ISSR analysis for determination of genetic diversity in 29 olive (*Olea europaea* L.) cultivars

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Abstract

Olive tree is one of the most important oleaginous crops in the world, and known for having large genetic variability. Application of molecular markers is a suitable tool to investigate the genetic pool in crops. Therefore, in the current study 29 olive cultivars were tested by ten primers constituted by inter simple sequence repeats (ISSR). Plant materials were obtained from the collection of Tarom Agricultural Research Center in 2015. Genomic DNA was extracted from fresh leaves of 29 cultivars by a modified CTAB method. Ten ISSR primers were used and obtained data were scored. An analysis of primer informativeness showed that all primers were productive and effective in separating olive cultivars. Nevertheless, some of them were more effective such as UBC 855 and UBC 825 primers. According to cluster analysis and genetic distance, the olive cultivars were separated into three major clusters. Majority of the cultivars from southern and central Mediterranean such as; Italian, Spanish and Grecian olive cultivars clustered in one group. Results of the current study suggest that Mediterranean olive germplasm were structured into two main gene pools, which strongly matched two distinct geographic areas, *i.e.* western and central as well as eastern Mediterranean regions.

Key words: Cluster analysis, Genetic distance, Inter simple sequence repeats, Olive.

INTRODUCTION

The common olive tree (*Olea europaea* L.), belonging to the family Oleaceae, is a diploid species with cross-pollination (Bartolini *et al.,* 2005). Olive cultivation in the Mediterranean region started 6000 years ago. However, it is commonly suggested that the dry lands extending from the southern Caucasus to the Iranian plateau, and the Mediterranean coasts of Syria to Lebanon, are the origins of the olive tree (Zohary and Spiegel-Roy, 1975; Kiristsakis, 1998). Most new olive cultivars have been obtained from crossing wild plants by empirical selection of the growers in different regions (Besnard *et al.,* 2001). Olive has determined high levels of heterozygosis (Rallo *et al.,* 2000) as well as the accumulation of a number of mutations (Lopes *et al.,* 2004; Baali-Cherif and Besnard, 2005).

Because of the large number of olive cultivars, some of them have been given the same name without being genetically similar (homonymy), while others were named differently although, they were genetically closer together (synonymy) (Besnard *et al.,* 2001), this causes numerous problems for the management and conservation of germplasm (Hakim *et al.,* 2010). Germplasm characterization is a first fundamental step in starting the pre-breeding process, and molecular markers are a valuable method to identify olive genotypes.

Recently, molecular markers have been developed in olive. Molecular markers are proving to be an important way to increase selection efficiency compared to conventional, morphologically based methods. They provide an easy and precise way to access the genetic variability as well as polymorphisms at the DNA level without environmental interference (do Val *et al*., 2012). The utility of molecular tools for evolutionary studies arises from the insensitivity of the

genetic markers to environmental factors (Hannachi *et al.,* 2010).

Inter simple sequence repeat (ISSR)-PCR is a technique oriented from microsatellite regions and uses microsatellite sequences as primers (16-18 bp) in a polymerase chain reaction to generate multi locus markers. ISSR markers are useful in studies on genetic diversity, phylogeny, gene tagging and evolutionary biology (Reddy *et al.,* 2002).

Genetic variability of olive germplasm has been investigated by ISSR molecular markers (Hess *et al.,* 2000; Vargas and Kadereit, 2001; Pasqualone *et al*., 2001; Gemas *et al.,* 2004; Terzopoulos *et al.,* 2005; Essadki *et al.,* 2006; Martins-Lopes *et al*., 2007, 2009; Gomes *et al.,* 2008, 2009; Linos *et al.,* 2014; Brake *et al.,* 2014). ISSR markers amplify hyper variable non-coding regions (Esselman *et al.,* 1999). ISSR is a multi-locus profiling technique being able to distinguish differences between the species, cultivars and genotypes and determine genetic diversity (Karp *et al.,* 1997).

In the present study, an analysis of polymorphism among 29 olive cultivars was undertaken by ISSR markers. This will enable us to determine genetic groups or clusters to establish breeding programs that encompass genetic diversity.

