IDENTIFICATION OF MOLECULAR MARKERS LINKED WITH SOME HORTICULTURE CHARACTERISTICS OF SOME OLIVE (*Olea europaea* L.) CULTIVARS

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Olive (*Olea europaea* L.) is considered one of the oldest cultivated trees in the world. Egypt, being one of the Mediterranean countries has large number of olive trees spread all over the east and west deserts since they are drought and salt tolerant trees. The olive genetic patrimony is very rich. The long living character of the tree together with low selection pressure has contributed to the preservation of variability within the species. Neverless, although cultivar diversity is very high, these cultivars are mainly local and old, having a limited diffusion area (Barranco and Rallo, 1985).

Differentiation of cultivars through morphological features is inefficient and inaccurate. This problem is further compounded by the perennial nature of the crop. The use of biochemical and genetic markers for identification of varieties offer a viable alternative method and RAPD technique has been employed to develop genetic fingerprints and to assess genetic diversity in a wide range of plants (Williams *et al*., 1990). Polymerase Chain Reaction (PCR) technology has led to the development of several novel genetic assays based on selective DNA amplification. A genetic assay was developed independently by two laboratories (Welsh and McClelland, 1990). RAPD (Random Amplified Polymorphic DNA) assay detects nucleotides sequence of polymorphisms of DNA using only a single primer of arbitrary nucleotide sequence. The protocol is quick, easy to perform and only nanograms of template DNA are required. The RAPD technique has been employed to determine linked markers in some fruits as Olive *(Olea europaea* L.) (Fabbri *et al*., 1995; Guillaume *et al*., 2001; Hamdy, 2003; Sesli and Yeğenoğlu, 2009), Date palm (*Phoenix dactylifera* L.) (Soliman *et al*., 2003), *A. comosus* (Tapia *et al*., 2005), *Citrus* cultivars (Baig *et al*., 2009), *Musa* spp. (Das *et al*., 2009) and *Mangifera indica* (Ismail, 2003; Damodaran *et al*., 2009; Maklad, 2012).

The main objective of this research was to study the effect of the environment of Abou Tesht region, Qena governorate on the behavior of a new olive vegetative clone (Clone AO13) which was compared with two olive cultivars (Manzanillo and Picual). Measurements included shoot length (cm.) and leaf area (m^2) was measured. In addition, the flower density, inflorescence length, total number of flowers per inflorescence, sexual expression%,

initial fruit set%, horticulture fruit set%, fruit drop%, fruit weight (g) and pulp/fruit%. RAPD analysis was tried to assess the genetic variation between them as well as the possibility of using RAPD-PCR analyzes to determine the specific markers for the three olive cultivars under study and that can be used to distinguish between cultivars, and also determine the degree of genetic relationship between these cultivars. In addition to these specific markers to determine the status of some horticulture characteristics such as sexual expression%, initial fruit set% and fruit drop%.

MATERIALS AND METHODS

This work was conducted in a private olive orchard located at the Abou Tesht region, Qena governorate, during two successive seasons i.e. 2011 and 2012 aiming to study some of the vegetative, flowering, fruit set characteristics and fruit properties affecting olive production in Egypt. To accomplish the purpose of this investigation. New olive vegetative clone namely (Clone AO13) and two olive cultivars namely (Manzanillo and Picual) were included in this study, five as far as possible trees in randomized complete block design from each cultivar were chosen, similar in vigor, size, each tree represented one replicate. The following measurements were recorded: Shoot length (cm.) and leaf area (m^2) were measured. In addition, the flower density, inflorescence length, total number of flowers per inflorescence, sexual expression%, initial fruit set%, horticulture fruit set%, fruit drop%

were calculated and fruit weight (g) and pulp/fruit% were determined according to (A.O.A.C., 1995). The obtained data were statistically analyzed according to the procedures outlined by Snedecor and Cochran (1972) using new LSD test at 5% to approve the differences between olive varieties statistically.

