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RESEARCH ARTICLE

ASSESSMENT OF GENETIC VARIATION AMONG EL-WADI EL-GEDID`S OLIVE TREES (*OLEA EUROPAEA* L.) USING INTER MICROSATELLITE MARKERS (ISSRs)

***Nahed, A. K. Rashed**

Plant Genetic Resources Department, Desert Research Center, El Mataria, Cairo, Egypt

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ABSTRACT

The present study was conducted to study the genetic diversity among sex (6) olive cultivars and one unknown genotype grown in El-Kharga Oasis, El-Wadi El-Gedid, Egypt using inter-simple sequence repeats (ISSR) markers. Ten (ISSR) primers amplified 92.00 fragments of which 46.00 fragments were polymorphic. The number of polymorphic bands per primer varied from 0.00 to 8.00 with 4.60 bands per primer on average. Genetic similarities were calculated using the Dice coefficient (Nei and Li., 1979). The resulting similarity matrix was subjected to the UPGMA clustering method for dendrogram construction and cultivar differentiation. ISSRs dendrogram obtained by UPGMA analysis grouped the local cultivar and the unknown local genotype grown in El-Kharga, El-Wadi El-Gedid very close to each other, in the same time grouped all foreign cultivars near each other's and a part from the local ones. The results obtained could be used for selection the promising parents to generate an effective breeding program and for conservation strategies to future olive propagation program under the promising area El-Wadi El-Gedid conditions. Also, show that ISSR markers could be useful for cultivar differentiation and genetic diversity of *Olea europaea* L. studies.

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INTRODUCTION

Schemes of Egypt development aim to expand the cultivated spot and release of the narrow strip on the banks of the present course of the River Nile. The New Valley (El-Wadi El- Gedid) and its oasis are the land of promise in modern revival and urbanization since, they occupy two thirds of Egypt`s area and all constituents of comprehensive development so as to make tomorrow better. Many of oasis have cultivated areas in-addition to the areas reclaimed from 1990, also, over two million, very much favorable to be cultivated feddans available for future reclamation (Ottosen and Niels, 2011 and Sayed, 2013). These areas were classified as arid and hyperarid with mild winters and very hot summer (Ayyad and Ghabbour, 1986), Monsoon hot air and sand carrying wind activity, is of frequent occurrence. Agriculture in these areas is mainly groundwater-dependent (Bornkamm & Kehl, 1990). The economical base of the oasis is agriculture of which olives are the principal producer (Abd El-Ghani and Fawzi, 2006). Local farmers consider their olives and extra virgin olive oil to be among the best in the world (Ilahiane, 2006). Olive (*Olea europaea* L.subspecies europaea) is an ancient oil producing crop of agriculture and economic significance worldwide. It not found wild in Egypt.

Corresponding author:* **Nahed, A. K. Rashed,
Plant Genetic Resources Department, Desert Research Center, El Mataria, Cairo, Egypt.

The unique group of Egyptian varieties had been cultivated at least since the mid first millennium BC; some seemed of astern origin, while the others could not be assigned to modern groups (Newton *et al.* 2006). It is a long-living diploid (2n =46) tree, most of are self-incompatible and out-crossing species with a very wide genetic patrimony. Since the beginning of its domestication, olive has been propagated vegetably to exploit the combination of genes which arose by random crosses or mutations (Carriero *et al*., 2002). This mode of production led to genetic erosion (Bronzini de Caraffa *et al*., 2002). To develop new El-Wadi El-Gedid`s olive varieties with goal of producing a very productive precocious, self fruitful, disease resistance tree that produce fruit that is easy to harvest and has excellent aromatic oil characteristics with good stability [protected Domination of Origin (PDO)], to target international markets, several crosses between wide genetic variation cultivars with superior characters in an elite breeding program should be make each year (Vossen, 2007). Assessment of the extent of genetic variability among El-Wadi El-Gedid`s olive cultivars is fundamental for olive breeding and conservation of these olive genetic resources which is particularly useful a general guide in the choice of parents for breeding hybrids. Molecular markers have been proved to be valuable and powerful tools in the evaluation of genetic diversity and differentiation within and between olive cultivars, characterization of germplasm collections and population, exploration of cultivar identity as well as genetic

