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Molecular Characterization of Local and Imported Olive Cultivars Grown in Egypt Using ISSR Technique

[1]El Saied S. Hegazi, [1]Ayman A. Hegazi, [2]Ahmed A. Tawfik and [2]Hossam A. Sayed

[1]Department of Pomology, Faculty of Agriculture, Cairo University, Giza, Egypt
[2]National Gene Bank and Genetic Resources, Giza, Egypt

Abstract: Olive *Olea europaea* L. is one of the most economically important crops in the Mediterranean area and known for having large genetic variability. Consequently, genetic variation among 22 olive cultivars (Twelve local cultivars grown in Egypt and ten foreign cultivars) was assessed using inter-simple sequence repeats (ISSRs) markers. Ten (ISSRs) primers amplified 71 fragments of which 38 were polymorphic. The number of polymorphic bands per primer varied from 4 to 10 with 7.1 bands per primer on average. Genetic similarities were calculated using the Jaccard similarity coefficient. The resulting similarity matrix was subjected to the UPGMA clustering method for dendrogram construction and cultivar differentiation. Our results indicate that ISSR can be useful for genetic diversity studies, to provide practical information for parental selection and to assist breeding and conservation strategies Also, the present results along with those of other researchers show that ISSRs can be used for cultivar differentiation in *Olea europaea* L.

Key words: Olive · *Olea europaea* L. · Genetic diversity · ISSR marker

INTRODUCTION

The genetic diversity could be an important resource for the development of modern olive culture towards typical olive oil and fresh productions. From here, the study of less common cultivars represents an important tool to preserve this genetic diversity in respect to genetic erosion due to the introduction of few commercial cultivars in the modern orchards. In fact, the modern olive oil industry and fresh consuming requires new and more productive cultivars to sustain the new trends in olive growing. This phenomenon implies that only a few commercial varieties are cultivated in the main production areas, whereas minor varieties are located in restricted areas and are sometimes threatened. Hence, the importance of these less common cultivars is in the conservation of several adaptive traits that could support olive growing, especially in relation to the effects of global change. Conservation programs could be useful tools for the management of this local genetic diversity. In this way, all accessions should be characterized to eliminate cases of mislabeling and redundancies (synonymy), identify the presence of different clones within the same cultivar (multi polyclonal populations) [1, 2] and safeguard all cultivars, in particular the minor ones, to avoid a loss in genetic diversity. PCR-based DNA markers, proved powerful tools for genetic analysis because of their simplicity and ease of handling. In the last years, molecular markers, such as AFLPs [3] and RAPDs [4, 5], have been used to characterize olive germplasm and are proved to be a powerful tools for genetic analysis which provide an opportunity for direct comparison and identification of olive tree material. Also, ISSRs method is based on the amplification of DNA segments between two microsatellite repeated regions [6] have been used to identify olive cultivars and olive drupes from different olive cultivars and to assess phylogenetic relationships in the *Olea europaea* complex [7-12].

MATERIALS AND METHODS

Plant Materials: The molecular characterization of Twenty-two cultivars (twelve local cultivars and ten foreign cultivars) of olive *Olea europaea* L. was carried out in this study on old olives trees grown at Horticulture Research Institute (HRI), Agricultural Research Center (ARC) and the Faculty of Agriculture, Cairo Univ., Egypt (Table 1).

Corresponding Author: El Saied S. Hegazi, Department of Pomology, Faculty of Agriculture, Cairo University, Giza, Egypt.

Table 1: List of Twenty-two olive cultivars studied and country of origin.

Code no.	Cultivar	Country of origin	Code no.	Cultivar	Country of origin
	Foreign cultivars			Local cultivars	
1	Frantoio	Italy	11	Teffahi	Egypt
2	Oblonga	U.S.A	12	Wateken	Egypt
3	Kalamata	Greece	13	Hamed	Egypt
4	Dolce	France	14	Wardan	Egypt
5	Picholine	Italy	15	Cairo 7	Egypt
6	Moroccan Picholine	Morocco	16	Maraki	Egypt
7	Chemlali Libyan	Libya	17	Melokey	Egypt
8	Chemlali Tunisia	Tunisia	18	Eggizi Shami	Egypt
9	Manzanillo	Spain	19	Balady	Egypt
10	Picual	Spain	20	Eggizi Oshem	Egypt
--	--	--	21	Eggizi Akse	Egypt
--	--	--	22	Senawe	Egypt

