MOLECULAR CHARACTERIZATION OF SOME EGYPTIAN OLIVE (*Olea europaea* L.) CULTIVARS AND THEIR TAXONOMIC RELATIONSHIP

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Olive belongs to the genus *Olea*, which is included within the Oleideae sub-family and in the Oleaceae tribe. Approximately, there are 30-40 *Olea* species, which are distributed in Oceania, Asia, Africa, and the Mediterranean region (Johanson, 1957). The genus is divided into two sub-genera, *Olea* and *Paniculatae*; sub-genus *Olea* is divided into 2 sections, *Olea* (including cultivated olive and its wild relatives) and *Ligustroides*. The section *Olea* includes the complex of *Olea europaea* L., the Mediterranean olive tree (Green and Wickens, 1989). The latter authors distinguished four sub-species according to their morphology and geographical distribution: 1) *O. europaea* subsp. Europaea, of the Mediterranean Basin; 2) *O. europaea* ssp. Laperrinei (Batt. and Trab.) Ciferri, of the Sahara Massifs; 3) *O. europaea* ssp. Cerasiformis (Webb. and Berth.) Kunk and sund of the Canary Islands and Madeira; 4) *O. europaea* ssp. Cuspidate (wall. Ciferri) of Asia (China, India, Pakistan, Iran and South Arabia) and Southeast Africa. *Olea europaea* subspecies *europaea* includes the cultivated varieties (var. europaea) and wild plants (var. sylvestris = Oleaster) of the Mediterranean Basin. Green and Wickens (1989) and Zohary and Spiegel (1975) hypothesized that the different wild forms of the *O. europaea* complex all have contributed to the evolution of cultivated olive.

Olive is a diploid species (2n=2x=46), but some tetraploid plants have been produced artificially (Rugini *et al*., 1996). The DNA content has been estimated for some cultivars to be approximately 2.2 pg (Rugini *et al*., 1996), corresponding to a genome size of 2,200 Mb (De la Rosa *et al*., 2003).

Over the centuries, hundreds of olive cultivars were selected for their qualitative and quantitative traits and for their adaptation to various microclimates in different regions of the Mediterranean basin (Bartolini *et al.*, 1993). However, the existences of synonyms and homonyms have resulted in considerable uncertainly about the current status of olive cultivars in many countries (Connell, 1994).

Wild plants occur in the same areas as the domesticated olive, in the maquis and in uncultivated sites and show some morphological differences with cultivars, i.e., smaller fruit size and lower oil content in the mesocarp (Terral and Arnold-Simard, 1996). Two distinct wild olives have been recognized: *Oleaster* and *feral* forms. *Oleaster* occupies primary niches in undisturbed area (Liphschitz *et al*., 1991) as a constituent of evergreen plant associations. Numerous studies confirm that olive is continuously present in the Mediterranean Basin for several thousand years, particularly in the Middle East, long before its domestication through palynological, anthropological and archeological evidences. Carrion and Dupre (1996) demonstrate the presence of some forms of olive during the last glaciations (18,000 B.C.) in the western and eastern Mediterranean regions.

Recently, Random Amplified Polymorphic DNA (RAPD) analysis described by Williams *et al*. (1990) and Welsh and McClelland (1990) have proven to be a useful tool for genetic typing and mapping. By using the genetic maps, it is possible to evaluate the correspondence of gene regulating phenotype in different populations (Mitton, 1994). The main advantage of RAPD markers over other molecular markers, in particular to markers involving DNA-DNA hybridization technique is the low technical input and small quantity of plant DNA needed for the analysis (Debener *et al*., 1996). Also use of this technique for olive cultivar studies has shown that RAPD analysis can be usefully applied in diagnosis of cultivar-species markers (Fabbri *et al*., 1995), diversity analysis of olive (Sanz-Cortes *et al*., 2001) and identification of genetic markers linked to suitable traits (Mekuria *et al*., 1999).

The aim of present study was to reveal the genetic characterization and relationships among four olive cultivars (Chemlali, Kalamata, Toffahi and Yonani) grown in Borq El-Arab region and six olive cultivars (Aspany, Hamady, Kosha, Marake, Petagn and Toffahi), in addition to a wild type grown in Siwa oasis by using RAPD markers as a commonly, fast and low-cost molecular marker technique.