mapping (Koehmstedt *et al*., 2011; Gomes *et al*,. 2012; Mohamed and Yacout, 2014 and Muzzalupo *et al*., 2014). Nowadays, inter-simple sequence repeats (ISSR) markers have shown to be powerful and efficient in assessing phylogenetic relationships in the o.europaea complex (Gemas *et al*., 2004), genetic structure of olive germplasm (Linos *et al*., 2014), genetic diversity in *Olea europaea* (Essadki *et al*., 2006 and Gomes *et al*., 2009), intra specific genetic diversity in wild olive (Noormohammadi *et al*., 2012a), inter-population genetic variation in *Olea cuspidate* (Noormohammadi *et al*., 2012b) as they are highly polymorphic, their use is cost effective, requiring no prior information of DNA sequence for primer design, re producible and their distribution in eukaryotic genome that makes them highly informative. The main goals of this work were: to assess the level of inter-cultivar polymorphism among seven olive cultivars and genotype grown under El-Kharga Oasis, El-Wadi El-Gedid stress conditions using ISSR markers, to investigate the genetic relationships among them and to develop cultivar- specific molecular markers characterizing each cultivar and genotype to put an essential step towards optimized conservation of these cultivars that located in remote regions support olive breeding program for newly reclaimed regions, particularly in relation to the effect of global changes and aridity.

MATERIALS AND METHODS

Plant Materials

Six olive (*Olea europaea* L.) cultivars and one unknown local genotype were collected from El-Kharga Oasis, El-Wadi El-Gedid, Egypt (Table 1).

ISSR Amplification

Inter-simple sequence repeats (ISSR) technique was carried out according to procedure described by Lopes *et al*. (2007). PCR reactions were performed in 25 µl volume containing 10mM Tris- buffer at pH 8.0; 50mM KCl; 1.5mM MgCl; 0,2mM of each dNTP; 0.3µM of a single primer; 20ng genomic DNA and 2 units of *Taq* DNA polymerase (Promega, USA). The used ISSR primers (5-3) sequences are shown in Table (2).

Amplification reactions were performed in a Perkin-Elmer/ Gene Amp PCR system 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after initial denaturation cycle for 5min at 94ºC. Each cycle consisted of denaturation step at 94ºC for 1 min, an annealing step at 50 ºC for 1min, and an elongation step at 72 ºC for 1.5min. The primer extension segment was extended to 7 min at 72 ºC in the final cycle. Amplification products were separated by electrophoresis on 1% agarose gel in 1x TBE buffer, stained by ethidium bromide and visualized under UV light. For the approximate quantification of the bands, we have been used a DNA GeneRuler^{TM}100 bp Plus Ladder was employed with primers ISSR-3, ISSR-5 and ISSR-10 and GeneRuler^{TM} DNA Ladder Mix was employed with the other primers. Fragments sizes were estimated relative to the DNA ladder.

Data Analysis

Scoring of ISSR data was achieved using 1% agarose gel electrophoresis profile. Clear and distinct fragments were scored as (1) for presence and (0) for absence.

Table 1. List of studied El-Wadi El-Gedid`s olive cultivars and genotype, country of origin and some traits

Code No.	Cultivar	Country of origin	Oil content	Fruit size	End use
1	Aggizi Aksi	Egypt	7-12%	Medium	Table
2	Unknown genotype	Unknown		Medium	Table
3	Coratina	Italy	18-22%	Medium	Oil
4	Koroneiki	Greece	18-24%	Small	Oil
5	Chemlali	Tunisia	15-20%	Very small	Oil
6	Manzanilla	Spain	15-20%	Medium	Table
7	Picual	Spain	15-22%	Medium	Oil&Table

Source: Ministry of Agriculture and land Reclamation. (2012) Agricultural Statistics, Volume: 2

Table 2. List of ISSR primers sequences and annealing temperatures Tm(Cº)

Primer	Sequence (5` - 3`)	Tm (Cº)
ISSR-3	$TTT(TCC)_5$	56
ISSR- 5	$(AGC)_4AC$	46
ISSR-10	$(TCC)_5AC$	56
H-1	$(AG)_9A$	56
H-2	$(AG)_9T$	56
H-3	$(AG)_9C$	58
H-4	$(AG)_9G$	58
H-10	$(AC)_9T$	56
H-21	$(AC)_9A$	56
H-24	$(CA)_9G$	58

DNA Extraction and Purification

Total genomic DNA for ISSR analysis, was extracted and purified from fresh young leaves of ten olives trees chosen randomly per cultivar and genotype according to DNeasy kit (Qiagen). All leaves of the ten plants for a single experiment were bulked prior to extraction. DNA was quantitated by UV-spectrophotometer at a wave length of 260-280 mm and 0.8% agarose gel electrophoresis.