Table 2: List of ISSR primers sequences and annealing temperatures

Primer	Sequence 5' - 3'	Ta	Primer	Sequence 5' - 3'	Ta
17898A	$(CA)_6AC$	40°C	HB9	$(GT)_6GG$	40°C
17898B	$(CA)_6GT$	40°C	HB10	$(GA)_6CC$	40°C
17899A	$(CA)_6AG$	40°C	HB11	$(GT)_6CC$	48°C
17899B	$(GA)_6GG$	42°C	HB12	$(CAC)_3GC$	45°C
HB8	$(GA)_6GG$	48°C	HB13	$(GAG)_3GC$	45°C

Isolation of Plant Genomic DNA: DNA extraction was carried out using young tissues collected from three trees per cultivar. Genomic DNA was extracted and purified according to Torres *et al.* [13].

DNA Extraction: Leaf tissue (0.5 g) was ground in liquid nitrogen and incubated at 65°C for 1 h in 1.5 ml extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA; 0.5 % SDS; 500 mM NaCl and 1% Polyvinylpyrrolidone). An equal volume of phenol/chloroform (24:1) was added and the whole mixture was centrifuged at 1000 rpm for 10 min. An equal volume of cold chloroform/ isoamylalcohol (24:1) was added to the supernatant and the mixture was centrifuged at 5000 rpm for 10 min. The precipitation of the upper phase was obtained by adding of 75 µl of 3 M ammonium acetate and 1 volume of cooled isopropanol and centrifugation at 1000 rpm for 10 min. The DNA pellet was washed with 70 % ethanol, then dried and dissolved into 400 µL of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). RNA was removed by incubation with 1 µL of RNase (10 mg/ml) at 37°C for 30 min. to have pure DNA and kept at -20°C until use. Estimation of DNA concentration and quality were based on Sambrook *et al.* [14]. DNA concentrations were measured by UV-spectrophotometer (Eppendorf Biophotometer Germany) at a wave length of 260-280 nm.

ISSR Amplification: Inter simple sequence repeats ($ISSR_s$) technique was carried out according to procedure described by Martins-Lopes *et al.* [11]. PCR reactions were performed in a 25 µL volume containing 10 mM Tris -HCl buffer at pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.3 µM of a single primer; 20 ng genomic DNA and 2 units of Taq DNA polymerase (Promega, USA). Amplification reactions were performed in a 96-well BioRad Thermal cycler (U.S.A) under the following conditions: 5 min. initial denaturation step (94°C), 35 cycles of 30 S at 94°C; 1 min at 50°C, 1 min at 72°C). The reaction was completed by a final extension step of 7 min at 72°C. Amplification products were separated by electrophoresis in 1 % agaros gels in 1x TBE buffer, stained by ethidium bromide and visualized under UV light. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Promega, U.S.A). The sequences of the ten $ISSR_s$ primers (5' - 3' anchored) are presented in Table 2.

Data Analysis: Scoring of $ISSR_s$ data was achieved using 1 % agarose gel electrophoresis profile. Clear and distinct fragments were scored as (1) for presence and (0) for absence. Cluster analysis of genetic distances among olive cultivars was performed using the unweighted pair group method with arithmetic average (UPGMA).

RESULTS AND DISCUSSION

Genotype Identification by Unique ISSR Markers: In the present study, Molecular fingerprinting of olive cultivars using 10 ISSRs were tested to explore the genetic diversity among different foreign and local olive genotypes based on the clear scorable band pattern and of good quality. Total number of amplified amplicons was 71 bands and the number of amplified DNA fragments by each primer ranged from 4-10 bands. The highest number of polymorphic bands was obtained by HB9 (10 bands), while HB12 produced the lowest number of polymorphic bands4 bands. The average number of bands/primer was 7.1 bands/template and the approximate size of amplification product ranged from 95-149 bp for the foreign cultivars. All primers produced polymorphic bands ranging in number from 2 to 6 fragments with an average polymorphism/primer of 3.8 (Tables 3 and 4). The percentage of polymorphism revealed by the different primers ranged from 37.5 to 85.7% with an average 53.3%. Seven out of seventy one ISSRs (about 9.8%) were found to be useful as cultivar specific markers which could be distinguish as 5 unique bands for foreign olive cultivars and 2 unique bands for local olive cultivars which some of them present in one cultivar and absent in the cultivars or vice versa. The number of ISSR-PCR fragments generated by using the ten primers could be used as cultivar specific markers both of Frantoio and Dolce cultivars characterized by unique fragments generated from 17898A, 17899B and HB11 primers. The ISSR markers varied in size between 96 and 1223bp for the local cultivars as shown in Table 5. Among these 4 genotypes, Frantoio, Oblonga Dolce and