RAPD - PCR analysis

Genomic DNA was extracted from the leaves of the three olive cultivars (*Olea europea* L.) using the Nucleon DNeasy plant mini Kit (Qiagen, Santa Clara, CA). The purified genomic DNA was subjected to PCR for RAPD analysis using 11 random primers (Table 1) each of operon 10-mer. The PCR reaction mixture consisted of 25 ng genomic DNA, 0.2 μM each of dNTPs, 1 μM primer, 1 x Taq DNA polymerase buffer and 1 units of Taq DNA polymerase in a final volume of 25 μl in sterile ultra-pure water. The PCR was performed in a Perkin Elmer 9700 thermal cycler for 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 1.5 min followed by final extension at 72°C for 7 min then

Data analysis

Each variable RAPD band was considered as a locus so that every locus had two alleles and scored as present (1) or absent (0). For data analysis, only polymorphic, reproducible, and clear-cut bands were kept. Dendrograms were constructed by the unweighted pair-group method using arithmetic averages

(UPGMA) algorithm as described by Sneath and Sokal (1973). The similarity value was calculated by SPSS program.

RESULTS AND DISCUSSION

Growth characters

Data in Table (2) showed that, the slight differences among the three olive cultivars concerning the two growth traits namely the shoot length and leaf area were statistically significant in most cases and during both seasons. The maximum value of the shoot length was recorded on the Manzanillo olive cv followed by the other cv's the leaf area was highest in Picual cv followed by the other two cultivars produced the smaller leaves. These results are in agreement with those obtained by El-Khawaga (2001)

Flowering aspects

It is clear from the data in Table (3) that great variation was observed on the flower density, inflorescence length, total number of flowers per inflorescence and sexual expression% among the three olive cultivars. The maximum of flower densities and the total number of flowers per inflorescence which varied significantly according to cultivars and seasons and were recorded higher value in Manzanillo cv. followed by the other two cultivars especially in the first season. These results were in agreement with those found by El-El-Khawaga (2001) who found Picual and Manzanillo cv's had the lowest values (14.51 and 15.39 flowers/inflorescence, respectively) compared to the other culti-

vars under this study. Clone AO13 produced the maximum of sexual expression% compared to the other two cultivars. This characteristic in these two cultivars may be under genetic and environmental effect according to (Hegazi and Stino, 1982; Fouad *et al*., 1992). There were no significant differences between the three cultivars in inflorescence length. Similar trend was observed during both seasons.

Fruit setting%

Table (4) obviously reveals that significant differences were observed on the percentages of initial fruit set%, horticulture fruit set%, fruit drop% among most olive cv's. The highest initial fruit set% was recorded in the Clone AO13, while olive cv's Manzanillo and Picual produced the lowest values of initial fruit set%. There were no significant differences between the three cultivars in horticulture fruit set% in the both seasons. The lowest values of fruit drop% were recorded in the Clone AO13. These results were true during both seasons.

Some physical characteristics of the fruits

It is evident from the data in Table (5) that fruit weight (g), and pulp/fruit% were not significantly varied among the three olive cultivars in fruit weight. The largest percent of pulp/fruit were recorded in Manzanillo olive cv while, the small percentage were recorded in the Clone AO13 and Picual olive cv's. These results were true the same during both seasons.

It could be concluded from the obtained results that the three olive cultivars are widely different in their growth, flowering and fruit setting aspects and fruit quality. This variations could be mainly due to their genetically differences as well as the reaction between genetically and environment. The Clone AO13, Manzanillo and Picual considered the best three promising olive cv's grown successfully under Abou Tesht region, Qena climatic conditions.

RAPD - PCR analysis

RAPD markers were used in order to identify the genetic relationships between olive cultivars (Fabbri *et al*., 1995). RAPD markers have greater utility than protein markers, because of their abundance in the genome, stability and their high level of DNA polymorphism (Arumuganathan and Earle, 1991; Lavi *et al*., 1994).

The three olive cultivars were tested using RAPD-PCR analysis to assess their genetic variation (Figs 1, 2 and 3). RAPD profile of each olive cultivar was generated using operon primers and compared them to each other. Out of 30 primers tested, eleven primers were selected as they gave clear, reproducible, and polymorphic banding profile. Using 11 arbitrary 10-mer primers, 102 distinct fragments of DNA were identified with an average of 9.27 DNA fragments per primer. Total of 43 DNA fragments was polymorphic with average 3.90 polymorphic

bands per primers. Fragment sizes ranged from 100 to 1200 bp.