MATERIALS AND METHODS

Plant materials

Field work was carried out for four olive cultivars (Chemlali, Kalamata, Toffahi and Yonani) collected from Borq El-Arab region, six cultivars (Aspany, Hamady, Kosha, Marake, Petagn and Toffahi) and wild type from Siwa Oasis, Egypt.

Methods

DNA extraction procedure

Collect unexpanded young leaves in liquid nitrogen or on ice and store at below -70°C until used. Grind 0.5 g of leaves into a very fine powder using mortar and pestle in the presence of liquid nitrogen. Add 5 ml of extraction buffer to

the ground leaves and mix in the mortar. Pour the slurry into clean 15 ml polypropylene centrifuge, rinse the mortar and pestle with 1 ml of extraction buffer and add to the original extract. Add 50 mg polyvinylpolypyrrolidone (PVP) and invert the tubes several times to mix thoroughly with the leaf slurry (100 mg PVP/g leaf tissue). Incubate at 60°C for 25 min and cool to room temperature. A volume of 500 SDS (20%) was added, incubated in water bath at 65°C for 20 min, followed by 15 min on ice, then centrifuged at 10.000 rpm, at 4°C for 10 min. Add 6 ml of chloroform : octanol and mix gently by inverting the tubes 20 to 25 times to form an emulsion. Spin at 6000 rpm for 15 min at room temperature. Transfer the top aqueous phase to a new 15 ml centrifuge tube with a wide-bore pipette tip. Add 0.5 volume of 5M NaCl to the aqueous solution recovered from the previous step and mix well. Add two volumes of cold (-20°C) 95% ethanol and refrigerate (4 to 6°C) for 15-20 minutes or until DNA strands begin to appear. Spin at 3000 rpm for three min and then at 5000 rpm for an additional three min at room temperature. Pour off supernatant and wash pellet with cold (0 to 4°C) 76% ethanol. Completely remove ethanol without drying the DNA pellet by leaving the tubes uncovered at 37°C for 20 to 30 minutes. Dissolve in 200 to 300 µL TAE. Treat with 1 µL RNAase A and 1 µL Proteinase K per 100 µL DNA solution and incubate at 37°C for 15 minutes. The DNA solution was then mixed with an equal volume of phenol-chloroform (1:1 v/v), vortexes briefly and centrifuged at 1400 rpm for 10 minutes. The DNA in the upper layer was reprecipitated over night at -20°C with 0.1 volume 2 M sodium acetate and 2 volumes absolute ethanol then centrifuged at 14000 rpm for 10 minutes and washed with 90% ethanol. The DNA was redissolved in 500 µL TAE. Quantify DNA in a spectrophotometer at A260. Keep DNA at -70°C for long term storage (Aitchitt *et al.,* 1993).

Polymerase chain reaction (PCR) conditions

Random Amplified Polymorphic DNA (RAPD) analysis described by Williams *et al*. (1990) was used. RAPD analysis was carried out using 10 (10-mer) oligonucleotide primers synthesized at (AGERI), ARC, Giza, Egypt, on an ABI 392 DNA/RNA Synthesizer Applied Biosystems, (Table 1). Polymorphisms detected as the presence or absence of a particular RAPD band. Amplification was performed in 25 µl reaction volume containing; Template (target DNA, about 10-100 ng) Oligonucleotides primers (5 µm), Taq polymerase (5 U µl-1), Taq polymerase buffer (10 x), MgCl2 (3 mM), dNTP (0.4 mM), Agarose, 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA), DNA molecular marker standards, Sterile distilled water (SDW), Gel loading buffer, Ethidium bromide (0.5 m g m l-1), 25 µl of mineral oil (Sigma).

The conditions for PCR amplification were: 40 cycles 1 minute at 94°C, 3 minutes at 37°C, 1 minute at 72°C, One cycle 10 minutes at 72°C, Hold at 4°C before analysis.

Gel electrophoresis of PCR products

Following amplification analyses PCR products by gel electrophoresis along side DNA ladder size standard on 1.5 % (w/v) agarose gel in the presence of ethidium bromide and visualized under UV light and photographed. DNA fragment sizes were determined by comparisons with the 100 bp+1.5 kb DNA Ladder marker.

