Molecular Phylogenetic Analysis of *Olea europaea* L. subsp. *europaea* Cultivars Grown in the Marmara Region, Turkey
(Analisis Filogenetik Molekul bagi Kultivar *Olea europaea* L. subsp. *europaea* Ditanam di Wilayah Marmara, Turki)

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ABSTRACT

*DNA markers based on the PCR techniques are being broadly utilized during the past two decades. Randomly Amplified Polymorphic DNA (RAPD) is one of these techniques employed for determining the polymorphisms. In this study, we performed a molecular analysis using DNA markers for some olive (*Olea europaea*) cultivars grown in the Marmara Region of Turkey. Our goal was to determine the genetic relationships between 12 olive cultivars and 'Delice', the outgroup. Seven RAPD primers out of 60 (10 base-pair long) amplified gDNAs with repeatable bands. These primers generated 84 characters / bands in total and 39 out of these were polymorphic. After a Branch-and-Bound analysis and a Neighbour Joining (NJ) analysis via PAUP* software, the smallest genetic distance was found between Gordales and Karamursel Su cultivars as 0.03571 whereas the greatest distances were found between Arbequina and Ascolana, Manzanilla, Hermandos, Gemlik, Verdial and Vegral cultivars as 0.17857, respectively. Maximum parsimony (MP) analysis yielded 8 equally most parsimonious trees with 65 steps. Alternatively, NJ analysis produced one tree mostly congruent with MP tree number one. In conclusion, Turkish olive cultivars grown in the Marmara Region appear to be sister to the Spanish olive cultivars based on the RAPD data.*

Keywords: Genetic distance; Marmara Region; Olea europaea *subsp.* Europaea; *olive cultivars; phylogenetic relationships; RAPD*

ABSTRAK

*Penanda DNA didasarkan pada teknik PCR telah digunakan secara kasar dalam dua dekad lepas. DNA Polimorfik Teramplifikasi Secara Rawak (RAPD) adalah salah satu daripada teknik yang digunakan untuk menentukan polimorfisme. Dalam kajian ini, kami telah menjalankan analisis molekul menggunakan penanda DNA bagi beberapa penanda kultivar zaitun (*Olea europaea*) yang ditanam di Wilayah Marmara, Turki. Tujuan kami ialah untuk menentukan perhubungan genetik antara 12 kultivar zaitun dan kumpulan luar 'Delice'. Tujuh primer RAPD daripada 60 (10 pasangan bes panjang) teramplifikasi gDNA dengan jalur boleh ulang. Primer ini telah menjana sejumlah 84 sifat/jalur dan 39 daripadanya ialah polimorfik. Selepas analisis Ranting-dan-Terikat dan suatu analisis Jiran Menyambung (NJ) melalui perisian PAUP*, jarak genetik paling kecil telah ditemui antara kultivar Gordales dan Karamursel Susebagai 0.03571 manakala jarak yang paling besar telah ditemui antara kultivar Arbequina dan Ascolana, Manzanilla, Hermandos, Gemlik, Verdial dan Vegral masing-masing pada 0.17857. Analisis Parsimoni Maksimum (MP) menghasilkan 8 pepohon yang paling sama dengan parsimoni dengan 65 langkah. Sebagai alternatif, analisis NJ menghasilkan satu pepohon yang paling kongruen dengan pepohon MP nombor satu. Sebagai rumusan, kultivar zaitun Turki yang ditanam di Wilayah Marmara dilihat sebagai adik beradik kepada kultivar zaitun Sepanyol didasarkan kepada data RAPD.*

Kata kunci: Jarak genetik; kultivar zaitun; Olea europaea *subsp*. europaea; *perhubungan filogenetik; RAPD*; *Wilayah Marmara*

