy® Plant Mini Kit (QIAGEN™, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were estimated by using a Gene Quant (Amessham pharmacia Biotech). The PCR conditions described by Cipriani *et al.* (2002) were used for the amplifications of the eight SSR (Table 3) primer pairs (Sefc *et al.*, 2000). Amplification reactions were performed in a final volume of 25µl in the presence of 20ng template DNA, 4pmol each primer, 0.2mM of each dNTP, 2mM $MgCl_2$, and 1U *Taq* polymerase (Sigma). Polymerase chain reaction (PCR) was carried out using a Biometra *T1* gradient thermalcycler (Biometra biomedizinische, Germany). After 5 min at 94°, 30 cycles were performed with 30s at 94°C, 30s at either 50 or 56 or 60°C and 30 s at 72°C, followed by a final extension step of 5 min at 72°C. The amplification products were resolved by electrophoresis in a 1.8% agarose gel in TAE buffers and revealed under UV illumination by ethidium bromide staining (Carriero *et al.*, 2002). RAPD's, AFLP's and SSR's fragments were scored as present/absent. Fragment scoring, and lane matching were performed automatically on digital images of the gels, using Phoretix 1D advanced Version 4.00 (Phoretix International, Newcastle upon Tyne, UK). All but the faintest bands were scored, where necessary scores and matches were corrected manually. Clustering methods and similarity coefficients were tested using the procedures SIMQUAL, SAHN, and TREE from the program NTSYSpc version 2.10 (Applied Biostatistics, Setauket, New York, USA). The clustering methods UPGMA, WPGMA, Complete-link, and Single-link were applied in all possible combinations with the similarity coefficients Dice, Jaccard and Simple matching. Rohlf (2000) describes clustering methods and similarity coefficients.

Table (3). Repeat motifs, primer sequences and annealing temperatures (Ta) microsatellite loci analyzed in Wetagen and Chemlali olive cultivars

Locus	Repeat motif	Primer sequence (5'–3')	*A.T*
DCA04	$(GA)_{16}$	CTT AAC TTT GTG CTT CTC CAT ATC C AGT GAC AAA AGC AAA AGA CTA AAG C	55°C
DCA05	$(GA)_{15}$	AAC AAA TCC CAT ACG AAC TGC C CGT GTT GCT GTG AAG AAA ATC G	50°C
DCA07	$(AG)_{19}$	GGA CAT AAA ACA TAG AGT GCT GGG G AGGGTAGTCCAACTGCTAATAGACG	60°C
DCA13	$(GA)_{15}$	GAT CAG ATT AAT GAA GAT TTG G AAC TGA ACC TGT GTA TCT TGC ATC C	55°C
DCA16	$(GT)_{13}(GA)_{29}$	TTAGGTGGGATTCTGTAGATGGTTG TTTTAGGTGAGTTCATAGAATTAGC	50°C
DCA17	$(GT)_9(AT)_7AGATA(GA)_{38}$	GATCAAATTCTACCAAAAATATA TAATTTTTGGCACGTAGTATTGG	50°C

RESULTS AND DISCUSSION

A- Random amplified polymorphism DNA (RAPD):