In the present study, RAPD-PCR was used to analyze the genetic diversity of the three studied olive cultivars, and to assess their genetic relationships using similarity indices and dendrogram tree. Eleven arbitrary random primers were used to determine RAPD polymorphism of the three olive cultivars (Table 1). The resulted amplified fragments are shown in Figs. (1, 2 and 3) and their densitometric analyses are illustrated in Table (6). Banding patterns were scored as present (1) or absent (0). All the 11 primers successfully amplified DNA fragments for all genotypes. A total number of fragments were visualized across the three investigated genotypes.

The results of the amplified fragments using the 10-mer arbitrary primers for the three olive cultivars revealed different levels of polymorphism from one primer to the other. The number of total amplified fragments (TAF), polymorphic bands (PB), amplified fragments (AF) and specific markers (SM) for each cultivar using the 11 primers are shown in Table (6). The Eleven primers produced number of fragments ranging from five (primers M17) to 13 (primers C12) across the three cultivars. Some of the eleven primers exhibited low value of polymorphism started from zero (M17), 20% (M02) and 27.27% (B18), medium value of polymorphism such as A4 (37.5%), C12 (38.46%), A17 and B12 (41.66%) and D20 (42.85%). On the other hand, some showed higher val-

ues of polymorphism as detected in primers: 50% (G19), 66.66% (C01) and 80% (C17). There were some specific fragments which can be used to discriminate each cultivar from the others. Some of these fragments were either absent in all cultivar known as exception cultivar (positive marker) or present in all cultivar but the assigned in one (negative marker) Table (6). These markers were distributed in the three olive cultivars as follows: Primer A04 showed three specific fragments, all of them as negative markers for Clone AO13 (700, 480 and 350 bp). Primer A17 showed five specific fragments, three of them as positive markers one for Manzanillo (1200 bp), one for Picual (300 bp) and one for Clone AO13 (190 bp) and also two as negative markers, one for Manzanillo (1000 bp) and one for Picual (650 bp). Primer B18 showed three specific fragments, two of them as positive markers for Clone AO13 (750 and 400 bp) and one for Manzanillo (200 bp) as a negative marker. Primer B12 showed five specific fragments two of them as positive markers for Clone AO13 (450 and 180 bp), and also three of them as a as negative markers, one for Manzanillo (250 bp) and two for Clone AO13 (430 and 190 bp). Primer C01 showed six specific fragments, five of them as positive markers three for Manzanillo (300, 200 and 170 bp), one for Picual (280 bp) and one for Clone AO13 (320 bp) and also one fragments as a negative marker for Clone AO13 (250 bp). Primer C12 showed five specific fragments all of them as negative markers three for Manzanillo (450, 370 and 260 bp) and two for Picual (700 and

390 bp). Primer C17 showed eight specific fragments, four of them as positive markers two for Manzanillo (380 and 280 bp), one for Picual (230 bp) and one for Clone AO13 (250 bp) and also four were as a negative markers three for Manzanillo (650, 550 and 320 bp) and one for Clone AO13 (300 bp). Primer G19 showed three specific fragments, one of them as positive markers for Manzanillo (300 bp) and two as a negative markers for Clone AO13 (700 and 380 bp). Primer M02 showed two specific fragments, all of them as negative markers for Clone AO13 (800 and 320 bp). Primer M17 there were no showed any specific fragments. Primer D20 showed three specific fragments, two of them as positive markers, one for Picual (400 bp) and one for Clone AO13 (240 bp) and also one as a negative markers for Clone AO13 (600 bp). RAPD technique has been employed to develop genetic fingerprints and to assess genetic diversity in a wide range of mango (Ismail, 2003). Several reports came out in which RAPD genetic markers were used in order to identify Olive cultivars and to determine genetic relationships among them (Hosseini-Mazinani and Samaee, 2004).