Binary matrix was used to estimate genetic similarity coefficient (GS) between two cultivars (Table 5) by employing Dice coefficient (Nei and Li, 1979). These similarity coefficients were used to generate a dendrogram (Fis 2) by mean of the unweighted pair group method of arithmetic means (UPGMA) (Sneath and Sokal, 1973). All of the above analysis was performed using SPSS computer program version 8.

Table 3. Total number of amplicons, size of amplified fragments, monomorphic amplicons, polymorphic amplicons and the percentage of polymorphism among El- Wadi El-Gedid`s olive cultivars and genotype as revealed by ISSR markers

Primer	Total number of amplicon	Size of amplified fragments (bp)	Monomorphic amplicons	Polymorphic amplicons	Polymorphism %
ISSR-3	9	800-300	5	4	44.40
ISSR-5	11	1250-300	4	7	63.60
ISSR-10	6	1100-300	3	3	50.00
H-1	7	700-300	4	3	42.90
H-2	9	650-280	5	4	44.40
H-3	9	700-250	4	5	55.50
H-4	3	600-250	3	0	00.00
H-10	12	1500-180	4	8	66.60
H-21	13	800-160	8	5	38.40
H-24	13	950-100	6	7	53.8
Total	92		46	46	
Average	9.2		4.6	4.6	45.96

markers identified each olive cultivar of El-Wadi El-Gedid (based on 10 primers)

Cultivars	Primer	ISSR markers	Marker size pb	Total numer of markers
Coratina	H-3	- ve	400	2
	H-21	- ve	550	
Koroneiki	H-3	- ve	250	2
	H-21	- ve	800	
Chemlali	H-21	- ve	600	3
	H-24	+ ve	100	
	H-24	+ ve	240	
Manzanilla	H-2	- ve	280	1
Picual	H-21	+ ve	160	2
	H-24	- ve	500	

Table 5. Similarity matrix among the studied El-Wadi El-Gedid`s olive cultivars and genotype based on ISSR-PCR primers analysis

	Aggizi Aksi	Unknown genotype	Coratina	Koroneiki	Chemlali	Manzanilla	Picual
Aggizi Aksi	100						
Unknown genotype	93	100					
Coratina	83	85	100				
Koroneiki	84	83	83	100			
Chemlali	83	85	79	81	100		
Manzailla	84	86	81	82	84	100	
Picual	79	82	87	82	84	82	100

RESULTS

Cultivar and Genotype Identification by ISSR

In this study, molecular fingerprinting of olive cultivars and unknown genotype using 17 ISSR primers were tested to investigate the genetic diversity among El-Wadi El-Gedid`s local and foreign olive cultivars based on the clear scorable band patterns. Only ten (10) ISSR primers used could be reproducible bands, ranging in size from 100 to 1500 bp. In total 92 polymorphic bands with an average of 9.2 bands for each primer (Tables 3 and 4). The highest number of polymorphic bands was obtained by primers H-21 and H-24 (13 bands), while primer H-04 produced the lowest number of polymorphic bands (3 bands). The mean value of polymorphism was 45.96%, with the highest value for the primers H-10 (66.60), ISSR-5 (63.60%) and H-3 (55.50%) and the lowest value for the H-4 (00.00%) and primer H-21 (38.40%). These results are in agreement with other studies (Essadki *et al*., 2006, Gomes *et al*., 2009, Noormohammadi *et al*., 2012a and Terzopoulus *et al*., 2005). Out of 10 ISSRs four markers were found to be useful as cultivar and genotype specific markers which could be distinguish as unique bands for foreign olive cultivars (Table 4).

The maximum number of unique markers was identified with Chemlali (Tunisian cultivar) [unique positive bands of sizes 100 and 240 pb with primer H-24, in-addition to negative band of size 600 pb presented with primer H-24]. Primer H-21 produced the highest number of unique negative bands which can be used as Coratina, Chemlali and Koroneiki and Picual specific markers (Table 4). These findings are in harmony with Hegazi *et al*. (2012).