Table 3: Cultivars characterised by unique positive and/or negative ISSR markers, marker size and total number of markers identified each olive cultivar (based on 10 primers)

	Unique Positive marker			Unique Negative marker			
Cultivars	Primer	Marker size	Total No. of markers	Primer	Marker size	Total No. of markers	Grand Total
			Foreign olive cultivars				
Frantoio	17898A	1466 bp	1	-	-	-	2
	17899B	1497 bp	1	-	-	-	
Oblonga	17898B	766 bp	1	-	-	-	2
	-	-	-	17899A	733 bp	1	
Kalamata	-	-	-	HB9	802 bp	1	1
Dolce	HB13	313 bp	1	-	-	-	3
	-	-	-	HB10	767 bp	1	
	-	-	-	HB11	200 bp	1	
Picholine	-	-	-	HB8	657 bp	2	2
					566 bp		
			Local olive cultivars				
Eggizi Oshem	17898B	448 bp	2	-	-	-	2
		323 bp					
Senawe	-	-	-	HB9	434 bp	1	1

Table 4: Total number of amplicons, size of amplified fragments, monomorphic amplicons, polymorphic amplicons and the percentage of polymorphism as revealed by ISSR markers among foreign cultivars.

Primer	Total number of amplicons	Size of amplified fragments (bp)	Monomorphic amplicons	Polymorphic amplicons	% polymorphism
17898A	7.0	96- 1466	1.0	6.0	85.7
17898B	7.0	323-909	1.0	6.0	85.7
17899A	7.0	166-733	4.0	3.0	42.9
17899B	8.0	384-1497	4.0	4.0	50.0
HB8	7.0	346-1223	4.0	3.0	42.9
HB9	10.0	146-1076	4.0	6.0	60.0
HB10	6.0	236-911	3.0	3.0	50.0
HB11	8.0	200-1013	5.0	3.0	37.5
HB12	4.0	504-1247	2.0	2.0	50.0
HB13	7.0	313-923	5.0	2.0	28.6
Total	71.0	-	33.0	38.0	-
Average	7.1	-	3.3	3.8	53.3

Table 5: Total number of amplicons, size of amplified fragments, monomorphic amplicons, polymorphic amplicons and the percentage of polymorphism as revealed by ISSR markers among local cultivars.

Primer	Total number of amplicons	Size of amplified fragments (bp)	Monomorphic amplicons	Polymorphic amplicons	% polymorphism
17898A	6.0	96 -1136	5.0	1.0	16.70
17898B	7.0	320 - 909	1.0	6.0	85.70
17899A	7.0	166 -733	6.0	1.0	14.30
17899B	7.0	383- 1077	4.0	3.0	42.90
HB8	7.0	346 -1223	6.0	1.0	14.30
HB9	9.0	146 - 967	4.0	5.0	55.60
HB10	6.0	236 - 911	5.0	1.0	16.70
HB11	7.0	200- 1013	6.0	1.0	14.30
HB12	3.0	504 - 741	2.0	1.0	33.30
HB13	6.0	487- 932	5.0	1.0	16.70
Total	65.0	-	44.0	21.0	-
Average	6.5	-	4.4	2.1	31.05

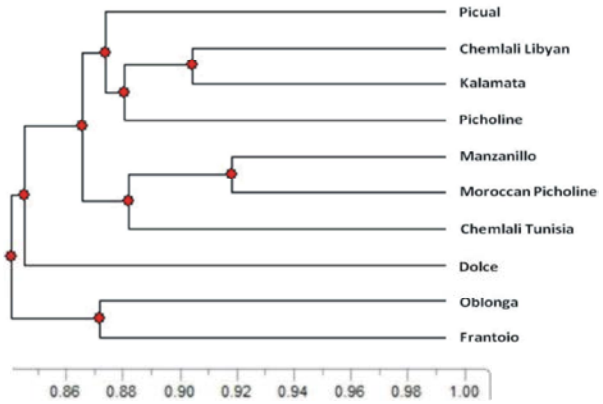

Fig. 1: UPGMA dendrogram based on the proportion of shared ISSR fragments obtained by using ten primers in the total DNA of the 10 introduce olive cultivars