The results of primer OPA-05 are presented in Table (4). The total fragments were 13 amplification products at the fragment lengths ranged between 150 bp to 1550 bp. For the first oil olive “Wetagen”, four of six fragments were monomorphic (150, 460, 900 and 1550 bp) and two fragments were polymorphic (350 and 700 bp). The percentage of the polymorphism was 33%. While for oil olive “Chemlali” four of seven fragments were monomorphic, and three fragments were polymorphic (290, 580 and 1400 bp). The percentage of the polymorphism was 43%. The results of primer OPA-10 are found in Table (4). The total fragments were 5 amplification products at the fragment lengths ranged between 400 bp to 900 bp. For oil olive “Wetagen”, two of three fragments were monomorphic (590 and 900 bp) and one fragments were polymorphic (400 bp). The percentage of the polymorphism was 33%. While for oil olive “Chemlali”, zero polymorphic amplification fragments were detected. The percentage of the polymorphism was zero. The results of primer OPA-15 showed that the total fragments were 9 amplification products at the fragment lengths ranged between 400 bp to 2100 bp. For “Wetagen” two of four fragments were monomorphic (350 and 1400 bp) and two fragments were polymorphic (2000 and 2100 bp). The percentage of the polymorphism was 50%. While for “Chemlali” two of five fragments were monomorphic, and three fragments were polymorphic (550 and 1500 bp). The percentage of the polymorphism was 60%. The results of primer OPB-07 showed that a total fragment were 7 amplification products at the fragment lengths ranged between 300 bp. to 1300 bp. For the oil olive “Wetagen” one of four fragments were monomorphic (400 bp) and three fragments were polymorphic. The percentage of the polymorphism was 75%. In “Chemlali” one of three fragments were monomorphic, and two fragments were polymorphic. The percentage of the polymorphism was 67%. The results of primer OPA-17 are found in Table (4). The total fragments were 11 amplification products at the fragment lengths ranged between 400 bp. to 3000 bp. For “Wetagen” one of five fragments were monomorphic (1600 bp) and four fragments were polymorphic. The percentage of the polymorphism was 80%. Also, for “Chemlali” one of six fragments were monomorphic, and five fragments were polymorphic. The percentage of the polymorphism was 83%. The primer OPC produced 9 fragments amplification products at the fragment lengths ranged between 500 bp to 2000 bp. For the “Wetagen” three of four fragments were monomorphic (500, 700 and 1350 bp) and one fragments were polymorphic (900 bp). The percentage of the polymorphism was 25%. While for and oil olive “Chemlali” three of five fragments were monomorphic, and two fragments were polymorphic (600 and 2000 bp). The percentage of the polymorphism was 40%. The total fragments for OPD-04 were 4 amplification products at the fragment lengths ranged between 400 bp. to 500 bp. For the first and second oil olive no polymorphic fragments were detected, and the percentage of the polymorphism was zero. The results of primer OPD-11

presented in Table (4). The total fragments were 10 amplification products at the fragment lengths ranged between 190 bp. to 1500 bp. For “Wetagen” three of four fragments were monomorphic, and one fragment was polymorphic (600 bp). The percentage of the polymorphism was 25%. While for “Chemlali” three of six fragments were monomorphic, and three fragments were polymorphic. The percentage of the polymorphism was 50%. Data in Table (٤) showed that the total amplification fragments for “Wetagen” were 32, 14 of them was polymorphic by 43.75% and for the second one was 36, 18 of them were polymorphic by 50%. The total fragments were 68 and the general genetic polymorphism was 47.05%. Data in Table (٥) for genetic similarity showed that the genetic similarity between the both oil cultivars was 0.13% based on the eight RAPD primers. Plant molecular geneticists are currently used RAPD markers routinely to identify genetic variations (Perron *et al.,* 1995; Lashermes *et al.* 1996; Irwin *et al.,* 1998 and Sun *et al.,* 1998). RAPD markers have been also used successfully in various taxonomic and Phylogenetic studies (Kazan *et al.,* 1993 and Wilkie *et al.* 1993). In addition, it locates regions of the genome linked to agronomically important genes (Reiter *et al.,* 1992; Martin *et al.,* 1991; Michelmore *et al.,* 1991; Pillay and Kenny 1996). Furthermore, it facilitates introgression of desirable genes into commercial accessions (Lavi *et al.* 1994).

