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Population genetic studies in wild olive (*Olea cuspidata*) by molecular barcodes and SRAP molecular markers

Rayan Partovi[1], Alireza Iranbakhsh[1,*], Masoud Sheidai[2], Mostafa Ebadi[3]

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2] *Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, Tehran, Iran*
[3] *Department of Biology, Islamic Azad University, Damghan Branch, Damghan, Semnan Province, Iran*
*Correspondign author. E-mail: iranbakhsh@iau.ac.ir

Abstract. Olive is an important horticultural plant having both cultivated and wild forms. The aim of the present study was investigating genetic diversity of 13 wild olive trees belonging four geographical populations in IRAN using SRAP neutral molecular markers as well as cp-DNA rpl intergenic sequences and ITS region. Genetic diversity parameters determined for 76 SRAP loci within the studied olive populations identified the most variable loci. Population differentiation parameters determined for SRAP loci, identified 13 SRAP loci with Gst value of 1, that means they differentiate the studied trees. PCoA analysis based on SRAP data separated olive trees from each other due to genetic difference. Distribution of the samples in PCoA plot indicated that the population 1 are more spread due to population genetic variability. However, the SRAP result reveals that these molecular markers can be used in population genetic investigations and germ plasm analysis. AMOVA showed significant genetic difference among the studied olive populations. Cp-DNA analysis produced 366 bp long sequences, out of which 224 sites were segregating among the studied plants. The mean nucleotide diversity was 0.32. TCS network based on cp-DNA separated most of the studied populations. Therefore, it seems that cp-DNA rpl sequences is a suitable barcode molecular marker for population genetic studies. Phylogenetic tree of ITS data could partially differentiate wild olive population. In conclusion, a combined use of SRAPs and cp-DNA sequences are suggested for wild olive population genetic investigation.

Keywords. SRAP, cp-DNA, ITS, Population genetic, Olive.

INTRODUCTION

Olive tree (*O. europaea* L.) of the genus Olea (*O. europaea* subsp. *europaea* var. *europaea*) is one of the most important horticultural crop plants. It is an ancient plant species with grate economic value (Zohary and Hopf 2000), and has both cultivated and wild forms. Oleaster (*O. europaea* sub-

sp. *europaea* var. *sylvestris* Miller) is the Mediterranean wild olive and is possibly the progenitor of the cultivated olive. The non-Mediterranean wild olives are geographically isolated from the oleaster and show different morphological characters. Green (2002) grouped all morphological forms of wild olive in a single aggregate i.e. *Olea europaea* subsp. cuspidata, but the other investigators consider these intra-specific forms as ecotypes both in Africa and Iran (Besnard *et al*. 2002; Sheidai *et al*. 2010).

The occurrence of natural hybridization has been reported between different sub spices within the genus *Olea*. This holds true also for *O. cuspidate* and *O. africana* (Besnard and Bervill 2000). Moreover, (Omrani-Sabbaghi *et al*. 2007) suggested hybridization of subsp. *cuspidata* and the cultivated olive in South Africa and Iran and (Sheidai *et al*. 2010) identified a population with intermediate morphological and molecular (RAPDs) characteristics.

The wild relatives of crop plants (CWRs) constitute an important resource for improving agricultural production and for maintaining sustainable agro-ecosystems. Genetic material from CWRs has been utilized by humans for to improve the quality and yield of crops. For example, wild maize (*Zea mexicana*) is routinely grown alongside maize to promote natural crossing and improve yields. More recently, plant breeders have utilized CWR genes to improve a wide range of crops like rice (*Oryza sativa*), tomato (*Solanum lycopersicum*) and grain legumes *(*Hajjar and Hodgkin 2007*).* Therefore, A CWR can be defined as "a wild plant taxon that has an indirect use derived from its relatively close genetic relationship to a crop. The CWRs comprise a wonderful gene pool for future crop breeding programs.