Results of similarity index based on RAPD-PCR with the 11 primers using UPGMA computer analysis are shown in Table (7). A distance matrix between cultivars showed a similarity distance ranged from 0.775 to 0.861 with a mean value of 0.818. Thus, the cultivars tested in this study were highly similar at the DNA level. The highest similarity value was rec-

orded between Manzanillo and Picual cultivars (0.861), the same conclusion was reached by Fabbri *et al.* (1995) for the above mentioned results for dendrogram analysis; who found that the similarity was scored between Manzanillo (Spain cv) and Picual (Spain cv) was (0.667). While the lowest similarity value for the above mentioned research was recorded between Manzanillo and the Clone AO13 (0.775). Dendrogram as shown in Fig. (4) illustrated the genetic relationships among the studied cultivars, where the two groups of Manzanillo and Picual were clustered in one group, while the Clone AO13 was clustered in the other group. In this study, OP-A, OP-Q and OP-I primer sets generated scorable bands in wild olive types, but 5 primers from the OP-J kit (OP-J 1 to OP-J 5) did not generate RAPD bands (Sesli and Yeğenoğlu, 2009a, b and 2010)

Molecular genetic markers related to some horticulture traits

In the present study, as shown in Table (8) some RAPD markers (10-mer) may be liked to some flowering and fruit set traits such as sexual expression% (two primers A17- 650 bp and C12- 700 bp), initial fruit set% (six primers, A17-190 bp, B18- 400 and 750 bp, B12-180 and 450 bp, C01- 320 bp, C17- 250 bp and D20-240 bp) and fruit drop% (seven primers, A04- 350 and 700 bp, B12- 190 and 430 bp ,C01- 250 bp, C17- 300 bp, G19- 380 and 700 bp, M02- 320 and 800 bp and D20- 600 bp)

DNA marker-assisted selection was established by Tanksley *et al.* (1989) in which genetic markers are likely to be useful not only for unambiguous cultivars identification but also for the purpose of mango breeding. Genetic markers can serve to detect linkages with agriculturally important trait.

SUMMARY

New olive vegetative clone namely (Clone AO13) and two olive cultivars namely (Manzanillo and Picual) grown in the orchard of the Abou Tesht region, Qena governorate, were examined and evaluated during 2011 and 2012 seasons for some of their vegetative, flowering, fruit set characteristics and fruit properties i.e. shoot length, leaf area, flower density, inflorescence length, total number of flowers per inflorescence, sexual expression%, initial fruit set%, horticulture fruit set%, fruit drop%, fruit weight (g) and pulp/fruit%. RAPD-PCR (polymerase chain reaction randomly amplified polymorphic DNA) analysis was performed to assess the genetic variation for these characteristics between two olive cultivars and clone AO13. The obtained results showed that, the leaf area was highest in Picual cv. followed by the other two cultivars. In Manzanillo cv. flower density and total number of flowers per inflorescence were the highest values compared to the other two cultivars, but, Clone AO13 gave the highest value of the sexual expression%, especially in the first season, Clone AO13 was the highest in initial fruit set followed by the other two cultivars. On the other

hand, there were no significant differences among studied cultivars in the horticulture fruit set% as well as fruit weight and pulp/fruit%. Dendrogram tree generated across RAPD analysis demonstrated that, the three tested olive cultivars in this study were highly similar at the DNA level. The highest similarity value was observed between Manzanillo and Picual cultivars (86.1%), while the lowest similarity was recorded between Manzanillo cv. and Clone AO13 (77.5%). Some RAPD markers may be linked to some flowering and fruit set characteristics such as sexual expression% (two primers A17- 650 bp and C12- 700 bp), initial fruit set% (six primers, from B12 at 180 bp to B18 at 750 bp) and fruit drop% (seven primers, from B12 at 190 bp to M02 at 800 bp)

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Table (1): Sequences and names of the selected primers.