Table (4). Number of amplified and polymorphic fragments for Wetagen and Chemlali olive oil based on RAPD-PCR

Cultivars		Primers								Total
			OPA-10	OPA-15	OPB-07	OPB-17	OPC-12	OPD-04	OPD-11	
Wetagen	**AF**	6	3	4	4	5	4	2	4	**32**
	PF	2	1	2	3	4	1	0	1	**14**
Chemlali	**AF**	7	2	5	3	6	5	2	6	**36**
	PF	3	0	3	2	5	2	0	3	**18**
Total	**TAF**	13	5	9	7	11	9	4	10	**68**
	TPF	5	1	5	5	9	3	0	4	**32**

- AF= No. Amplified Fragments, PF= Polymorphic Fragments, TAF= Total no. Amplified Fragments. TPF= Total no. Polymorphic Fragments.

Table (5). Similarity indices (%) calculated by NTSYS program among the Wetagen and Chemlali olive oil based on RAPD-PCR analysis

	Wetagen
Chemlali	0.13

b- Amplification Fragments Length Polymorphism (AFLP)

The results of primer *EcoRI*- ACA and *MseI*-CAT (A) are found in Table (6). The total fragments were 68 amplification products at the fragment lengths ranged between 90 bp to 3100 bp. For "Wetagen" 21 of 39 fragments were monomorphic and 18 fragments were polymorphic. The percentage of the polymorphism was 46%. Also, for "Chemlali" 21 of 29 fragments were monomorphic and 8 fragments were polymorphic. The percentage of the polymorphism was 27%. The results of primer *EcoRI*- AAG and *MseI*- AAG (B) in Table (6). The total fragments were 24 amplification products at the fragment lengths ranged between 110 bp to 2100 bp. For the first oil olive "Wetagen" 7 of 11 fragments were monomorphic and four fragments were polymorphic. The percentage of the polymorphism was 36%, for "Chemlali" 7 of 13 fragments were monomorphic and 6 fragments were polymorphic. The percentage of the polymorphism was 46%. The results of primer *EcoRI*- ACT and *MseI*- CAT (C) are presented in and Table (6). The total fragments were 28 amplification products at the fragment lengths ranged between 90 bp to 3200 bp. For "Wetagen" 7 of 13 fragments were monomorphic and six fragments were polymorphic. The percentage of the polymorphism was 46%, for "Chemlali" 7 of 15 fragments were monomorphic and eight fragments were polymorphic. The percentage of the polymorphism was 53%. Data in Table 6 showed that the total amplification fragments for "Wetagen" were 73, 10 of them was polymorphic by 13.69% and for the second one "Chemlali" were 75, 12 of them were polymorphic by 16%. The total fragments were 148 and the general genetic polymorphism was 14.86%. Data in Table (7) for genetic similarity showed that the genetic similarity between the both oil cultivars was 0.07% based on the three AFLP primers.

Table (6). Number of amplified and polymorphic fragments for the Wetagen and Chemlali olive oil based on AFLP analysis.

Cultivars		Primers			Total
		A	B	C	
Chemlali	**AF**	39	11	73	73
	PF	18	4	10	10
Wetagen	**AF**	29	13	75	75
	PF	8	4	12	12
Total	**TAF**	68	24	148	148
	TPF	26	8	22	22

- AF= No. Amplified Fragments, PF= Polymorphic Fragments, TAF= Total no. Amplified Fragments, TPF= Total no. Polymorphic Fragments, A: primer combination *Eco*RI-ACA/*Mse*I-CAT, B: primer combination *Eco*RI-AAG/*Mse*I-CTG, C: primer combination *Eco*RI-ACT/*Mse*I-CAT.