Since natural populations of CWRs are at risk and are threatened by habitat loss, deforestation, etc., population genetic study of these natural populations is important task as it provides insight about the genetic variability, population genetic structure, gene flow versus population fragmentation as well population genetic differentiation. The obtained information can be utilized in both breeding as well as conservation strategies of the CWRs.

Recent population genetic studies use different molecular markers to investigate the genetic diversity as well as other population genetic features. This is also true for olive (Bracci *et al*. 2011), for example, Random Amplified Polymorphic DNA (RAPDs) (Sheidai *et al*. 2010) microsatellite (simple sequence repeat; SSRs) and inter simple sequence repeat; ISSR markers, Amplified Fragments Length Polymorphic markers (AFLPs) (Baldoni *et al*. 2006), cp-DNA (Besnard *et al*. 2011).

In the present study, genetic diversity, genetic divergence and genetic structure of four populations of *O. europaea* subsp. *cuspidata* from different localities are investigated using nrDNA ITS (Internal Transcribed Spacer) and SRAP (sequence-related amplified polymorphism) markers.

Since, SRAP marker technique combines easiness, reliability, high variability, moderate throughput ratio and superficial sequencing of the selected bands, we used this technique to amplify coding regions of DNA to target open reading frames.

MATERIALS AND METHODS

Thirteen specimens belonging to four geographical populations of subspecies *Olea europaea* subsp. *cuspidata* L. were collected from different localities that were placed between three provinces Bakhtiari, Boyer-Ahmad and Khuzestan. Details of geographical populations are given in Table 1.

DNA extraction and PCR reactions

DNA was extracted from dried leaf specimens (approximately 0.5 g material per sample) using CTAB (Cetyl trimethyl-ammonium bromide) activated charcoal protocol (Krizman *et al*. 2006 and Sheidai *et al*. 2013). Extracted DNA was run on 0.8% agarose gel. PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 mg genomic DNA and 1 U of Taq DNA polymerase (Bioron, Germany).

Table 1. List of 13 specimens of *Olea europaea* subsp. *cuspidata* L. from four populations accompanied by their distribution, altitude, longitude and herbarium number.

No.	Localities	Altitude	Latitude	Longitude	Voucher no.
1	Chaharmahal and Bakhtiari Province, Dehedz – Lordgan, Iran	1713	31°31'18"	50°28'26"	HSBU2018700
2	Kohgiluyeh and Boyer-Ahmad Province, Khersaan Road, Iran	1380	31°26'59"	50°28'57"	HSBU2018705
3	Chaharmahal and Bakhtiari Province, Lordgan, Monj, Gachahan, Iran	1151	31°26'48"	50°32'19"	HSBU2018711
4	Chaharmahal and Bakhtiari Province, Lordgan, Monj, Gachahan, Iran	1592	35°55'41"	57°41'53"	HSBU2018712

SRAP study

Five sequences related amplified polymorphism (SRAP) primer pairs including forward primers: Me1, Me2, Me3, Me4, Me5 and reverse primers: Em1, Em2, Em3, Em4, Em5 were used (Feng *et al*. 2014). PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 1 U of Taq DNA polymerase (Bioron, Germany). Following programs used for amplification of SRAP region in a PCR reaction: 94°C, 1 min, 35°C, 1 min, and 72°C, 1 min for first five cycles then 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 55°C and 2 min at 72°C and a final extension at 72°C for 7-10 mi

ITS study

The complete ITS region was amplified using forward ITS5 (5'- GGA AGT AAA AGTCGT AAC AAG G- 3') and reverse primers ITS4 (5'- TCC GCT TAT TGA TAT GC-3') (White *et al*. 1990). Following program used for amplification of nuclear region in a PCR reaction: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 53.5°C and 2 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C.