Primer	Sequence (5' - 3')	Primer	Sequence (5' - 3')
A-04	5'-AATCGGGCTG-3'	C-17	5'-TTCCCCCCAG-3'
A-17	5'-GACCGCTTGT-3'	G-19	5'-GTCAGGGCAA-3'
B-18	5'-CCACAGCAGT-3'	M-02	5'-ACAACGCCTC-3'
B-12	5'-CCTTGACGCA-3'	M-17	5'-TCAGTCCGGG-3'
C-01	5'-TTCGAGCCAG-3'	D-20	5'-ACCCGGTCAC-3'
C-12	5'-TGTCATCCCC-3'		

Table (2): Shoot length and Leaf area of the three olive cultivars grown under Abou Tesht condition in 2011 and 2012 seasons.

Cultivars	Shoot length (cm)		Leaf area (m^2)	
	2011	2012	2011	2012
Manzanillo	15.23	13.42	3.40	3.12
Picual	10.42	8.69	4.00	3.62
Clone AO13	12.78	10.54	3.42	3.31
LSD at 0.5%	2.83	2.46	1.62	1.52

Table (3): Flower density, inflorescence length, total no of flowers/per inflorescence and sexual expression% of the three olive cultivars grown under Abou Tesht condition in 2011 and 2012 seasons.

Cultivars	Flower density		Inflorescence length (cm)		Total no of flowers per inflorescence		Sexual expression%	
	2011	2012	2011	2012	2011	2012	2011	2012
Manzanillo	33.31	21.72	2.4	2.3	9.4	8.63	59.73	56.28
Picual	29.16	15.87	2.3	2.2	8.56	8.44	51.28	48.11
Clone AO13	31.24	19.45	2.3	2.2	8.21	7.32	62.14	57.24
LSD at 0.5%	1.83	1.7	N.S	N.S	1.2	1.1	3.01	2.72

Table (4): Initial fruit set%, horticulture fruit set% and fruit drop% of the three olive cultivars grown under Abou Tesht condition in 2011 and 2012 seasons.

Cultivars	Initial fruit set%		Hort. fruit set%		Fruit drop%	
	2011	2012	2011	2012	2011	2012
Manzanillo	18.36	22.63	3.21	4.41	81.63	75.14
Picual	16.85	19.44	3.24	4.45	82.22	77.16
Clone AO13	28.40	30.31	3.36	4.62	72.18	68.54
LSD at 0.5%	1.56	1.92	1.73	1.12	0.587	1.09

Table (5): Fruit weight (g) and pulp/fruit% of the three olive cultivars grown under Abou Tesht condition in 2011 and 2012 seasons.

Cultivars	Fruit weight (g)		Pulp/fruit%	
	2011	2012	2011	2012
Manzanillo	4.50	4.30	88.60	87.50
Picual	4.70	4.50	85.20	84.70
Clone AO13	4.30	4.10	86.10	85.20
LSD at 0.5%	0.41	0.38	0.90	0.80

Table (6): Number of amplified fragment and specific markers of the three olive cultivars based on RAPD-PCR analysis using 11 primers.

Primers	Manzanillo				Picual		Clone AO13		TSM
	TAF	PB	AF	SM	AF	SM	AF	SM	
A-04	8	3	8	-	8	-	5	3(-)	3
A-17	11	5	8	1(+) 1(-)	8	1(+) 1(-)	9	1(+)	5
B-18	11	3	8	1(-)	9	-	11	2(+)	3
B-12	12	5	9	1(-)	10	-	10	2(+) 2(-)	5
C-01	9	6	7	3(+)	5	1(+)	4	1(+) 1(-)	6
C-12	13	5	10	3(-)	11	2(-)	13	-	5
C-17	10	8	5	2(+) 3(-)	7	1(+)	6	1(+) 1(-)	8
G-19	6	3	6	1(+)	5	-	3	2(-)	3
M-02	10	2	10	-	10	-	8	2(-)	2
M-17	5	0	5	-	5	-	5	-	0
D-20	7	3	5	-	6	1(+)	5	1(+) 1(-)	3

TAF = Total amplified fragments   PB = Polymorphic bands for each primer
AF = Amplified fragments   p = (+) ve   n = (-) ve
SM = Specific markers including either the presence or absence of a fragment
TSM = Total number of specific markers

Table (7): Similarity indices among the three olive cultivars based on RAPD-PCR using 11 primers.