Table (7). Similarity indices (%) calculated by NTSYS program among the Wetagen and Chemlali olive oil based on AFLP analysis

	Wetagen
Chemlali	0.07

c- Simple Sequence Repeat (SSR)

Primer DCA04 produced two detectable alleles in both olive oil (Table, 8) the length ranged from 90 to 150 bp, all the alleles were polymorphic, and no monomorphic ware detect. The total amplification alleles were four and both olive oil gave the same number of alleles for DCA13 were 2 as shown in Table (8). DCA05 primer detects one allele for Wetagen and two alleles for Chemlali. While the DCA16 showed just one allele for both olive oil cultivars. The lengths for both primers ranged from 195 and 140 to 229, in respect. DCA17 and DCA07 showed that these two primers showed two alleles for the current samples ranged in length from 100, 110 to 121 and 150, respectively. Data in Table (8) showed that he average numbers of alleles was 2.83 ranged from the lowest one allele in DCA16 to the highest four alleles in DCA04 and DCA13. Based on the data in Table (9) the genetic similarity between both olive oil was 0.03%. Finally, the previous data for RAPD, AFLP and SSR showed that the genetic similarity between the “Chemlali” and “Wetagen” oil was 0.17% (Table, 10) and this due to that both olive cultivars growing at the same area and same conditions. But we can conclude that these three different techniques can draw DNA fingerprint for both olive oil. Molecular markers based on PCR amplification are widely used and their number is continuously increased. This is due to their capability to detect extremely low amounts of DNA and the easiness of use. Currently, these markers are tested against genomic DNA for plant breeding programs, genetic relations, or identification of varieties or species for legal rights or scientific knowledge. We have been able to use these markers with oil to fingerprint and differentiate olive oils in markets. The differences found in the DNA patterns can be used as a characteristic fingerprint of each oil with the certainty that these amplifications are only obtained with olive DNA and not with DNA from any other contaminant origin.

Table (8). List of the Amplified SSR Alleles

Locus	Range of Allele sizes (bp)	No. of alleles
ssrOeUA-DCA04	090-150	4
ssrOeUA-DCA05	195-229	2
ssrOeUA-DCA07	100-121	3
ssrOeUA-DCA13	93-161	4
ssrOeUA-DCA16	140	1
ssrOeUA-DCA17	110-150	3
Average		**2.83**

Table (9). Similarity indices (%) calculated by NTSYS program among the Wetagen and Chemlali olive oil based on SSR -PCR analysis.

	Wetagen
Chemlali	0.03

Table (10). Similarity indices (%) calculated by NTSYS program among the "Wetagen" and "Chemlali" olive oil based on combined data analysis.

	Wetagen
Chemlali	0.17

CONCLUSION

Molecular markers as a tool could be helpful to screening the olive oil by DNA fingerprint and differentiate the olive oils in markets. The differences found in the DNA patterns can be used as a characteristic fingerprint of each oil with the certainty that these amplifications are only obtained with olive DNA and not with DNA from any other contaminant origin.

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الملخص العربى

البصمة الوراثية لصنفين زيت زيتون إعتمادا على معلمات جزيئية مختلفة

زهراء سعيد سلطان[١] – احمد السيد عبد الله[١] – نادر رجب عبد السلام[٢]

[١]قسم علوم الاغذية – كلية الزراعة سابا باشا – جامعة الاسكندرية ، [٢]قسم النبات الزراعى – كلية الزراعة سابا باشا– جامعة الاسكندرية

يعتبر زيت الزيتون من أجود أنوع الزيت عالميا لما لة اهمية اقتصادية وتغذوية عالية. تم خلال هذة التجربة إستخدام صنفين من زيت الزيتون البكر هما وطيقين وشيملالى وذلك بغرض عمل البصمة الوراثية لتلك الزيوت باستخدام مجموعة من التكنيكات المختلفة مثل القطع العشوائية وتكرار التراكيب البسيطة والتعدد فى أطوال القطع الناتجة. وتم عزل المادة الوراثية لكلا من الصنفين وإختبرت بالتكنيكات السابقة. أوضحت النتائج أن هناك إختلاف فى الأشكال المظهرية بين نوعى الزيت المستخدم وتم تحديد عدد من المواقع والأليلات المتخصصة التى تميز كل صنف عن الاخر الأمر الذى قد يعتبر مرجعا حال الكشف عن جودة الزيوت المباعة فى المحلات التجارية بالاعتماد على تلك الواسمات الجزيئية.