Cp- DNA study

The intergenic spacer of chloroplast genome rpl16 was amplified and sequenced with universal primers following the methodology of (Shaw *et al*. 2005; Timme *et al*. 2007). Each 20 µl of PCR tube contained 10 µl of 2x PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 Unit of Taq DNA polymerase (Bioron, Germany), and 1 µl of template genomic DNA at 20 ng µl-1 . The amplification reaction was performed in Techne thermocycler (Germany) with the following program: 2 min 94°C, 1 min at 94°C; 1 min at 54°C and 1min at 72°C. The reaction was completed by final extension step of 6 min at 72°C.

Data Analyses

SRAP bands were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Data obtained were analyzed for the genetic diversity parameters like, Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Weising *et al*. 2005; Freeland *et al*. 2011). Principal coordinate analyses (PCoA) were performed using PAST ver. 2.17 (Hammer *et al*. 2012).

Nei's genetic distance was used among populations. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006).

The phylogenetic methods used to investigate the species relationships were Maximum parsimony (MP), Maximum likelihood (ML), Networking and Bayesian approaches. The ITS sequences were firstly aligned and used to test the proper nucleotide substitution model as applied in MEGA 7. Program (Tamura *et al*. 2012). Networking was performed using Splits Tree 4 program (Huson and Bryant 2006) and Bayesian analysis was done using BEAST software v1.6.1 (Drummond *et al*. 2012a, b).

RESULTS

In total, 76 SRAP bands were obtained in olive trees studied. Some of these bands were common while, few bands were private in these trees. For example, SRAP bands 51, 52 and 76 occurred only in trees of population 4, while SRAP band 7 happened only in one of the trees in population 1. Similarly, SRAP band 4 was observed in the tree of population 3.

Genetic diversity parameters determined for all SRAP loci within the studied *olive* populations identified the most variable loci. The loci with highest value of gene diversity (H) and Shanon information index (I) are the most diverse SRAP loci (Table 2). The mean value obtained for H = 0.34, while I = 0.52.

Population differentiation parameters determined for SRAP loci in the studied olive trees (Table 3), identified the loci with highest migration/ exchange value (Nm) and also SRAP loci with the highest differentiation value (Gst). In total, 13 SRAP loci had Gst value = 1, that means they differentiate the studied trees. Similarly, SRAP loci with Nm>1 are considered highly migrated among the populations.

PCoA analysis of the studied olive trees after 99 times permutation, based on SRAP data is presented in Figure 1. PCoA plot clearly separates olive trees of the studied populations from each other due to genetic difference. Distribution of the samples in PCoA plot indicates that olive trees of population 1 are more spread due to within population genetic variability. However, in general, the SRAP result reveals that these molecular markers can be used in population genetic investigations and germ plasm analysis of olive.

Nei' genetic distance determined among olive trees based on SRAP data (Table 4), revealed that the genetic distance among trees of the population 1 varies from

Table 2. Genetic variability parameters for SRAP loci studied in *olive* populations.

Locus	Sample Size	Ne	H	I
5	13	1.9187	0.4788	0.6718
8	13	1.9299	0.4818	0.6749
30	13	1.9683	0.4920	0.6851
36	13	1.9882	0.4970	0.6902
39	13	1.8989	0.4734	0.6663
53	13	1.9882	0.4970	0.6902
54	13	1.8943	0.4721	0.6650
55	13	1.9928	0.4982	0.6913
57	13	1.9562	0.4888	0.6819
58	13	1.9216	0.4796	0.6726
59	13	1.8943	0.4721	0.6650
64	13	1.8943	0.4721	0.6650
65	13	1.9865	0.4966	0.6898
67	13	1.9562	0.4888	0.6819
72	13	1.8943	0.4721	0.6650
Mean	13	1.5918	0.3492	0.5242
St. Dev	0.2911	0.1268	0.1506	

Ne = Effective number of alleles.
H = Nei's (1973) gene diversity.
I = Shannon's Information index [Lewontin (1972)].