Data analysis

RAPD bands were scored as 1 (present) or 0 (absent) in a binary matrix for each primer. A conservation criterion for the selection of bands was used. Only reproducible and well-defined bands were considered as potential polymorphic markers. The identification of olive cultivars was carried out by the combination of different primers (Belaj, 2004). For the numerical analysis the NTSYS – pc, version 1.50 program (Rohlf, 1989) was used. The following steps were carried out for each data matrix: Average taxonomic distance (DIST) was generated by using the similarity of interval data program (SIMINT) in order to measure the dissimilarity between cultivars. Clustering was performed using the unweighted - pair-group method with arithmetic mean (UPGMA) by using sequential, agglomerative, hierarchial and nested clustering method (SAHN) as defined by Sneath and Sokal (1973). The output of SAHN-clustering program was presented in the form of a phenogram by using the tree display graphic (TREEG).

RESULTS AND DISCUSSION

RAPD analysis technique has been used extensively for varietal identification, phylogenetic relationships, parentage determination and marker assisted selection in a wide range of plant species because of its simplicity.

Figure (1) shows PCR-RAPD pattern of different olive cultivars: six from Siwa region (Aspany, Hamady, Kosha, Marake, Petagn and Toffahi), four from Borq El-Arab region (Chemlali, Kalamata, Toffahi and Yonani) and the wild type of Siwa Oasis, Egypt, while, Table (2) shows the unique positive and negative RAPD markers respectively for the same olive cultivars characterizations.

RAPD assay permitted the identification of the ten olive cultivars besides wild type by unique positive and negative markers. Twenty-one positive unique RAPD markers were identified to characterize the selected cultivars. Forty-three negative unique RAPD markers were identified to characterize the selected cultivars. Certain primers were more informative than the others e.g., OPB-12 and OPC-05 which, together, had the potential to identify eight and seven respectively. In this respect, Radwan (2004) found that twenty-three positive unique RAPD markers were identified to characterize seven olive cultivars. Nevertheless, negative RAPD markers were also able to characterize the selected olive cultivars. Certain primers were more informative than the others e.g., OPA-01 and OPG-16 that, together, had the

potential to identify four cultivars.

Hu and Quiros (1991) stated that Broccoli and Cauliflower could be distinguished by using only two and three arbitray primers, respectively. Similarity, Wolf and Rijn (1993) demonstrated that, only two primers were necessary to distinguish 18 *Chrysanthemum* cultivars. Aruna *et al*. (1995) presented a key for 7 blackberry genotypes based on 11 markers amplified by four primers.

The presence of unique RAPD markers among the various olive cultivars indicates the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications including the determination of cultivar's purity, efficient use and management of genetic resources collection, particularly in identification of mislabeled accessions (Ahmed, 1999).

The output of SAHN-clustering program was presented in the form of a phenogram by using the tree display graphic (TREEG). The resulting dendrogram, (Fig. 2), shows that cultivar 1 (Siwa Toffahi) is separated from other olive cultivars it may be referred to changing of environmental factors which made Siwa Toffahi is different from Borq El-Arab Toffahi. The remaining cultivars were differentiated into two main groups cultivar 4 (Kosha) and other cultivars which divided into two groups; group 1 includes wild and Hamady and group 2 includes two subgroups; subgroup1 includes Chemlali and Kalamata and subgroup2 includes other cultivars.

The dendrogram shows that the studied cultivars have an average taxonomic distance of about 0.52. At this level, the branch of cultivar 1 (Siwa Toffahi) is split off from the cluster whose the branch of cultivar 4 (Kosha) is split off at a distance of about 0.45. At a distance close to 0.42 a two cultivars were also delimited as one group these are cultivars wild and Hamady, which are split off at the 0.33 level. At a distance level of about 0.40 cultivars Chemlali and Kalamata were also delimited from the remaining cultivars and regarded as distinct groups which are split off at the 0.30. At a distance level of about 0.35 the cultivar 8 (Borq El-Arab Toffahi) is separated as a single taxa.

The remaining is split at a distance of about 0.28 into a branch of cultivar 7 (Aspany) and a subgroup which is delimited in two cluster, one includes cultivar 11 (Yonani) at a distance of about 0.26 and the second includes cultivar 5 (Petagn) and 6 (Marake) as one subgroup at a distance of 0.14. Radwan, (2004) found that the genetic similarity ranged from 65.5% to 81.7%. Constantly, the highest genetic similarity (81.7) revealed by the RAPD analysis was that between Yonani and Kalamata, both belonging to the same geographical location (Greece). This was followed by 78.8% and 78.7% between Toffahi and Chemlali and between Manzanello and Kalamata, respectively.