INTRODUCTION

Olive, *Olea europaea* (Oleaceae) is an important tree species and one of the most frequently used food source for its valuable oil, fruits, flowers, leaves and wood in the world, especially in the circum-Mediterranean region (Efe et al. 2011). Wide genetic variations in olives occur in the Mediterranean region with about 2000 cultivars (Albertini et al. 2011). Olive oil was an important commodity in the past restricted to the Mediterranean basin but is a widespread product today in the world due to its economically important properties such as being a food source, fuel for lighting and also as an ointment (Sanchez & Spener 2002). The principle fat source of the Mediterranean diet is olive oil and it has favourable and desirable effects on human health due to its health-beneficial nutrients such as monounsaturated fatty acids, the high oleate and significant linoleate contents, vitamins, minerals, dietary fibers, complex carbohydrates and minor components (Harwood & Yaqoob 2002; Wahrburg et al. 2002). Random Amplified Polymorphic DNA (RAPD) is a molecular

marker that has been widely employed to understand genetic diversity in various plants (Belaj et al. 2008; Besnard & Berville 2000; Bracci et al. 2011; Busconi et al. 2003; Khan et al. 2013; Marieschi et al. 2011; Martins-Lopes et al. 2009; Meng et al. 2010; Ozkaya et al. 2006; Parmaksiz & Ozcan 2011; Sadder & Ateyyeh 2006; Yildiz et al. 2011; Zitoun et al. 2008). RAPD markers can be used in population genetics studies, in systematic analyses and in taxonomical classification studies. Development of the first linkage map in olive used RAPD, AFLP and SSR markers (Belaj et al. 2008). In recent years, some olive searches included AFLP markers (Owen et al. 2005; Strikic et al. 2010) and SSR markers (Ipek et al. 2009). RAPD markers are also useful for developing SCAR markers (Marieschi et al. 2011). Although use of this marker has many advantages (fast, cheap, does not require prior sequence information, can resolve the relationships among closely related taxa and requires small amount of DNA), it also has several disadvantages (being a dominant marker and having a repeatability problem).

The objective of this work was to determine the phylogenetic and phenetic relationships among twelve olive cultivars commonly grown in the Marmara Region of Turkey and between cultivated taxa and the 'wild' olive taxon since this region contains economically important and famous table olives (Gemlik, Karamürsel & Edincik). This study will contribute to better understanding of the relationships of these important cultivars.

MATERIAL AND METHODS

PLANT MATERIAL

All plant samples used in this study were collected as thirteen accessions from Edremit Olive Reproduction and Breeding Station in Balıkesir, Turkey in July of 2005 (Table 1). Herbarium specimens were prepared from the plant materials and deposited in the Herbarium of Balıkesir University, College of Arts & Sciences at Çağış Campus. Plant leaves from the accessions were kept in dry ice between the Edremit Olive Reproduction and Breeding Station and molecular biology laboratory of the College of Arts & Sciences at Balıkesir University. They were reserved frozen at -89°C in a freezer until DNA isolation step. Table 1 shows all ingroup and outgroup olive cultivars grown in Marmara region of Turkey selected for this study.

TABLE 1. Twelve olive cultivars and 'Delice', the outgroup (*O. europaea* subsp. *europaea* var. *sylvestris*), collected from Edremit Olive Reproduction and Breeding Station in Edremit, Balıkesir, Turkey and their collection times

Olive Cultivars	Collection Times
Samanlı	13.07.2005
Edincik Su	13.07.2005
Vegral	13.07.2005
Domat	13.07.2005
Manzanilla	13.07.2005
Arbequina	13.07.2005
Gordales	13.07.2005
Karamürsel Su	13.07.2005
Ascolana	13.07.2005
Hermandos	13.07.2005
Gemlik	13.07.2005
Verdial	13.07.2005
Delice	21.07.2011

Twelve cultivars of *Olea europaea* subsp. *europaea* (var. *europaea*) were used as ingroup taxa and a 'wild' olive taxon, 'Delice', was chosen as an outgroup for this work. The 'wild' olive taxon, 'Delice' (*Olea europaea* subsp. *europaea* var. *sylvestris*, (Green 2002), was chosen as the outgroup since it is the closest taxon to the cultivated olive taxa and has been used for grafting purposes in the region.

DNA ISOLATION AND PCR AMPLIFICATION

Total genomic DNA, gDNA, isolation was carried out using DNeasy Plant Mini Kit (Catalog No: 69104) of the Qiagen Company. The company's protocol was followed for the isolation procedure. In order to visualize gDNAs, 0.8% standard agarose gel electrophoresis procedure was performed. Isolated gDNAs were measured using a spectrophotometer with the following ratio: OD260/OD280. RAPD-PCR amplifications of gDNAs followed Williams et al.'s procedure (Williams et al. 1990). RAPD primers used in this work were obtained from Integrated DNA Technologies Company (Coralville Integrated DNA Technologies, Inc. 1710 Commercial Park Coralville, Iowa 52241 USA) using OPERON Incorporation's (Alameda, CA, USA) RAPD primers list. About 60 primers screened and primers with repeatable bands were chosen for the analysis. Table 2 shows the primer list used in this study with repeatable bands and their sequences with