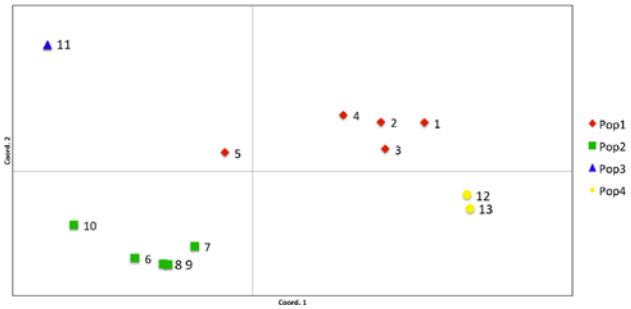

Figure 1. PCoA plot of olive trees based on SRAP data revealing genetic separation of populations.

0.30 to 0.54, while it varies from 0.46 to 0.76 in olive trees of population 2.

AMOVA showed significant genetic difference (Phipt = 0.43, P =0.01) among the studied olive populations. It also revealed that 43% of total genetic variation was due to among population genetic difference, whereas, 57% occurred due to within population genetic variability.

Cp-DNA analysis

We obtained 366 bp long sequences, out of which 224 sites were segregating among the studied plants. The

Table 3. Genetic differentiation parameters in the olive trees studied based on SRAP loci.

Locus	Sample Size	Ht	Hs	Gst	Nm
2	13	0.2633	0.2381	0.0958	4.7202
3	13	0.3921	0.3145	0.1981	2.0240
4	13	0.3750	0.0000	1.0000	0.0000
7	13	0.0514	0.0472	0.0813	5.6481
10	13	0.2633	0.2381	0.0958	4.7202
13	13	0.3750	0.0000	1.0000	0.0000
14	13	0.1669	0.1162	0.3036	1.1472
17	13	0.2000	0.1746	0.1270	3.4365
22	13	0.0514	0.0472	0.0813	5.6481
23	13	0.3750	0.0000	1.0000	0.0000
24	13	0.1064	0.0873	0.1791	2.2910
28	13	0.1794	0.1508	0.1595	2.6340
31	13	0.2086	0.1634	0.2164	1.8105
32	13	0.3750	0.0000	1.0000	0.0000
34	13	0.1000	0.0944	0.0557	8.4721
36	13	0.5000	0.0000	1.0000	0.0000
39	13	0.3750	0.0000	1.0000	0.0000
41	13	0.1064	0.0873	0.1791	2.2910
43	13	0.2086	0.1634	0.2164	1.8105
44	13	0.5000	0.0000	1.0000	0.0000
45	13	0.3750	0.0000	1.0000	0.0000
47	13	0.1794	0.1508	0.1595	2.6340
48	13	0.1669	0.1162	0.3036	1.1472
50	13	0.3750	0.0000	1.0000	0.0000
51	13	0.3750	0.0000	1.0000	0.0000
52	13	0.3750	0.0000	1.0000	0.0000
53	13	0.5000	0.0000	1.0000	0.0000
58	13	0.4226	0.3434	0.1875	2.1669
62	13	0.3625	0.2744	0.2431	1.5564
68	13	0.2086	0.1634	0.2164	1.8105
69	13	0.1000	0.0944	0.0557	8.4721
75	13	0.5000	0.0000	1.0000	0.0000
76	13	0.3750	0.0000	1.0000	0.0000
Mean	13	0.3680	0.1308	0.6445	0.2758
St. Dev	0.0172	0.0085			

mean nucleotide diversity (p) was 0.32. TCS network of the studied olive trees Figure. 2) separated most of the studied populations. For instance, trees of the population 2 and the population 4 were grouped together, while trees of population 1 were scattered in between these two populations. Therefore, it seems that cp-DNA (rpl16) sequences are a suitable barcode molecular marker for population genetic studies of olive.

There has been no report of rpl16 sequences for the cultivates olive. Therefore, we could not compare these two forms together.

Table 4. Nei genetic distance among the studied olives.