MATERIALS AND METHODS

Plant material

A total of 29 olive cultivars from *O. europaea* L. were provided by Tarom Agricultural Research Center (Table 1).

DNA extraction

Genomic DNA was extracted from 100 mg of fresh leaves of 29 olive cultivars by modified CTAB extraction method (Doyle and Doyle, 1987). Leaf material was ground to a fine powder in liquid nitrogen in a 2 ml tube. After the addition of 1 ml of extraction buffer [100 mM Tris-HCl, 2 M NaCl, 20 mM EDTA, 2% (w/v) PVP, pH 8], and 50 µl β-mercaptoethanol, the mixture was homogenized and incubated at 65˚C for 60 min, mixed and vortexed thoroughly. Then, an equal volume of 24:1 (v/v) mixture of chloroform: isoamyl alcohol was added. After centrifugation at 11000 g for 20 min, the supernatant was separated and mixed with 0.7 (v/v) volumes of cold isopropanol (-20˚C) and after centrifugation at 10000 g for 5 min, the upper aqueous phase was decanted. Precipitated DNA was washed in 70% (v/v) ethanol, dried, and dissolved in 0.2 ml of double-distilled water. The quality of the DNA was determined on a 0.8% agarose gel.

ISSR fingerprinting

Ten ISSR primers were used (Table 2). The choice was based on the degree of polymorphism, as well as on clearness and reproducibility of the amplified DNA fragments. The 10 µl volume PCR reactions contained 10 ng of genomic DNA, 5 µl of a PCR kit (Sigma, St. Louis, MO, USA), 1.1 µl of primer, (100 pmol) and 2.5 µl of double distilled water. The amplification was performed in a Q-cycler thermocycler (HainLifescience, UK). The temperature profile consisted of an initial denaturation step at 94◦ C for 5 min followed by 35 cycles of denaturing step at 94 C◦ for 45 s, primer annealing at 52◦ C for 30 s (depending on the type of primers) and extension step at 72◦ C for 1 min. The final elongation step was set at 72◦ C for 10 min. The amplified products were separated on a 2% agarose gel. The agarose gel was stained with fluorescent dye (4 µg/ ml) in 1×TBE buffer. The ISSR bands were visualized under UV

Table 1. The studied cultivars and their origin.

NO.	Cultivar name	Origen	Use	NO.	Cultivar name	Origen	Use	NO.	Cultivar name	Origen	Use
1	Kalamata	Greece	double	11	Koroneiki	Greece	Oil	21	Roghani	Iran	Oil
2	Karydolia	Spain	double	12	M. de sevilla	Spain	Table	22	Mary	Iran	Table
3	Souri	Lebanon	Oil	13	Konservolia	Greece	double	23	Picudo	Spain	Table
4	Grossane	French	double	14	Voliotiki	Greece	Table	24	Shengeh	Iran	Table
5	Valanolia	Greece	Oil	15	Verdial de jaen	Spain	Table	25	Dezful	Iran	double
6	Amygdalolia	Greece	double	16	Frantoio	Italy	Oil	26	Cailetier	French	Oil
7	Kayssi	Syria	Table	17	Arbequina	Spain	Oil	27	Sevillana	Spain	Table
8	Abou-satl	Syria	Table	18	Picual	Spain	double	28	Oblonga	Greece	Oil
9	Mavi	Spain	double	19	M. de kaserna	Spain	Table	29	Coratina	Italy	Oil
10	Jlot	Syria	Table	20	Zard	Iran	double				

Table 2. Selected primers for ISSR analyses in 29 olive cultivars.

ISSR - primers	Repeat motif	Ta (℃)	Reference
UBC808	(AG)8C	54	Bahmani *et al*., 2015
UBC 811	(GA)8C	54	Brake *et al*., 2014
UBC 814	(CT)8A	52	Brake *et al*., 2014
UBC834	(AG)8YT	56	Bahmani *et al*., 2015
UBC 823	(TC)8C	54	Brake *et al*., 2014
UBC 860	(TG)8RA	54	Pivoriene *et al.,* 2008
UBC 826	(AC)8C	54	Brake *et al*., 2014
UBC 855	(AC)8YT	56	Brake *et al*., 2014
UBC 825	(AC)8T	52	Brake *et al*., 2014
UBC 827	(AC)8G	54	Pivoriene *et al*., 2008

Y = (CT); R = (AG); Ta= annealing temperature.

light and photographed with a digital camera. A 100 bp DNA molecular weight marker (New England Bio Labs, USA) was used as the standard size marker.