These results are consistent with the predominantly allogamous nature of

Olea europaea L. species. This work indicates the importance of the study of the amount and distribution of genetic diversity for a better exploration of olive genetic resources and the design of plant breeding programs.

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Table (1): Sequence of arbitrary (10-mer) RAPD primers used for the identification of the eleven olive cultivars.

Number	Primers	Sequence 5'-3'
1	OPC-05	GATGACCGCC
2	OPB-08	GTCCACACGG
3	OPA-10	GTGATCGCAG
4	OPB-15	CCAGGGTGTT
5	OPB-12	CCTTGACGCA
6	OPA-11	CAATCGCCGT
7	OPB-16	TTTGCCCGGA
8	OPA-17	GACCGCTTGT
9	OPA-18	AGGTGACCGT
10	OPB-18	CCACAGCAGT

Table (2): The ten olive cultivars besides wild type characterized by unique positive and negative RAPD markers.

Cultivars	Unique positive RAPD markers			Unique negative RAPD markers		
	Size of marker band (bp)	Primer	Totat # of marker/cultivar	Size of marker band (bp)	Primer	Total number of marker/ cultivar
Toffahi (Siwa)	2059 984 209 79 87	OPA-18 OPA-18 OPA-18 OPA-17 OPB-12	5	1539 212 1453 573	OPB-18 OPB-18 OPB-08 OPB-15	4
Wild	105 2207 787	OPA-11 OPB-15 OPB-12	3	1040 965 614 788 320	OPB-12 OPB-15 OPA-18 OPC-05 OPB-16	5
Hamady	-	-	-	1164 706 386 134 374 651	OPB-16 OPC-05 OPA-18 OPA-18 OPB-08 OPB-12	6
Kosha	2106	OPB-12	1	113 522 255 688 525 416	OPA-17 OPA-10 OPA-10 OPB-18 OPB-18 OPA-11	6
Petagn	1721 293	OPB-15 OPA-18	2	859 827 1123	OPB-16 OPA-11 OPB-18	3
Marake	115 1080	OPB-18 OPA-17	2	1583	OPB-12	1
Aspany	1299 1116	OPA-17 OPC-05	2	2096 462 892	OPC-05 OPB-08 OPB-12	3
Toffahi (Borg)	-	-	-	163 1640 1180 1308 908 209 945	OPB-15 OPA-10 OPB-18 OPA-11 OPA-11 OPA-11 OPC-05	7
Chemlali	404 640	OPA-10 OPC-05	2	106 1200 463	OPB-15 OPA-17 OPA-17	3
Kalamata	185 885	OPC-05 OPB-15	2	563 147 595 426	OPB-12 OPB-12 OPB-08 OPC-05	4
Yonani	467 775	OPB-15 OPC-05	2	1324	OPB-12	1
Total			21			43