Pop	1	2	3	4	5	6	7	8	9	10	11	12
2	0.30											
3	0.39	0.42										
4	0.44	0.46	0.33									
5	0.54	0.40	0.52	0.42								
6	0.65	0.54	0.57	0.62	0.40							
7	0.56	0.62	0.66	0.64	0.65	0.54						
8	0.76	0.70	0.81	0.77	0.71	0.44	0.69					
9	0.65	0.64	0.68	0.73	0.67	0.49	0.57	0.57				
10	0.60	0.53	0.60	0.68	0.50	0.41	0.48	0.55	0.43			
11	0.60	0.55	0.73	0.60	0.57	0.65	0.80	0.82	0.69	0.53		
12	0.41	0.43	0.57	0.62	0.63	0.60	0.72	0.65	0.56	0.65	0.67	
13	0.47	0.46	0.57	0.52	0.63	0.63	0.68	0.69	0.56	0.68	0.71	0.17

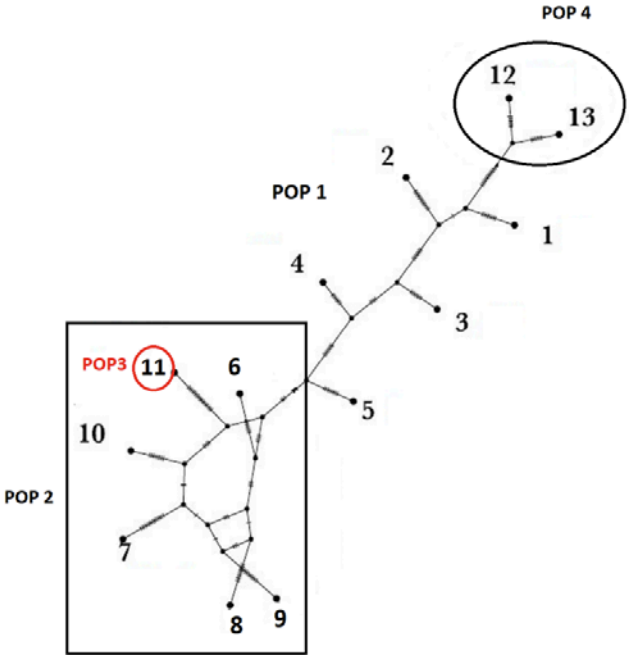

Figure 2. TCS network of olive trees based on cp-DNA sequences revealing almost separation of the studied populations.

ITS sequence analysis

We obtained 183 bp long sequences in ITS region with 150 variable sites. The analysis revealed the presence of 8 haplotypes in ITS with haplotype diversity, Hd: 0.80.

An Olive tree 7, 9, 11 and 13 had similar sequences and forms a single haplotype group.

The nucleotide distance (p distance) of the studied trees (Table 5), revealed that p distance among olive trees of population 1 varied from 0.36 to 0.54, while the same value in population varied from 0.01 to 0.49.

Maximum likelihood phylogenetic tree (ML) (Figure 3) of the studied olive trees based on ITS sequences revealed that, trees of population 1, differ in their ITS sequences and were grouped in a separate clade. However, trees of populations 2, 3, and 4 were placed together in a single unresolved clade. This result indicates that ITS sequences can be used along with cp-DNA barcodes in olive population genetic studies.

Comparing phylogenetic trees of SRAP markers, Cp-DNA and ITS sequences produced quart let distance = 0.56 and test performed based on the most agreeable sub-trees (MAST) (Figure. 4) revealed that, these markers do differentiate some of the olive trees and place them in distinct clades.

Joint phylogenetic ITS analysis of wild populations and randomly selected cultivated olives (Figure. 5) revealed the genetic separation of these olives from each other. ITS sequences could differentiate different olive trees of wild populations but not the cultivars from each other.