TABLE 2. Primers used in the RAPD-PCR reactions and their Tm degrees with GC contents

Primer	DNA sequences	Tm	% GC
OPC-01	TTC GAG CCA G	34°C	60%
OPC-02	GTG AGG CGT C	37°C	70%
OPC-11	AAA GCT GCG G	36°C	60%
OPA-10	GTG ATC GCA G	33°C	60%
OPA-12	TCG GCG ATA G	34°C	60%
OPA-13	CAG CAC CCA C	37°C	70%
OPB-12	CCT TGA CGC A	35°C	60%

their properties.

PCR Master Mix Kit (Catalog No: RP02) of Gene Mark Company was used for RAPD-PCR reactions. Kit contents were as follows: 1.25 unit Taq DNA polymerase, PCR reaction buffer, MgCl$_2$ (final concentration 1.75 mM), 200 μM dNTP. The company's protocol was followed. Tables 3 and 4 show the RAPD-PCR contents for each reaction and PCR cycles with their conditions, respectively.

In order to test the repeatability of each RAPD band, amplifications were repeated at least three times. For the contamination risk of the reactions, a negative control with no DNA added in it, was used for each application. A DNA standard from Roche Company (DNA molecular weight marker III) was loaded to a well for each gel electrophoresis to determine the size of the DNA bands.

DATA ANALYSIS

Phylogenetic and phenetic analyses of the RAPD data were assessed using PAUP* (Phylogenetic Analysis Using Parsimony and other methods, Version 4.0b10) software (Swofford 2002). 84 characters, gel bands/fragments, produced by RAPD primers were used to construct the data matrix. Data was analyzed first using Branch-and-Bound algorithm under parsimony criterion and minimum evolution parameter was used for Neighbour Joining search under genetic distance criterion.

RESULTS AND DISCUSSION

Most of the gDNAs extracted had the purity around 1.8-ratio at OD260/280, which was very good for PCR reactions. Amounts of the gDNA extracts used in PCR amplifications were about 30 ng/μL.

RAPD-DNA PROFILES

After a careful screening process for 60 RAPD-PCR primers, seven primers generated repeatable band profiles. Some of the RAPD-DNA profiles with repeatable bands along with their particular primers used to amplify are shown on Figure 1. One-letter or two-letters representations of all taxa were indicated above gel electrophoresis photos in all figures. These letter-indications are explained in Figure 1. The olive cultivars, in-group taxa, were amplified first and the out-group taxon was amplified later during experimental studies.

DATA ANALYSIS

Analysis of data was based on the results taken from the agarose gel photos for each primer. Only visible bands were evaluated for data analysis. Phylogenetic and phenetic analyses were performed on a data matrix comprising characters scored as '1' for presence of the band and '0' for absence of the band on the gel photos. For the analysis of RAPD-DNA data, in all seven primers with 84 characters/ bands were attained. Primers generated maximum twelve characters. Data matrix can be presented upon request by the corresponding author. Data matrix was converted to #NEXUS format for the application in PAUP* software, Version 4.0b10.

During the analysis, parsimony and genetic distance (minimum evolution) criteria were used. Parsimony analysis used Branch-and-Bound algorithm and revealed 45 constant characters out of 84 characters, 20 variable parsimony-uninformative characters and 19 variable parsimony-informative characters. All characters were weighted equally and 10000 trees were kept in memory through the analysis, while all tree topologies were evaluated without constraints. Analysis yielded 8 most parsimonious trees with 65 steps. Figure 2 shows tree

TABLE 3. RAPD-PCR contents

PCR buffer	5 μL
MgCl2	(5 × PCR Master Mix Kit)
dNTP	
Taq DNA Polymerase	
Primer	1 μL
gDNA	2.5 μL
Distilled water	16.5 μL