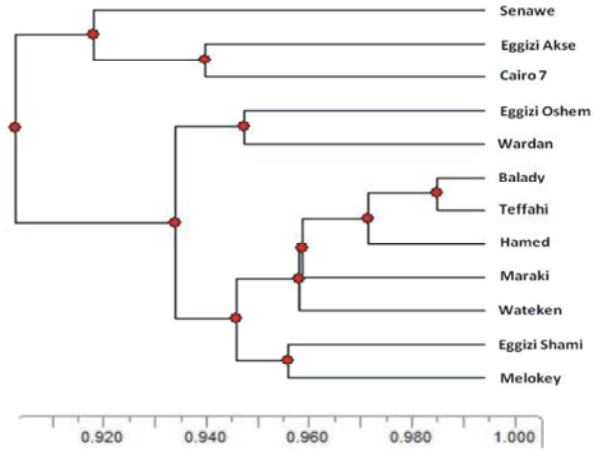

Fig. 2: UPGMA dendrogram based on the proportion of shared ISSR fragments obtained by using ten primers in the total DNA of the 12 local olive cultivars

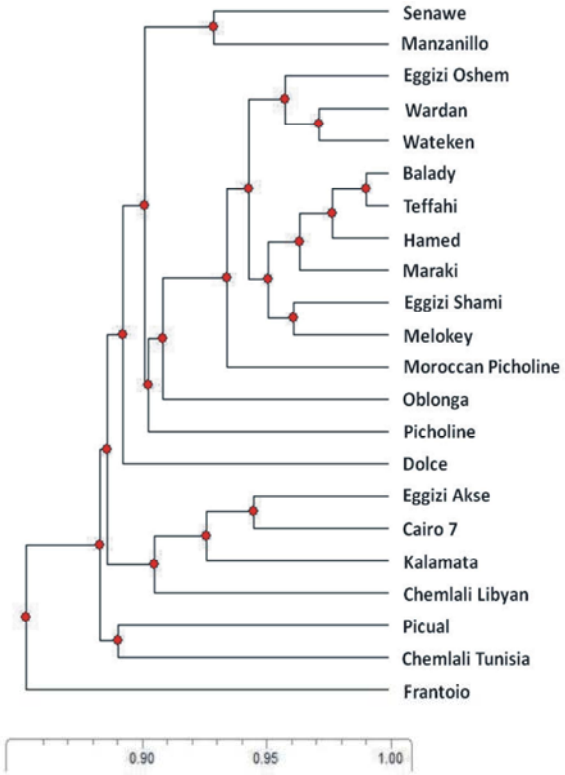

Fig. 3: UPGMA dendrogram based on the proportion of shared ISSR fragments obtained by using ten primers in the total DNA of the 22 olive cultivars

Eggizi Oshem were characterized by both positive ISSR markers. Fourteen unique markers were generated from 9 primers out of 10 tested ISSR primers. The maximum number of unique markers was identified with Frantoio and Eggizi Oshem genotypes which reflect their genetic diversity among all tested cultivars. Several authors

reported on the usefulness of ISSR for cultivar identifications. ISSRs are ideal as markers for genetic mapping and population studies because of their abundance and the high degree of polymorphism between individuals within a population of closely related genotypes [15].

Molecular markers have been extensively used to derive genetic relationships between olive cultivars [4, 9, 11, 16, 17, 18]. ISSR analysis was used for the DNA profiling and differentiation of total 31 *Olea europaea* L. cultivars grown in Greece [10]. Also, (g) applied ISSR technique for phylogenetic analysis within the *O. europaea* L. species and cultivar identification respectively and for the study of intra-cultivar variability of 201 accessions belonging to 11 Portuguese cultivars [9]. Moreover, the high level of polymorphism observed in our study was consistent with other comparable studies [9, 11, 12, 19, 20].

ISSR Clustering Analysis: The ISSR dendrogram obtained by UPGMA analysis grouped the ten foreign cultivars into two main clusters and three minor subclusters. The Jaccard's coefficient ranged from 0.86 to 1.00 (Fig. 1). The lowest similarity coefficient was observed between two cultivars Oblonga and Frantoio and 'Dolce' cultivar (0.84). While, the highest similarity coefficient was obtained between 'Manzanillo' and 'Moroccan Picholine' (0.93). Frantoio and Oblonga were ranked in a separated cluster and the other 8 cultivars were clustered into 3 sub-clusters, sub-cluster I comprised one cultivar (Dolce), sub-cluster II grouped three cultivars: 'Manzanillo', 'Moroccan Picholine' and 'Chemlali Tunisia'. Sub-cluster III grouped four cultivars: 'Picholine', 'Kalamata', 'Chemlali Libyan' and 'Picual'.