Data analysis

The data obtained from ISSR primers were scored according to the presence (1) or absence (0) of amplified products. Genetic distance was calculated using the Ward (minimum spherical cluster) dissimilarity index and Dice's similarity coefficient. The dissimilarity matrix was calculated, a weighted neighbor-joining tree obtained (Saitou and Nei, 1987) using the dissimilarity analysis and representation for windows (DARwin5) software (Perrier and Jacquemoud-Collet, 2006). Several genetic parameters were determined by GenALEx 6 (Peakall and Smouse, 2006). Polymorphism information content (PIC) of a band was computed according to Roldan-Ruiz *et al.* (2000) as follow:

$$PIC = 2fi\,(1 - fi)$$

Where PICi is the polymorphic information content of the locus i, fi is the frequency of the amplified fragments (band present) and 1 - fi is the frequency of non-amplified fragments (band absent). The frequency was calculated as the proportion between the number of amplified bands at each locus and the total number of accessions (excluding missing data). The PIC of each primer was calculated using the average PIC value from all loci of each primer:

$$PIC = \frac{1}{n}\sum_{i=1}^{n} PIC$$

Where n is the NPB for that primer. The marker index (MI) was calculated as described by Varshney *et al.* (2007):

$$MI = EMR \times PIC$$

$$EMR = \partial \times \beta$$

Where EMR is the product of the fraction of polymorphic loci (∂) and the number of polymorphic loci for an individual test (β).

The resolving power (RP) of each primer was calculated according to Prevost & Wilkinson (1999):

$$PR = \sum I_b$$

Where Ib represents the informative fragments. The I_b can be represented on a scale of 0-1 by the following formula:

$$I_b = 1 - [2 \times (0.5 - p)]$$

The genotype and allelic frequency data were used to compute the genetic diversity indices, i.e., (1) percentage of polymorphic loci [p%=(polymorphic loci/total loci)×100], (2) observed (na) and effective number of alleles (ne), where ne in the number of equally frequent alleles and (3) Shannon's Information Index (I) (Shannon and Weaver 1949) was calculated as:

$$I = -\sum pi \ln pi$$

Where pi is the allelic frequency of the ith allele in question for the specific cultivar and (iv) Nei's genetic diversity (h) (Nei 1973) derived from:

$$H_i = 1 - \sum_{j=1}^{n} {P_{ij}}^2$$

Where pi is the frequency of the ith allele at the locus and was calculated by PopGene program version 1.31 (Yeh *et al.,* 1999). For each locus, the Nei's index produces values between 0 and 0.5, while the Shannon index varies from 0 to 0.73 according to a natural log scale (Lowe *et al.*, 2004):

$$\%\, p = \frac{np}{n} \times 100$$

Where p is percentage of polymorphic bands, np is the number of polymorphic bands and n is polymorphic loci.

RESULTS

Ten primers were able to generate 55 scorable markers (51 of which were polymorphic) with 92.73% polymorphism and an average of 5.5 polymorphic bands per primer (number of loci/assay unit). The primers UBC 855 and UBC 825 produced the highest NPB (9 and 8, respectively), and primers UBC834 produced the smallest (3), with an average of 5.5 NPB per primer. The most of the primers showed 100% polymorphism with the exception of UBC 814, UBC 860, and UBC 826 (60%, 75% and 75%, respectively).

In ISSR analysis, the mean value of Nei's gene diversity index (H) varied from 0.11 (UBC 814) to 0.34 (UBC 823), (Figure 1) with an average of 0.23. The Shannon's index average was 0.37 for all primers, the lowest value belonged to UBC 814 (0.16), and the highest value belonged to UBC834 (0.50). The prominent mean number of effective alleles (Ne) was obtained by UBC 825 and UBC 855 (13.38 and 12.62, respectively, Table 3). ISSR markers efficiency can also be evaluated by parameters such as PIC, MI and RP for ISSR. Parameters such as PIC have been used increasingly for assessing the informative potential of ISSR markers (Gomes *et al*., 2009), these values can range from 0 for monomorphic markers to 0.5 for markers that are present in 50% of accessions and absent in the other 50% (Thimmappaiah *et al*., 2009).