	Manzanillo	Picual
Picual	86.1	
Clone AO13	77.5	83.4

Table (8): Performance of different olive cultivars against three horticulture characters.

	Flowering and fruit set characteristics	Genotypes			Marker linked	
		Manzanillo	Picual	Clone AO13	Primer	MW bp.
Sexual expression%	High	+	-	+	A17 C12	650 700
Initial fruit set%	High	-	-	+	A17 B18 B12 C01 C17 D20	190 750, 400 450, 180 320 250 240
Fruit drop%	Low	+	+	-	A04 B12 C01 C17 G19 M02 D20	700, 350 430, 190 250 300 700, 380 800, 320 600
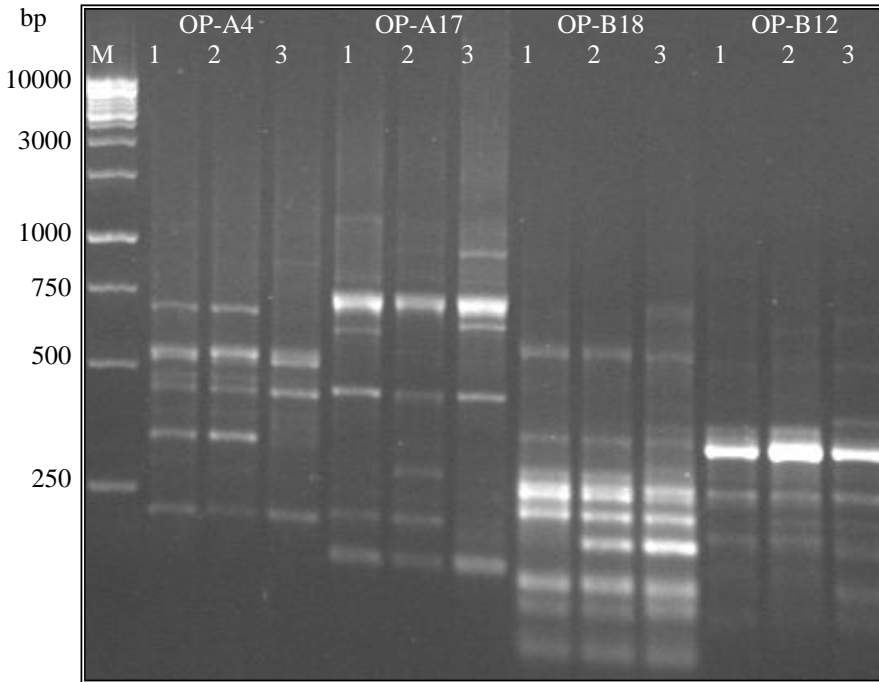
Fig. (1): DNA polymorphism of the three olive cultivars amplified with primers OP-A4, A17, B18 and B12 using RAPD-PCR (M) DNA ladder marker (bp). (1) Manzanillo, (2) Picual and (3) Clone AO13.
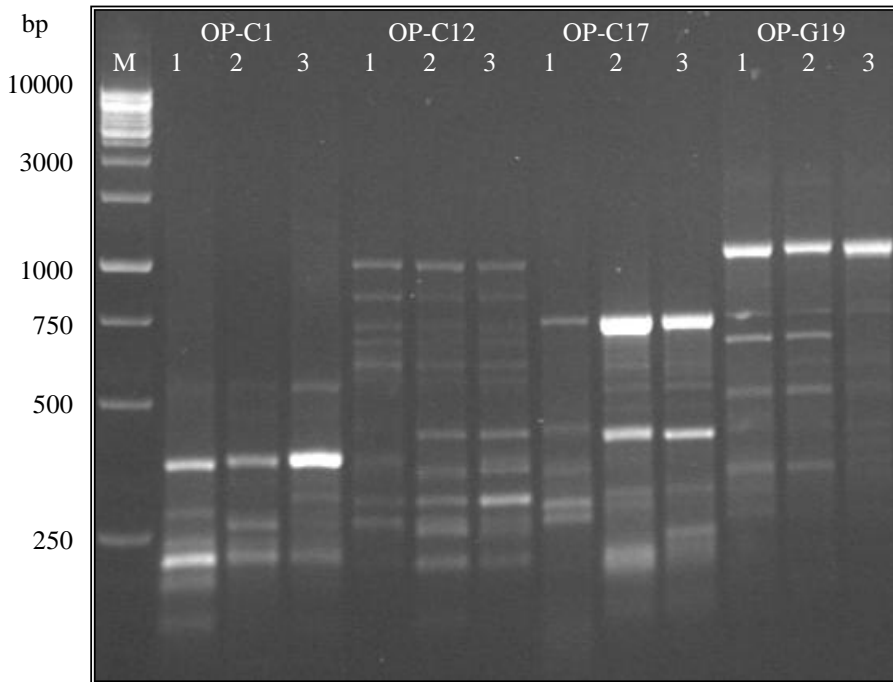
Fig (2): DNA polymorphism of the three olive cultivars amplified with primers OP-C1, C12, C17 and G19 using RAPD-PCR (M) DNA ladder marker (bp) (1) Manzanillo, (2) Picual and (3) Clone AO13.
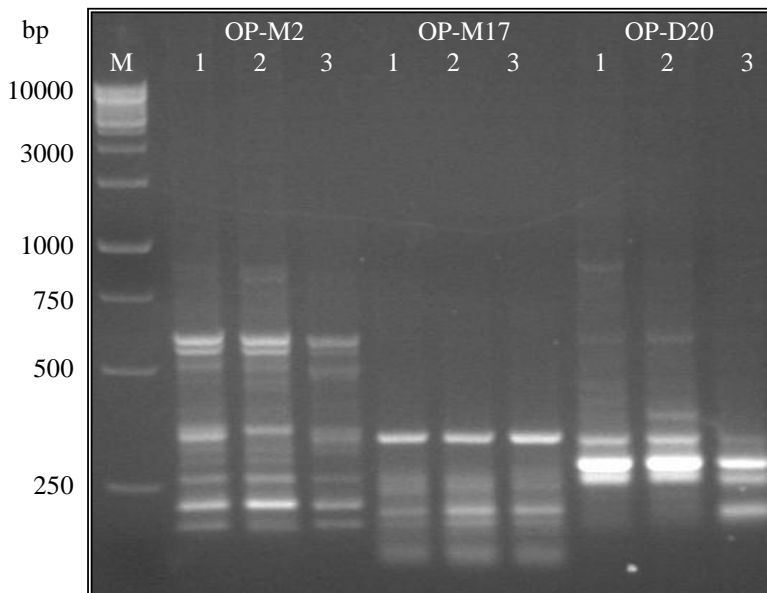