DISCUSSION

Genetic structure analysis of both cultivated and wild olive is important for breeding and conservation purposes (Baldoni *et al.* 2006). Olive cultivars can be considered as varieties of unknown origin, currently propagated vegetative by cutting or grafting. Analysis of nuclear and cytoplasmic DNA polymorphisms in Mediterranean oleaster populations has shown that eastern oleaster populations differ greatly from those of the west Mediterranean (Besnard *et al.* 2001), while the genet-

Table 5. P nucleotide distance among olive plants based on ITS sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	-												
2	0.53	-											
3	0.52	0.43	-										
4	0.56	0.50	0.36	-									
5	0.54	0.51	0.46	0.45	-								
6	0.49	0.36	0.25	0.36	0.33	-							
7	0.48	0.35	0.24	0.35	0.32	0.01	-						
8	0.48	0.35	0.24	0.35	0.33	0.01	0.00	-					
9	0.48	0.35	0.24	0.35	0.33	0.01	0.00	0.00	-				
10	0.48	0.35	0.24	0.35	0.33	0.01	0.01	0.01	0.01	-			
11	0.48	0.35	0.24	0.35	0.33	0.01	0.00	0.00	0.00	0.01	-		
12	0.48	0.35	0.24	0.35	0.32	0.01	0.00	0.00	0.00	0.01	0.00	-	
13	0.48	0.35	0.24	0.35	0.32	0.01	0.00	0.00	0.00	0.01	0.00	0.00	-

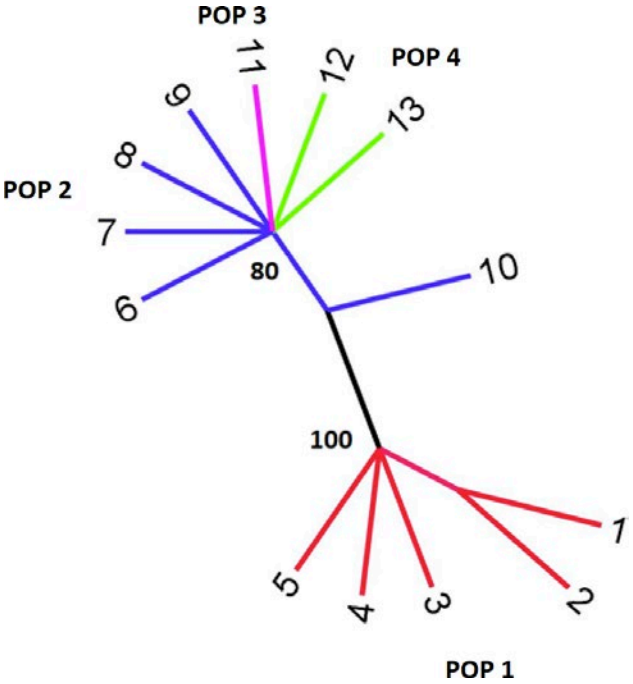

Figure 3. ML phylogenetic tree of the studied olive trees based on ITS sequences.