Genetic Relationships and Cluster Analysis

The ISSR- derived data were subjected to calculate the genetic distances among studied cultivars and unknown genotype, the similarity matrix was calculated using Dice coefficient (Nei and li, 1979) (Table 5). These similarity coefficients were used to generate a dendrogram (Fig.2) by UPGMA analysis in order to determine the grouping of different cultivars and genotype. From similarity matrix, the genetic similarity coefficient varied between 0.79 and 0.93 with the average of 0.876. The lowest genetic similarity (GS) value 0.79 derived between Coratina (Italian cultivar) and Chemlali (Tunisian ones) while, the highest GS value 0.93 found between the local variety Aggizi Aksi and the unknown genotype. ISSRs dendrogram obtained by UPGMA analysis grouped the local cultivar and the

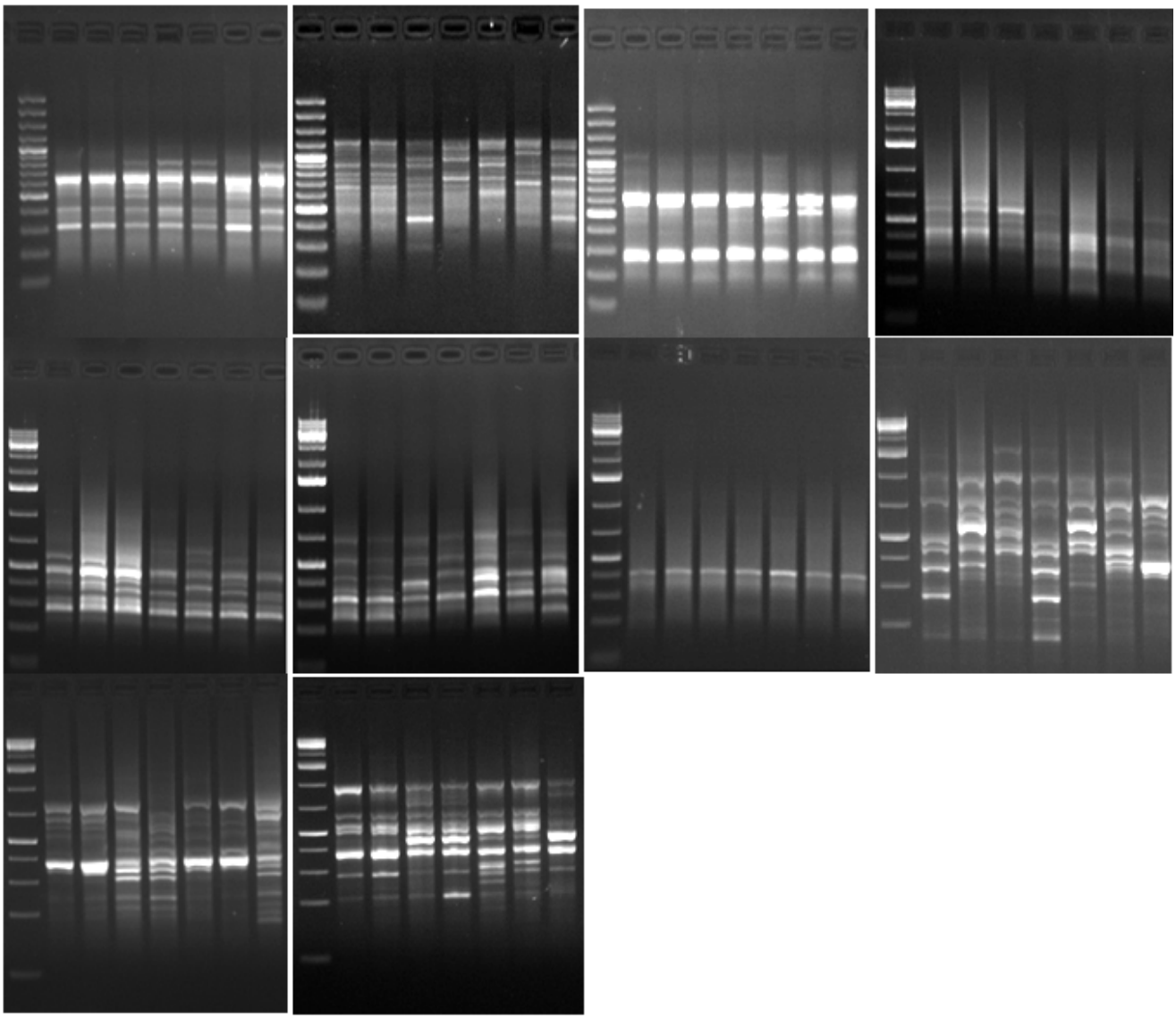