TABLE 4. Cycles and conditions of RAPD-PCR reactions

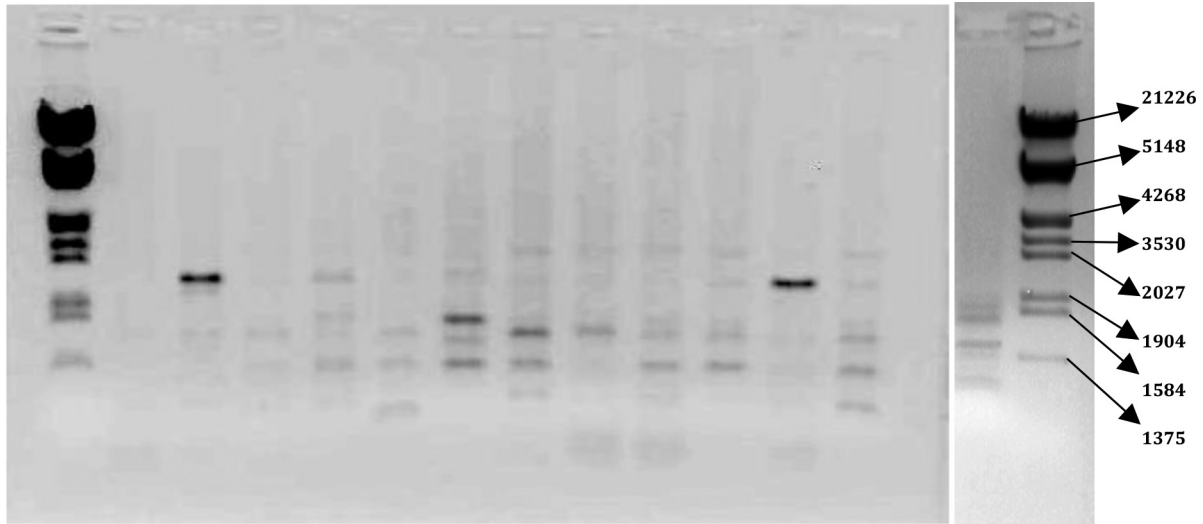|			
Pre-heating	94ºC	5 min	1 cycle
1. step	94ºC	45 s	
2. step	37ºC	45 s	35 cycles
3. step	72ºC	2 min	
4. step	72ºC	10 min	1 cycle
5. step	4ºC	25 h	

FIGURE 1. RAPD-PCR gel electrophoresis photo amplified with OPC-01 primer. The letter representations of all olive cultivars above gel photo are explained as follows: M-DNA marker, S-Samanlı, E-Edincik Su, V-Vegral, Do-Domat, Mn-Manzanilla, A-Arbequina, G-Gordales, K-Karamürsel Su, As-Ascolana, H-Hermandos, G-Gemlik, Vr-Verdial, De-Delice. Numbers next to the marker on the right side show the sizes of the bands on the gel

number 1 of 8 most parsimonious trees. These 8 trees were equally most parsimonious and the tree number one was chosen among them due to its similarity to neighbor joining (NJ) tree (cf. Figures 2 and 3). In order to assess the statistical supports on individual branches, a Bootstrap analysis (Felsenstein 1985) was performed and branches getting Bootstrap supports higher or lower than 50% were shown in numbers below branches on Figure 2.

As a phenetic approach, Neighbour Joining algorithm was used to assess genetic distances and to generate a NJ tree. Figure 3 shows the NJ tree. Parsimony tree number 1 and NJ tree are mostly congruent to each other (cf. Figures 2 & 3). Parsimony tree number 1 indicates that cultivars 'Domat' and 'Arbequina' are significantly supported that these are very closely related with a Bootstrap support of 77%. Also pair wise genetic distance between these two taxa was significantly low comparing to other genetic distances for the other taxa (0.09524, Table 5). Bootstrap support for the ancestral branch yielding to 'Gordal' and 'Karamursel Su' received another significant confirmation
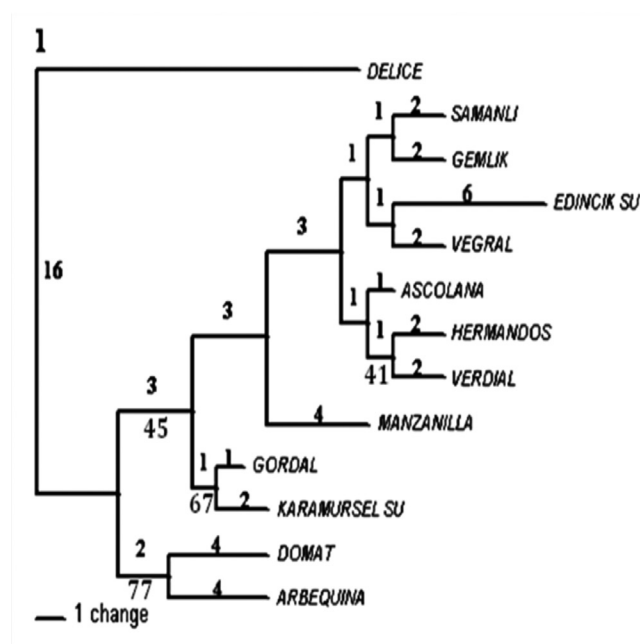