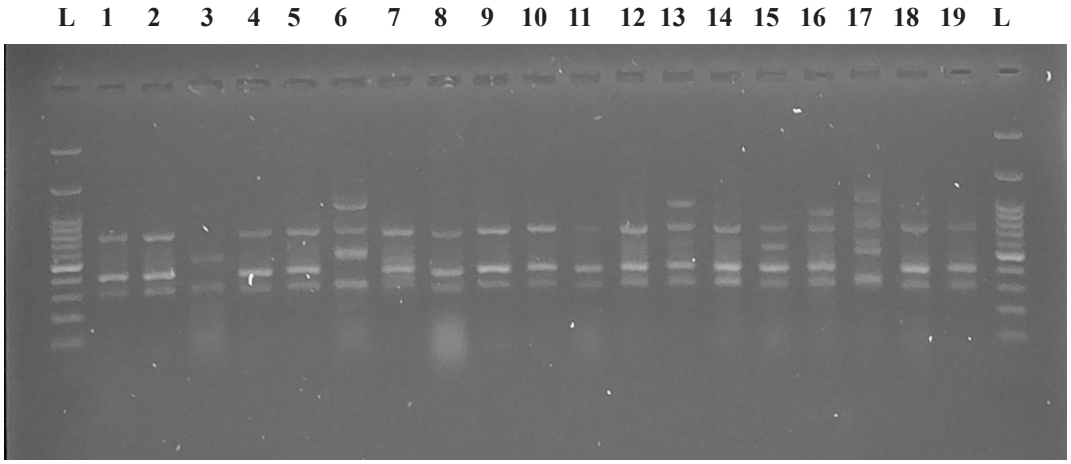

Figure 1. Agarose gel showing the electrophoretic patterns of the examined *O. europaea* cultivars with primer UBC 823.

Table 3. Comparison of infromativeness obtained with ISSR markers in 29 *Olea europaea* cultivars.

ISSR primers	NSB	NPB	Na	Ne	H	I	PIC	EMR	P (%)	MI	RP
UBC808	5	5	10	7.21	0.29	0.46	0.41	5	100 %	2.05	3.4
UBC 811	5	5	10	7.74	0.31	0.47	0.25	5	100 %	1.25	5.6
UBC 814	5	5	8	6.03	0.11	0.16	0.09	5	60 %	0.45	5.7
UBC834	5	3	6	4.4	0.33	0.50	0.35	3	100 %	1.05	2.8
UBC 823	6	6	12	8.84	0.34	0.45	0.34	6	100 %	2.04	4.9
UBC 860	5	4	7	5.02	0.19	0.31	0.29	3.2	75 %	0.928	3.7
UBC 826	5	4	7	5.73	0.13	0.23	0.21	3.2	75 %	0.672	3.2
UBC 855	9	9	18	12.62	0.26	0.42	0.38	9	100 %	3.42	5.5
UBC 825	10	10	20	13.38	0.21	0.33	0.16	10	100 %	1.6	7.9
UBC 827	4	4	8	5.01	0.18	0.32	0.16	4	100 %	0.64	1.6
mean	5.9	5.5	10.6	7.59	0.23	0.37	0.27	5.12	92.73 %	1.38	4.4

NSB: Number of scored bands; NPB: Number of polymorphic bands; Na=observed number of alleles, ne=effective number of alleles (Kimura and Crow, 1964) H=Nei's (1973) gene diversity, I=Shannon's Information index [Lewontin (1972)], PIC= polymorphic information content, P=percentage of polymorphic bands, MI=Marker index, EMR=effective multiplex ratio, RP= Resolving power.