Figure 1. Inter simple sequence repeat (ISSR) amplification pattern obtained from DNA of the studied El-Wadi El-Gedid`s olive (*Olea europaea* L.) cultivars and unknown genotype (Lanes 2-8) generated by 10 ISSR primers (ISSR-3, ISSR-5, ISSR-10,H-1,H-2,H-3,H-4,H-10,H-21 and H-24,respectevily) M: GeneRuler^{TM}100 bp Plus DNA Ladder was used with primers ISSR-3, ISSR-5 and ISSR-10 & GeneRuler^{TM} DNA Ladder Mix was used with the other primers

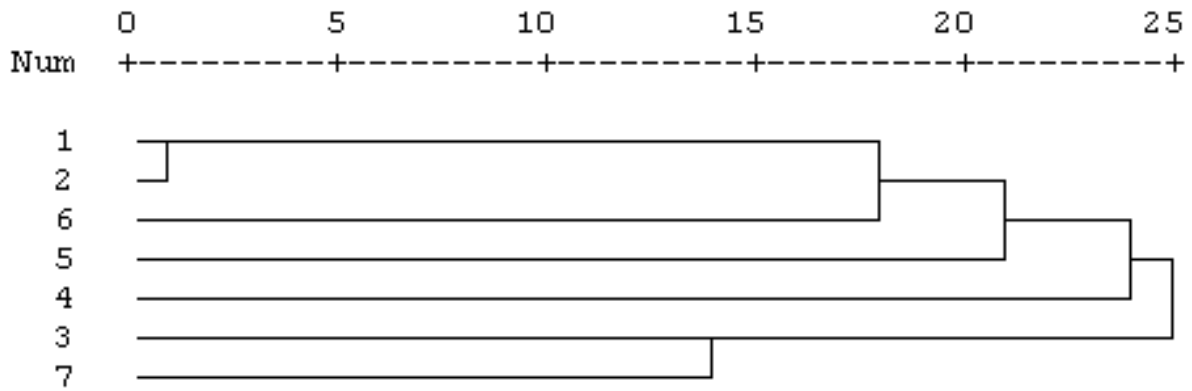

Fig. 2. UPGMA dendrogram showing genetic similarities among the studied El- Wadi El-Gedid`s olive (*Olea europaea* L.) cultivars and unknown genotype constructed using Nei & Li similarity coefficients based on ISSR markers.

unknown local genotype grown in El-Kharga, El-Wadi El-Gedid very close to each, in the same time grouped all foreigner cultivars near each other's and a part from the local ones. Several authors reported on the usefulness of ISSR for cultivar identifications (Hegazi *et al*., 2012; Belaj *et al.* 2002; Essadki *et al*. 2006; Omrani-Sabbaghi *et al.* 2007).