FIGURE 2. Phylogram of the tree number 1 out of 8 trees after a Branch-and-Bound analysis under parsimony criterion. Numbers above and right to the branches show branch lengths and numbers below indicate Bootstrap values

with 67% Bootstrap value. On the other hand, the branch between the ancestors of 'Domat & Arbequina' clade and the ancestors of 'Gordal & Karamursel Su' clade and rest of the cultivars received relatively significant Bootstrap value (45%, Figure 2). These are mostly congruent with the pair wise genetic distances (cf. Table 5 with Figures 2 & 3).

NJ analysis yielded a tree with similar topology to parsimony tree (Figure 3). In that tree, branch lengths are proportional to the changes in RAPD-PCR data per site. Maximum Parsimony and NJ trees were different at one branch e.g., Edincik Su and Vegral were sister taxa in maximum parsimony tree while Edincik Su was sister to the clade comprising Verdial, Hermandos, Ascolana, Vegral, Gemlik and Samanli in NJ tree. This result showed us that the position of the Edincik Su is not clear since there is no significant Bootstrap support.

Ipek et al. (2009) used a different marker (SSR) to investigate olive cultivars in which seven out of 13 taxa used in this study were included in their work from the same region of Turkey. Results of both markers are not congruent in general because of the differences in the markers and the cultivars used in both studies. However, their finding with 'Karamursel Su' is similar to our results (i.e. 'Karamursel Su' is closely related with another cultivar from the Spain, 'Gordales' in this study and 'Hojiblanca' in their work).

Based on these results, it would be anticipated that native olive cultivars grown in Marmara region show sister group relationship with the cultivars native to Spain (see the position of Domat and Arbequina to rest of the taxa on Figures 2 & 3). Vegral and Gordales also showed close relationship with the cultivars from the Marmara Region. For instance, a native cultivar, namely 'Karamursel Su',
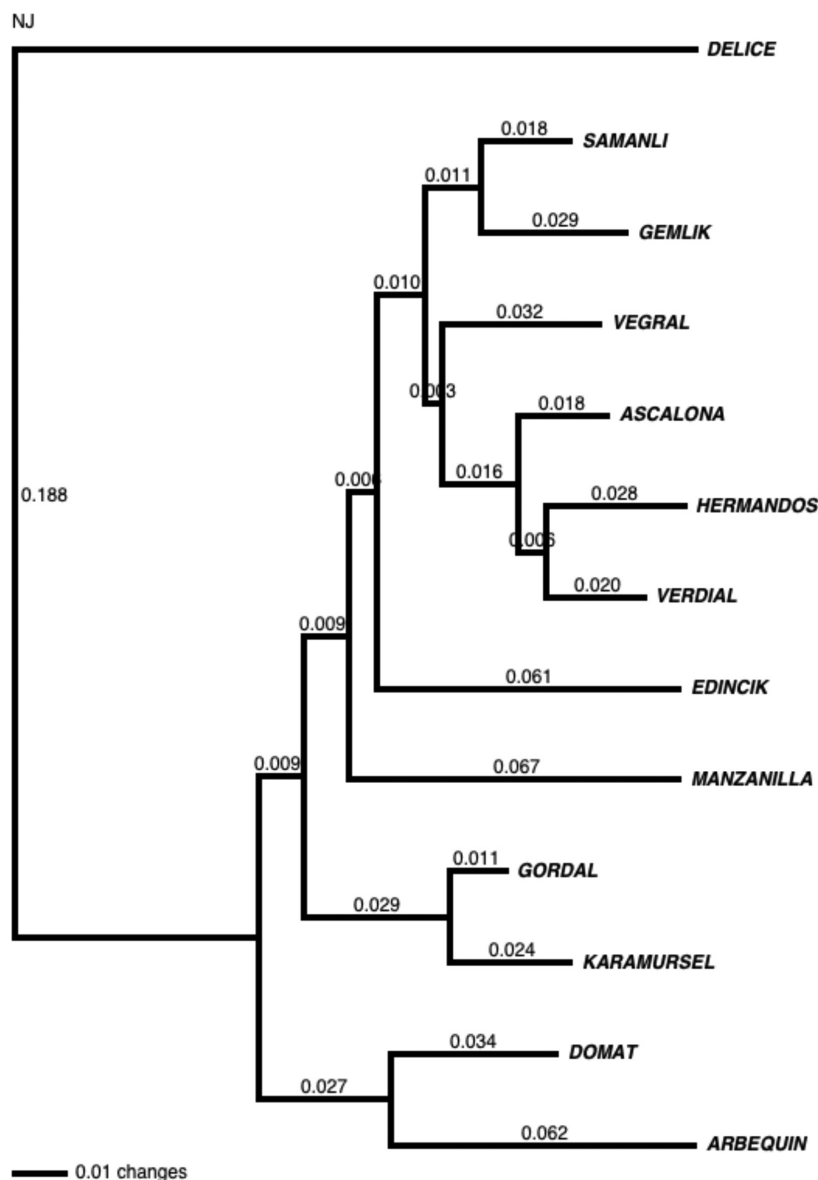