The mean of PIC values for all loci of each ISSR primer was analyzed to determine PIC values of each ISSR primer. The range of PIC was between 0.09-0.41. This factor must be between 0-0.5 when using dominant marker (RAPD-ISSR), high value means the primer is good for genetic diversity among samples (Kayis *et al*., 2010). The highest PIC obtained was 0.41 and 0.38; by the primers UBC808, UBC 855, and an average of 0.27 per primer.

To determine the general usefulness of markers, MI (marker index) was calculated for each ISSR primer. The marker index (MI), which can be considered to be general measure of efficiency for detecting polymorphism, ranged from 0.45 to 3.42 for UBC 814 and UBC 855 primers (average 1.38) in different ISSR primers. There was a positive correlation between the values of MI and PIC, which means a greater MI was associated with greater values of PIC.

An important property of a good marker system is the capacity to distinguish among different accessions. The resolving power (RP) indicates potential of the primers chosen. The average RP was obtained 4.4 per ISSR primer (Table 3).The highest RP value was observed with the ISSR primer UBC 825 (7.9) and the lowest with the ISSR primer UBC 827 (1.6). ISSR marker data showed that all of them were productive and effective for separating olive cultivars. However, some of them were more effective such as UBC 855, and UBC 825 primers. Finally, it can be stated that, the ISSR technique provided an efficient assessment of genetic variability in *Olea europaea*.

Cluster analysis

According to Figure 2 the olive cultivars were separated into three main clusters. The first cluster (A) contained 20 cultivars, subdivided into two subgroups. Interestingly, the first one (A1) included twelve cultivars such as; Zard, Arbequina, Amygdalolia, Picual, Roghani, Oblonga, Koroneiki, Cailetier, M. de kaserna, Coratina and Karydolia. These cultivars originated from Iran, Spain, Greece, Italy and France. The remaining cultivars constituted the second subgroup (A2) such as; Picudo, Kalamata, M. de sevilla, Sevillana, Souri, Dezful, Grossane and Mary. These cultivars originated from Spain, Greece, Lebanon, and France. The second cluster (B) composed of five cultivars such as; Verdial de Jaen, Voliotiki, Konservolia, Frantoio and Mavi. These cultivars originated from Greece, Spain, Lebanon, and France. The third included (C) three cultivars such as; Kayssi, Abou-satland Jlotall originating from Syria as well as Valanolia from Greece.

Results of the current study suggest that Mediterranean olive germplasm was structured into two main gene pools, which strongly matched two distinct geographic areas, i.e. western and central as well as eastern Mediterranean regions. On the basis of our study, most cultivars clearly clustered according to their geographic origin. Majority of the cultivars from southern and central such as; Italian, Spanish and Grecian olive cultivars clustered in one group. However, some cases are excluded due to human migration from one area to another. Also, this variation could be due to the fact that woody perennial outbreeding species maintain most of their variation within a population by cross pollination (Belaj *et al.,* 2003).

Numerous genetic studies have reported genetic differentiation between western and eastern Mediterranean areas (Besnard *et al*., 2002, 2007; Breton *et al*., 2006; Sarri *et al*., 2006; Linos *et al*., 2014; Breton *et al*., 2006; Erre *et al*., 2010). Besnard *et al.* (2002b) stated that chloroplast sequences highlighted a strong differentiation between eastern and western parts of the Mediterranean area. There is a clear distinction between western (Spanish cultivars) and eastern Mediterranean (Syrian cultivars) gene pools (Besnard *et al*., 2002, 2007; Breton *et al*., 2006). The cluster analysis based on ISSR markers (Figure 2) showed a similar topology. For instance, most of Spanish cultivars clustered in Group A as well as Syrian cultivars clustered in Group C. Therefore, ISSR markers can be valuable for distinguishing olive cultivars based on their origin. Besnard *et al.* (2001c) cited that cultivated olive has been selected from different gene pools from both eastern and western regions of the Mediterranean Basin. Also, evolution of oleaster populations (wild olive) in the western and eastern Mediterranean by allozymes (Lumaret and Ouazzani, 2001; Lumaret *et al.*, 2004), inter-simple sequence repeats (ISSRs; Vargas and Kadereit, 2001) and simple sequence repeats (SSRs) (Breton *et al.*, 2006), have shown a clear distinction between eastern and western Mediterranean oleasters (Besnard *et al.*, 2002*b*; Lumaret *et al.*, 2004). But some of western cultivars did not separate according to their cultivation area. This could be the result of allogamous, self-incompatible, cross-incompatible, natural selection and vegetative propagation by growers (Guerin and Sedgley, 2007).