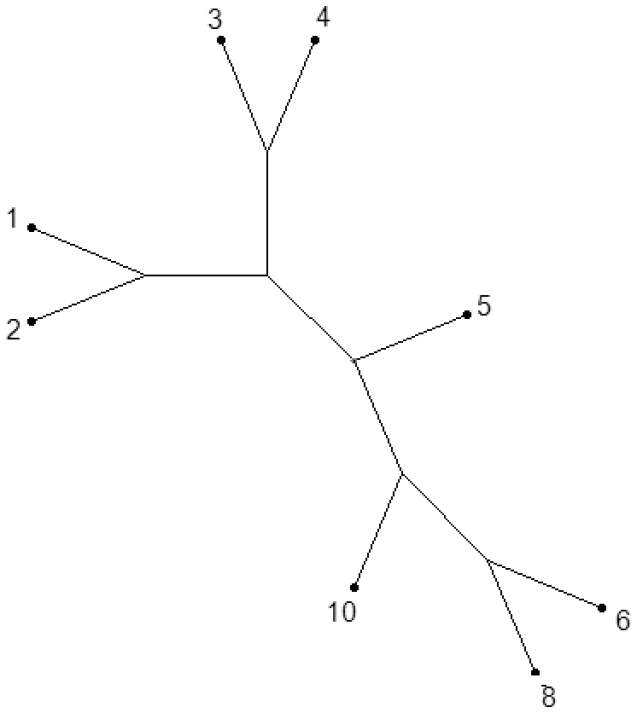

Figure 4. Most agreeable sub-trees (MAST) plot showing the common clades differentiated by ITS, Cp-DNA and ISSR trees.

ic diversity of cultivated populations shows a complex patchy pattern (Owen *et al.* 2005). The present investigation also revealed genetic difference between Iranian wild populations and the cultivated olive forms.

Based on the frequency and distribution of polymorphisms, several authors suggested that many olive cultivars have been produced from naturally cross-bred genotypes (Besnard *et al.* 2001), while, others, due to the great genetic distance between populations of wild olives and cultivars, suggested that many local cultivars may have an allochthonous origin (Angiolillo *et al.* 1999; Bronzini de Caraffa *et al.* 2002).

Genetic diversity of both cultivated and wild olives has been investigated by using different molecular markers (Bracci *et al.* 2011), revealing the genetic structure of these olive forms. In the present study, SRAP and cp-DNA rpl sequences could be used in wild olive dif-

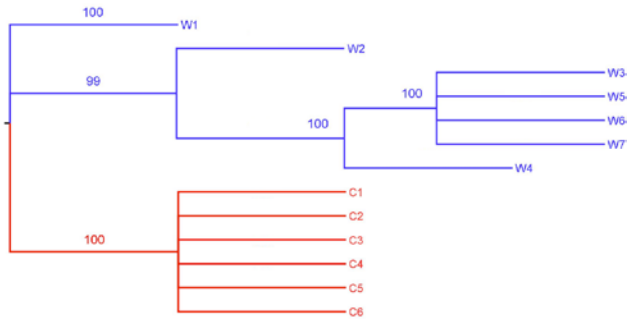

Figure 5. Maximum parsimony phylogenetic tree of wild and cultivated olives based in ITS sequences, revealing genetic distinctness of the two groups.

ferentiation. Cp-DNA polymorphisms is used for phylogeographic, population genetic and forensic analyses in plants, but detecting cp-DNA variation is sometimes challenging, limiting the applications of such an approach (Besnard *et al.* 2011). According to our knowledge rpl16 sequences were only used in genetic variability assessment of tissue culture regenerated olive plants (Kangarloo *et al.* 2016) and not in olive population genetic investigation. Therefore, our study is the first time report on application of the cp-DNA sequences for wild olive population differentiation.

Besnard *et al.* (2001) used eight complete sequences of cp-DNA genomes of *Olea* in their study. The reported low nucleotide divergence between olive cp-DNA lineages, not exceeding 0.07%. Based on these sequences, markers were developed for studying two single nucleotide substitutions and length polymorphism of 62 regions (with variable microsatellite motifs or other indels). They used these markers to study the cp-DNA variation in cultivated and wild Mediterranean olive trees. The discriminating power of cp-DNA variation was particularly low for the cultivated olive tree with one predominating haplotype, but more diversity was detected in wild populations. This is almost in agreement with present study findings. Besnard *et al.* (2001) and Pérez-Jiménez *et al.* (2013) suggested that cp-DNA markers will have applications for a comparative study of the dynamic of wild olive tree populations in different environments, such as archipelagos and Saharan mountains. Such information may be relevant for defining appropriate strategies of prospection and *in situ* conservation of the wild olive tree.

In conclusion, the present study revealed that a combination of neutral molecular markers, like SRAPs and cp-DNA sequences are powerful markers to differentiate wild olive populations.

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