FIGURE 3. Neighbour Joining (NJ) genetic distance tree with objective function set to 'minimum evolution' and distance measure was set to mean character differences

TABLE 5. Pairwise genetic distances between the olive cultivars grown in Marmara Region of Turkey. Numbers above diagonal shows pairwise genetic distances and below diagonal indicates number of variable characters out of total number of characters. Higher and lower distances were shown in bold numbers

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 DELICE (outgroup)	-	0.25000	0.29762	0.25000	0.26190	0.28571	0.26190	0.22619	0.26190	0.25000	0.27381	0.25000	0.25000
2 SAMANLI	21	-	0.09524	0.07143	0.10714	0.10714	0.15476	0.09524	0.08333	0.07143	0.09524	0.04762	0.09524
3 EDINCIK	25	8	-	0.09524	0.13095	0.13095	0.15476	0.14286	0.10714	0.11905	0.14286	0.11905	0.11905
4 VEGRAL	21	6	8	-	0.10714	0.13095	0.17857	0.11905	0.13095	0.07143	0.09524	0.07143	0.04762
5 DOMAT	22	9	11	9	-	0.16667	0.09524	0.13095	0.14286	0.13095	0.15476	0.10714	0.13095
6 MANZANILLA	24	9	11	11	14	-	0.16667	0.10714	0.11905	0.10714	0.10714	0.13095	0.13095
7 ARBEQUINA	22	13	13	15	8	14	-	0.13095	0.11905	0.17857	0.17857	0.17857	0.17857
8 GORDALES	19	8	12	10	11	9	11	-	0.03571	0.07143	0.09524	0.11905	0.09524
9 KARAMURSEL	22	7	9	11	12	10	10	3	-	0.10714	0.13095	0.13095	0.13095
10 ASCOLANA	21	6	10	6	11	9	15	6	9	-	0.04762	0.07143	0.04762
11 HERMANDOS	23	8	12	8	13	9	15	8	11	4	-	0.07143	0.04762
12 GEMLIK	21	4	10	6	9	11	15	10	11	6	6	-	0.07143
13 VERDIAL	21	8	10	4	11	11	15	8	11	4	4	6	-

was closely allied with the cultivar 'Gordales'. The highest genetic variation was between 'Delice' and 'Edincik Su' (0.29762, Table 5 and Figures 2 & 3).

In conclusion, based on parsimony and genetic distance trees it can be stated that native olive cultivars/taxa grown in Marmara Region are sister taxa to the Spanish olive cultivars, based on our RAPD-PCR DNA data. However, with this limited data on hand, it may not be reasonable to draw a clear conclusion on the origin of the olive cultivars grown in the Marmara Region since adding or deleting any taxon or taxa might change the tree topology in the analysis. Additionally, we have yet to know the native range of all these cultivars. Nevertheless, this investigation would provide some insights on the relationships of the cultivars grown in the region. Certainly, more data (e.g. morphological and/or DNA sequencing data) with more taxa would improve this inference and would result in more reliable conclusions.

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