On the contrary to the foreign cultivars, ISSR based phylogenetic tree showed a high degree of genetic similarities among the Egyptian cultivars (Fig. 2) UPGMA analysis grouped the twelve local cultivars into two main clusters and eight sub-clusters. The Jaccard's coefficient ranged from 0.90 to 1.00 (Fig. 2). The lowest similarity coefficients were observed among the three cultivars "Cairo 7, Eggizi Akse and Senawe and Melokey cultivar (0.91) while the highest similarity coefficient was obtained between Balady and Teffahi (0.99). Cluster I comprised three cultivars Cairo 7, Eggizi Akse and Senawe, the second cluster II grouped nine cultivars divided into two sub-clusters Fig. 2: Balady, Teffahi and Eggizi Oshem, Hamed and Wardan in sub-cluster. While, the other

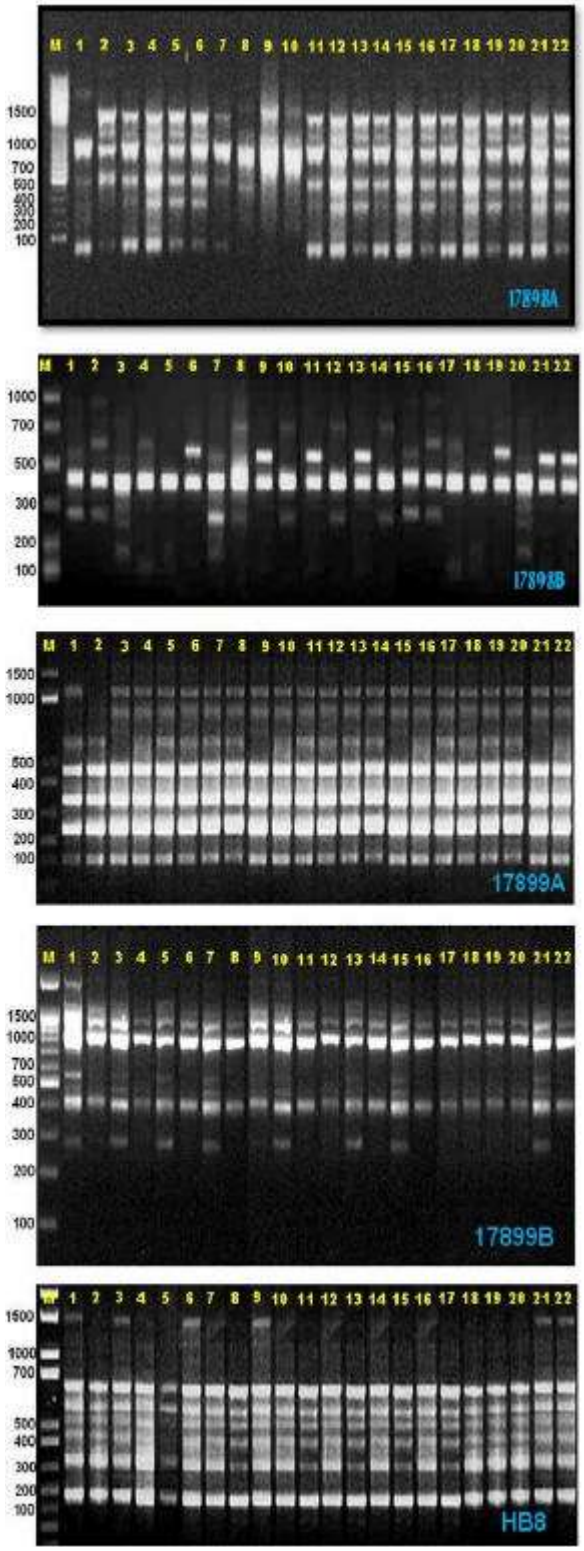

Fig. 4.a: ISSR patterns obtained from the 22 olive cultivars analyzed by using five primers (17898A, 17898B, 17899A, 17899B and HB_8)