Fig (3): DNA polymorphism of the three olive cultivars amplified with primers OP- M2, M17 and D20 using RAPD-PCR (M) DNA ladder marker (bp). Manzanillo, (2) Picual and (3) Clone AO13.
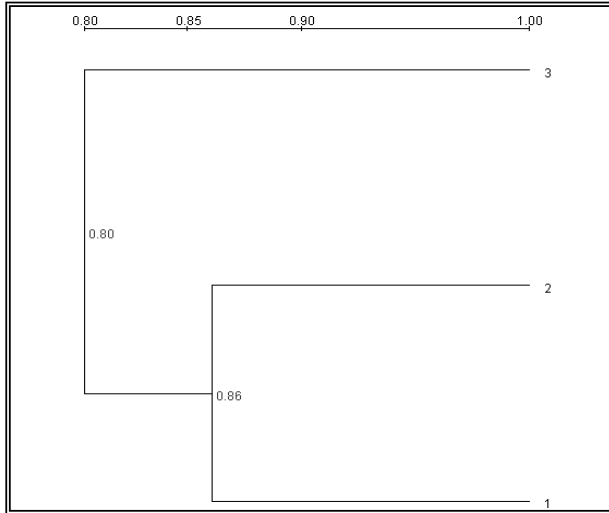
Fig (4): Dendrogram for the genetic distances relation-
ships among the three olive cultivars based on
similarity indices data of RAPD 11 analyses.
(1) Manzanillo (2) Picual and (3) Clone AO13