Genetic distance

The similarity coefficient among cultivars of *O. europaea* ranged from 0.12 (between Kayssi and Abou-satl as well as Cailetier and M. de kaserna; Syrian cultivars) to 0.68 (between Picudo and Kayssi).

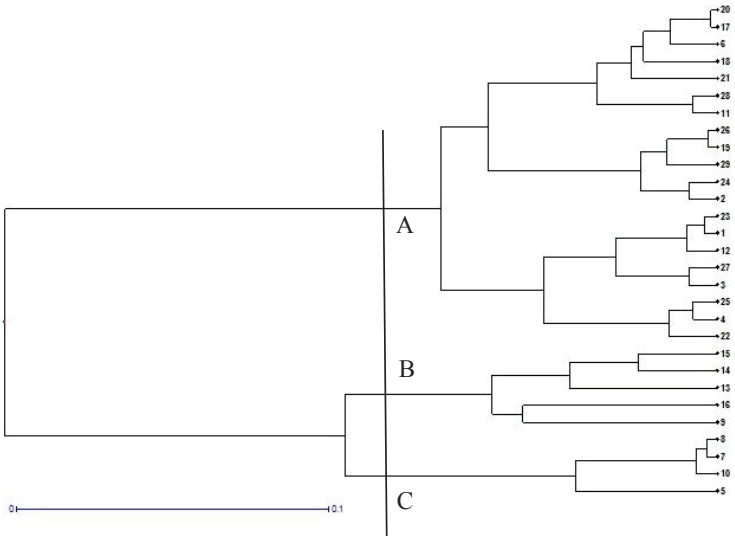

Figure 2. Dendrogram of 29 *Olea europaea* cultivars based on Ward's similarity coefficient.

These results are in agreement with cluster analysis data since these cultivars are from western and eastern Mediterranean area (Table 4). Belaj *et al*., 2002 cited that differences obtained between the eastern and western regions by RAPD marker were significant (φst=0.055; p<0.001). More recently, data from DNA markers have been used in estimating genetic distances and forecasting heterosis in plant breeding. For breeding programs, parents which have a high genetic distance from each other would help design breeding programs. Genetic distance is a valuable yardstick for selecting parents in hybrid breeding. The chance of heterosis increases with increasing genetic distance of the parents. Heterosis, or hybrid vigor, is used to describe the phenomenon that the hybrid progenies of diverse inbred varieties present an increase in growth rate, yield, fertility, tolerance to disease and pests, adaptations environmental stress, and other changes in desirable agronomic traits (Shull, 1988). ISSR markers will be useful for identifying olive cultivars and perform genetic studies interest to breeding and conservation programs. In recent times, the diversification of olive varieties, have advanced genetic implement at the molecular level in most of the olive-growing countries (Baldoni and Belaj, 2010).

Genetic structure

To identify the genetic structure in olive germplasm, a model-based analysis was performed using STRUCTURE 2.3.4 (Pritchard *et al*., 2000). The STRUCTURE algorithm was run using 10 independent replicate runs per *K* value from 1 to 10. Each run involved a burning period of 50 000 iterations. Based on the highest DK obtained from structure harvester, *K*=2

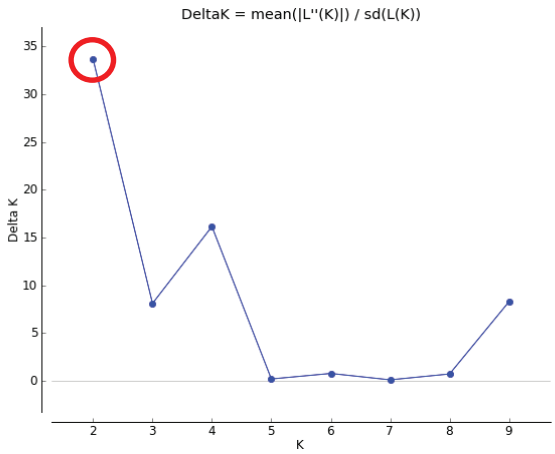

Figure 3. Subgroup determination using the Structure harvester. The vertical and horizontal axes show the values of the ΔK and number of sub-populations respectively.