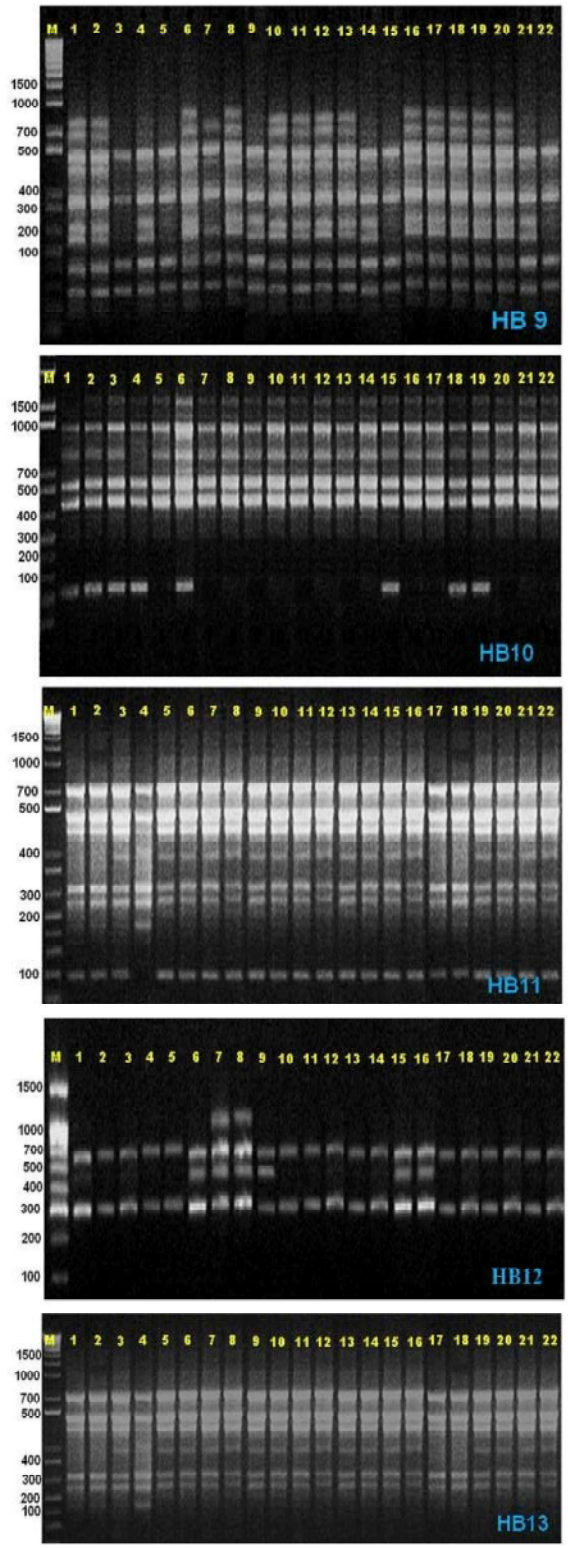

Fig. 4b: ISSR patterns obtained from the 22 olive cultivars analyzed by using five primers (HB_9, HB_{10}, HB_{11}, HB_{12} and HB_{13})

sub-cluster grouped four cultivars: Wateken, Maraki, Eggizi Shami and Melokey, on the contrary to the foreign cultivars, ISSR based phylogenetic tree showed a high degree of genetic similarity among the Egyptian cultivars (Fig. 2).

Combined dendrogram analysis grouped the twenty two cultivars into five main clusters and eight sub-clusters. The Jaccard's coefficient ranged from 0.85 to 1.00 (Fig. 3). The lowest similarity coefficient was observed among two cultivars Frantoio and Dolce (0.81), while the highest similarity coefficient was obtained between Balady and Teffahi (0.99). Cluster I comprised one cultivar Frantoio, the second cluster II grouped two cultivars Chemlali Tunisia and Picual. While, Cluster III grouped four cultivars Chemlali Libyan, Kalamata, Cairo 7 and Eggizi Akse. Cluster IV included one cultivar Dolce, whereas Cluster V grouped the rest of the cultivars. The results confirm that the olive is a highly variable species which reflect the genetic diversity among olive cultivars. The high diversity found between olive cultivars is probably due to a diverse germplasm origin, which presumably results from crosses between wild and cultivated olives, resulting in new cultivars in different parts of the Mediterranean and low breeding pressures [4, 16, 21]. This may also explain why in the combined dendrogram (Fig. 3) the foreign cultivars could be intermixed with the Egyptian ones because of the wide exchange of plant material in the Mediterranean basin and the natural or artificial breeding of olive over centuries. The same pattern was observed between Iranian and foreign cultivars [22].

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