DISCUSSION

Olive is widely distributed and grown successfully under the prevailing conditions of El-Kharga Oases El-Wadi El-Gedid due to its ability to grow under the stress conditions. Using molecular markers techniques for studying genetic diversity among these cultivars is very important for any breeding program to achieve genotypes with higher olive oil quantity and quality and for conservation of these elite cultivars and genotypes. ISSR marker system was used to study the genetic diversity among sex (6) olive cultivars and one unknown genotype grown in El-Wadi El- Gedid, Egypt and showed high polymorphism rate (Table3) that is consistent with the results from previous reports on olive genetic diversity based on different molecular markers (Gomes *et al*., 2009; Souza *et al*., 2012; Abdelhamid *et al*., 2013; Asadiar *et al*., 2013; Trujillo *et al*., 2014). These results were tested repeatedly in different laboratories and showed stable results. This phenomenon clearly demonstrated that there is great diversity within El-Kharga,El- Wadi El-Gedid`s olive germplasm (foreign and local). Of the ten ISSR primers tested, 45.96% of polymorphic fragments were observed. The high efficiency of ISSR markers to identify cultivars has been previously reported in the genetic diversity of olive cultivars (Essadki *et al*., 2006; Gemas *et al*. 2004; Terzopoulos *et al*. 2005; El Saied *et al*., 2012, Yegenoglu1 and Sesli, 2017**,** Zhan *et al* 2015) and assess phylogenetic relationships in the *Olea europaea* complex (Hess *et al*. 2000; Gemas *et al*. 2004). 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 that the olive tree is a predominant allogamous species showing a high degree of out crossing, which leads to considerable levels of heterozygosity and DNA polymorphism among individuals (Carolyn *et al*., 2005, Khierallah *et al*., 2013, Lopes *et al*., 2007, Mohamed & yakoot 2014). Another contributing mechanism adding up to germplasm diversity could be the appearance of bud sports or somatic mutations occurring in such long-lived trees and reproduced through vegetative propagation.

ISSRs dendrogram obtained by UPGMA analysis grouped the local cultivar and the unknown local genotype grown in El-Kharga, El-Wadi El-Gedid very close to each, in the same time grouped all foreign cultivars near each other's and a part from the local ones. In this regard, Hegazi *et al*. (2012) showed that a high degree of genetic similarities among the Egyptian cultivars. Also, a correlation among olive cultivars and their putative areas of origin has also been reported (Angiolillo *et al*., 1999; Nikoloudakis *et al*., 2003; Hagidimitriou et al., 2005; Montemurro et al., 2005; Baldoni *et al*., 2006; Belaj *et al*., 2010; Albertini *et al*., 2011; Marra *et al*., 2013). In-addition, Newten 2006 reported that the modern Egyptian cultivars are significantly distinct from the rest of the foreign olive varieties. The clustering of the cultivars from the same or nearby region suggests a common genetic base and an autochthonous origin for these cultivars. This result agrees with the hypothesis of autochthonous origin of most of the olive cultivars as well as their limited diffusion from their centres of origin (Barranco and Rallo, 2000; Belaj *et al*.2001; Besnard *et al*. 2001a).So, the unknown genotype may be an Egyptian genotype. On the other hand, clustering of foreign olive cultivars in one cluster, indicate that grouping genotypes based on the geographic. In this regards, Besnard *et al.* (2001) found that olive genotypes from different countries clustered together within a group and they did not find any grouping based on their geographical origins. The result was similar to Poljuha *et a*l. (2008) who studied genetic diversity among Slovenian and Croatian olive cultivars and found that Croatian olive cultivars clustered with olive cultivars from Slovenia. Previous studies indicated that olive genotypes have been freely exchanged among collectors in different countries for centuries origin is not useful in olive. Fayek *et al*. (2014) Besnard *et al*. (2001), Contento *et al*. (2002) and Belaj *et al.* (2003) interpreted the high diversity found between Egyptian and foreign genotypes under study is probably due to a diverse germplasm origin that presumably results from crosses between wild and cultivated olive resulting in new cultivars in different parts of the Mediterranean, and low breeding pressures.

Conclusion

In conclusion, this study showed a high degree of genetic diversity among the studied olive (*Olea europaea* L.) (local and introduced) cultivars cultivated in El-Kharaga Oasis, El-Wadi El- Gedid and that ISSR markers are useful molecular tool to discriminate olive cultivars and evaluate genetic variation among studied cultivars. The high genetic 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 pressure (Besnard *et al*., 2001, Belaj *et al.*, 2003a, Contento *et al.*, 2002 and Hegazi *et al*., 2012). Genetic similarities also showed that the unknown genotype may be a local Egyptian one. The study of locally cultivated varieties is of immense importance, due to the fact that regional cultivars have been selected over the centuries for their adaptation to microclimate and soil type, resistance to parasites, and nutraceutical and gustative properties (Gismondi and Canini, 2013).This study is important for conservation of the studied elite local and introduced olive cultivars and genotype that sustain El-Kharga Oasis, El-Wadi El-Gedid harsh conditions and global changes through their precious genetic background. In the main time, these results are pre-requisite to develop more improved olive cultivars for new orchard establishment especially in new reclaimed area.

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