(Δ*K*=) appeared to be the best model for olive genetic structure (Figure 3). According to the *K*=2 model, olive germplasm structured into two gene pools (Figure 4). Twenty cultivars were assigned to the first group (I, red) such as; Kalamata (Greece), Karydolia (Spain), Grossane (French), Mavi (Spain) Koroneiki (Greece), M. de sevilla (Spain), Konservolia (Greece), Verdial de jaen (Spain), Arbequina (Spain), Picual (Spain), M. de kaserna (Spain), Zard (Iran), Roghani (Iran), Mary (Iran), Picudo (Spain), Shengeh (Iran), Dezful (Iran), Cailetier (French) Sevillana (Spain), Oblonga (Greece) and Coratina (Italy). This group was the main group by 68.96% of cultivars. The second group (II, green) contained four cultivars such as; Kayssi (Syria), Abou-satl (Syria), Jlot (Syria) and Frantoio (Italy) and

Table 4. Dice's similarity coefficient among 29 *Olea europaea* cultivars based on ISSR data analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
2	0.33																											
3	0.29	0.33																										
4	0.38	0.29	0.24																									
5	0.61	0.66	0.51	0.49																								
6	0.31	0.27	0.31	0.26	0.38																							
7	0.39	0.51	0.27	0.35	0.33	0.28																						
8	0.32	0.43	0.29	0.36	0.43	0.23	**0.12**																					
9	0.53	0.53	0.37	0.50	0.48	0.59	0.54	0.52																				
10	0.40	0.44	0.24	0.36	0.30	0.22	0.14	0.17	0.45																			
11	0.35	0.43	0.43	0.35	0.54	0.36	0.36	0.37	0.56	0.42																		
12	0.18	0.32	0.27	0.32	0.62	0.33	0.42	0.35	0.53	0.43	0.29																	
13	0.44	0.56	0.44	0.40	0.59	0.51	0.41	0.50	0.54	0.47	0.35	0.44																
14	0.38	0.33	0.33	0.38	0.58	0.39	0.39	0.47	0.49	0.40	0.44	0.46	0.33															
15	0.40	0.32	0.40	0.45	0.49	0.49	0.45	0.45	0.35	0.35	0.46	0.43	0.47	0.32														
16	0.40	0.56	0.48	0.38	0.49	0.45	0.41	0.41	0.50	0.42	0.58	0.48	0.63	0.40	0.42													
17	0.27	0.36	0.41	0.26	0.41	0.24	0.38	0.38	0.59	0.35	0.19	0.35	0.44	0.46	0.43	0.57												
18	0.30	0.26	0.30	0.30	0.56	0.27	0.45	0.33	0.39	0.38	0.32	0.23	0.42	0.40	0.42	0.51	0.28											
19	0.30	0.17	0.39	0.30	0.54	0.28	0.49	0.41	0.56	0.38	0.36	0.24	0.47	0.44	0.25	0.54	0.29	0.22										
20	0.35	0.26	0.30	0.20	0.44	0.19	0.40	0.41	0.61	0.38	0.23	0.33	0.47	0.35	0.50	0.58	0.10	0.27	0.32									
21	0.30	0.21	0.26	0.35	0.61	0.32	0.55	0.53	0.52	0.42	0.41	0.33	0.48	0.35	0.42	0.56	0.33	0.32	0.37	0.22								
22	0.37	0.37	0.32	0.26	0.47	0.43	0.38	0.47	0.29	0.40	0.38	0.41	0.45	0.37	0.35	0.58	0.30	0.33	0.38	0.33	0.33							
23	0.13	0.39	0.26	0.30	0.64	0.36	**0.68**	0.30	0.39	0.38	0.41	0.19	0.53	0.49	0.38	0.50	0.38	0.37	0.36	0.45	0.41	0.28						
24	0.28	0.19	0.40	0.22	0.65	0.33	0.46	0.45	0.57	0.51	0.33	0.35	0.43	0.32	0.35	0.59	0.30	0.38	0.24	0.29	0.38	0.35	0.33					
25	0.24	0.38	0.29	0.18	0.42	0.35	0.30	0.32	0.49	0.40	0.30	0.22	0.45	0.48	0.36	0.53	0.22	0.30	0.26	0.30	0.45	0.21	0.21	0.32				
26	0.38	0.24	0.38	0.18	0.42	0.35	0.39	0.40	0.54	0.40	0.35	0.32	0.45	0.48	0.28	0.36	0.27	0.30	**0.12**	0.30	0.45	0.26	0.35	0.23	0.14			
27	0.27	0.27	0.19	0.30	0.60	0.21	0.28	0.23	0.48	0.30	0.28	0.25	0.50	0.35	0.44	0.44	0.29	0.28	0.36	0.24	0.36	0.42	0.28	0.33	0.31	0.43		
28	0.30	0.30	0.39	0.35	0.54	0.28	0.40	0.33	0.50	0.33	0.18	0.24	0.47	0.44	0.42	0.50	0.24	0.27	0.23	0.27	0.37	0.38	0.36	0.33	0.30	0.35	0.24	
29	0.43	0.27	0.43	0.40	0.57	0.32	0.48	0.40	0.54	0.41	0.32	0.29	0.57	0.57	0.41	0.65	0.33	0.32	0.19	0.32	0.45	0.43	0.40	0.33	0.39	0.26	0.32	0.23