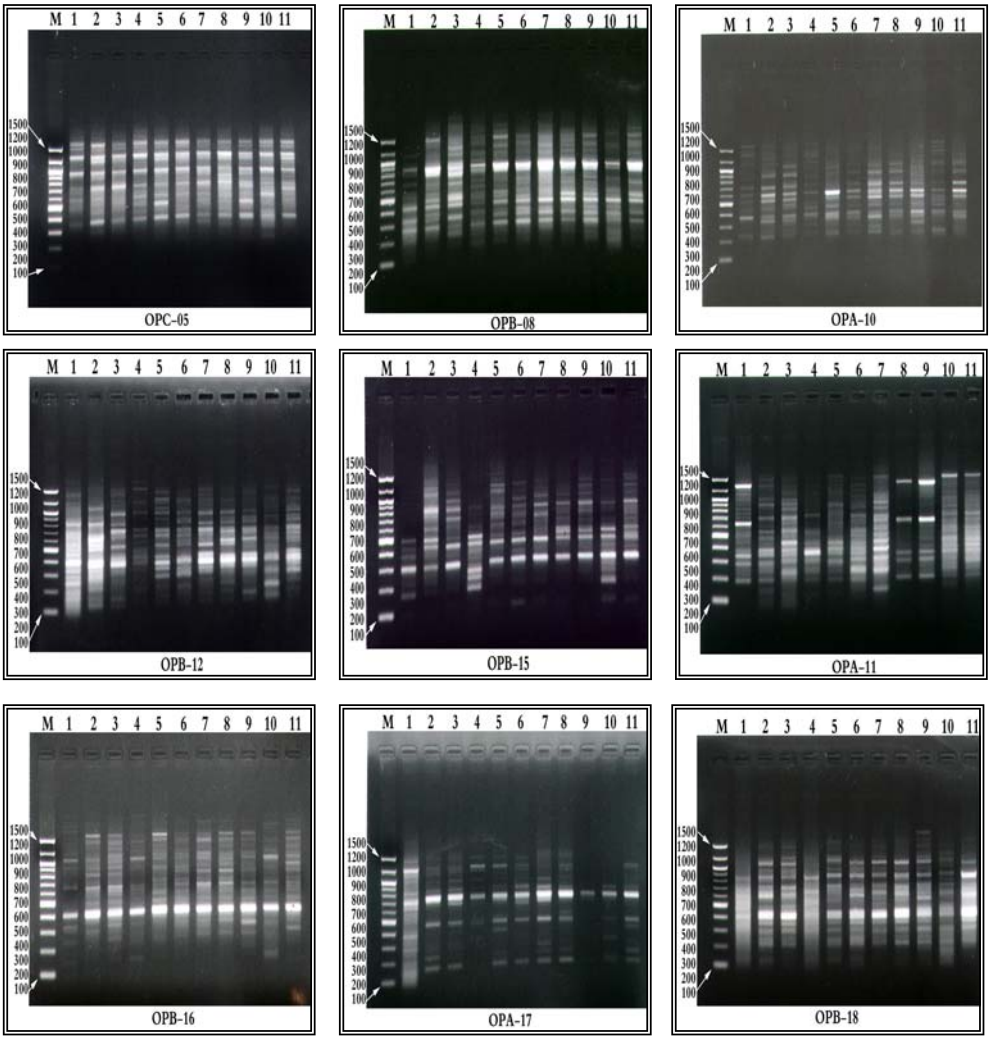
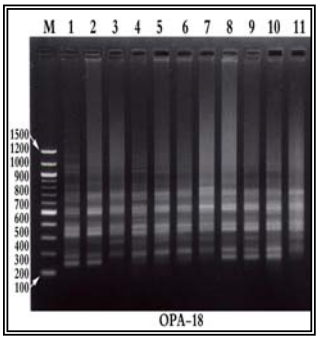


Fig. (1): PCR-RAPD pattern of different olive cultivars: six from Siwa region (Toffahi, Wild, Hamidy, Kosha, Petagn, Marake and Aspany) “Lanes 1-7 respectively”; four from Borq El-Arab region (Toffahi, Chemlali, Kalamata and Ynani) “Lanes 8-11, respectively”



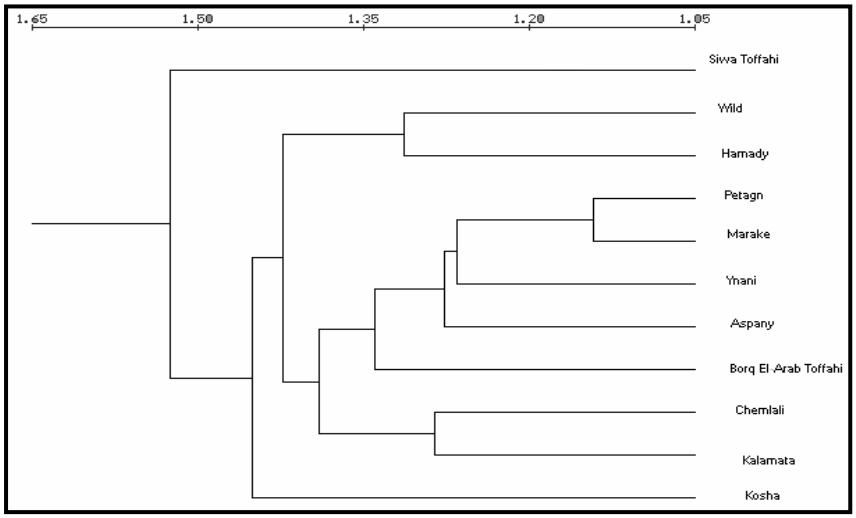


Fig. (2): Dendrogram of 11 Egyptian olive cultivars using PCR-RAPD analysis.