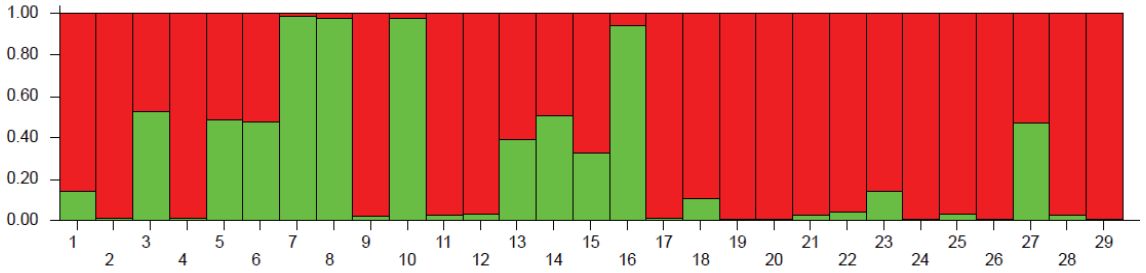

Figure 4. BAPS analysis on *Olea europaea* L. genotypes. Each vertical bar represents one individual genotype.

mixture cultivars were Souri (Lebanon), Valanolia (Greece), Amygdalolia (Greece), Voliotiki (Greece) and Sevillana (Spain).

The result showed that, most of the cultivars in the first and second groups were Spanish and Syrian, respectively. Structure analysis reflected the WARD clustering and genetic distance. The results of structural analysis showed differences between the western (Spain) and eastern (Syria) genetic pools. BAPS analysis evidenced a certain distance between the gene pools. Our results are in overall agreement with previous result of Besnard *et al*. (2002, 2007); Breton *et al*. (2006); Sarri *et al*. (2006); Linos *et al.* (2014), Breton *et al.* (2006); Erre *et al.* (2010).

The genetic diversity data are helpful for the verification of synonyms and homonyms and determination of misidentified cultivars. ISSR markers can be used in order to specify the gaps in the gene pools and organize the future additions. The application of the ISSR approach enables us to predict confident correlation between molecular marker data and morpho-agronomical descriptors on a species that is expected to attract much attention in the near future. Grouping genotypes based on cluster analysis and genetic structure indicated that genetic variations were in agreement with the geographical distribution of cultivars. Finally, the results of the genetic diversity would be useful to develop a breeding program. In conclusion, the molecular markers used here were shown to be useful for the identification of olive cultivars. They demonstrated that the western (Spanish cultivars) and eastern Mediterranean (Syrian cultivars) gene pools were separate.

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