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Genetic stability in a predominating Turkish olive cultivar, Gemlik, assessed by RAPD, microsatellite, and AFLP marker systems

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Abstract: Olive (*Olea europaea* L.) is an important fruit crop, with many health promoting properties being continuously discovered. World demand for both the fruit and olive oil is increasing in pace and hence countries try to meet the demand by disseminating 1 or 2 productive and adaptive cultivars to different regions formerly dominated by local cultivars. This study was performed to investigate the level of diversity in a very productive and adaptive cultivar, Gemlik, which has started to dominate most of the olive growing regions of Turkey. Three different marker systems, namely microsatellites (SSR), RAPD, and AFLP, with 15, 20, and 3 primer combinations, respectively, could not detect any genetic variation among 60 olive cv. Gemlik specimens sampled from 5 different regions, except several rare and random polymorphisms. The results showed that the expanding cultivar most possibly belonged to the clones of a single tree and revealed 100% sample identity.

Key words: AFLP, genetic variation, microsatellites, *Olea europaea*, RAPD

1. Introduction

The olive tree (*Olea europaea* L.) is known as one of the oldest cultivated fruit trees. Although it originated from the Mediterranean Basin, including a part of Anatolia and Syria, its cultivation took place approximately 5000 years ago in Syria, from where the tree in cultivated form dispersed to all other parts of the Mediterranean (Özbek, 1975). Unlike other crops, olive germplasm has not suffered any genetic erosion, since turnover with new genotypes has not occurred and old plants are able to survive for a long time without cultivation (Angiolillo et al., 1999). However, recently the situation has started to change. With many health-promoting effects revealed, olive oil has become a highly demanded product. The plant's distribution area is currently being extended worldwide (Rugini and Gutierrez-Pesce, 2006; Cerezo et al., 2011) and economically important cultivars with high adaptability are being disseminated throughout new locations, replacing local cultivars, usually through government policies, as in the case of cv. Gemlik.

Turkey contains 88 different local olive cultivars. The country's average olive oil production during the years 2004 to 2010 was 128.5 thousand tons, placing it in sixth position among the major oil producing countries and third worldwide in terms of table olive production with 330 thousand tons (IOOC, 2011). The most important Turkish olive cultivars in terms of prevalence and fruit/oil production capacity include Gemlik, Ayvalık, and Memecik, which altogether constitute nearly 95% of all olive plantations in Turkey (Dıraman, 2007). Gemlik does not show intensive periodicity; it has a high adaptation capacity and cold/disease resistance. It is easily propagated vegetatively by cuttings and easily processed to be consumed as a fruit and in the form of olive oil. Therefore, it has a distinguishing place among the olive cultivars grown in Turkey. Based on the above-mentioned characteristics and through support by government policies, the cultivation area of the Marmara originated Gemlik cultivar has expanded throughout the country in the last 20 years. Currently, Gemlik constitutes 80% of the

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olive plantations in the Marmara region and 11% of the olive plantations across the country. Besides the Marmara region, economically important plantations of Gemlik are mostly found in Manisa and Aydın provinces, the East Mediterranean region of Turkey, and Southeast Anatolia (Dıraman, 2007). Moreover, its cultivation area continues to expand across the country at a great pace, wherever the cultivar can adapt and fruit.

With the advent of PCR-based molecular markers, it is possible to make direct inferences about genetic diversity and interrelationships among organisms at the DNA level without the confounding effects of the environment and/or faulty pedigree records (Agarwal et al., 2008). RAPD markers are polymorphic DNA separated by gel electrophoresis after PCR using short random oligonucleotide primers. It has been particularly used for genetic and molecular studies, as it is a simple and rapid method for determining genetic diversity and similarity in various organisms (Salem et al., 2007). AFLPs are generated by complete restriction endonuclease digestion of total genomic DNA, followed by selective PCR amplification and electrophoresis of a subset of the fragments, resulting in a unique, reproducible profile for each individual (Meudt and Clarke, 2007). Microsatellite marker techniques utilize the intra- as well as interindividual variation in microsatellites or simple sequence repeat region for fingerprinting analyses (Agarwal et al., 2008). These techniques have been used individually or in combination for the detection of genetic variability between cultivars in various plant species (Moslemi et al., 2010; Baraket et al., 2011; Gürkök et al., 2013; Mukherjee et al., 2013), including olives (Angiolillo et al., 1999; Mekuria et al., 2002; Belaj et al., 2003).

There are limited studies on the genetic stability of Turkish cultivars. A recent study showed that about 8% of the olive samples collected from the southern Marmara region, which were identified as Gemlik by the growers, did not share the same SSR alleles with the Gemlik cultivar present at Atatürk Central Horticultural Research Institute, Yalova (Ipek et al., 2009, 2012). Since the origin of Gemlik is around the Marmara region and it is deliberately being disseminated to various locations across the country, the aim of this study was to investigate the potential genetic variability in Gemlik sampled from 5 different orchards representing different regions of Turkey.

2. Materials and methods

2.1. Plant materials

Young *Olea europaea* L. cv. Gemlik leaves of 60 individuals were collected from 5 main locations of olive production in Turkey: Balıkesir (Marmara), Aydın (South Aegean), Manisa (North Aegean), Antalya (West Mediterranean), and Hatay (East Mediterranean) (Figure 1), with 12 individuals representing each region. All the samples were collected from young orchards composed of olive trees 5–10 years old.

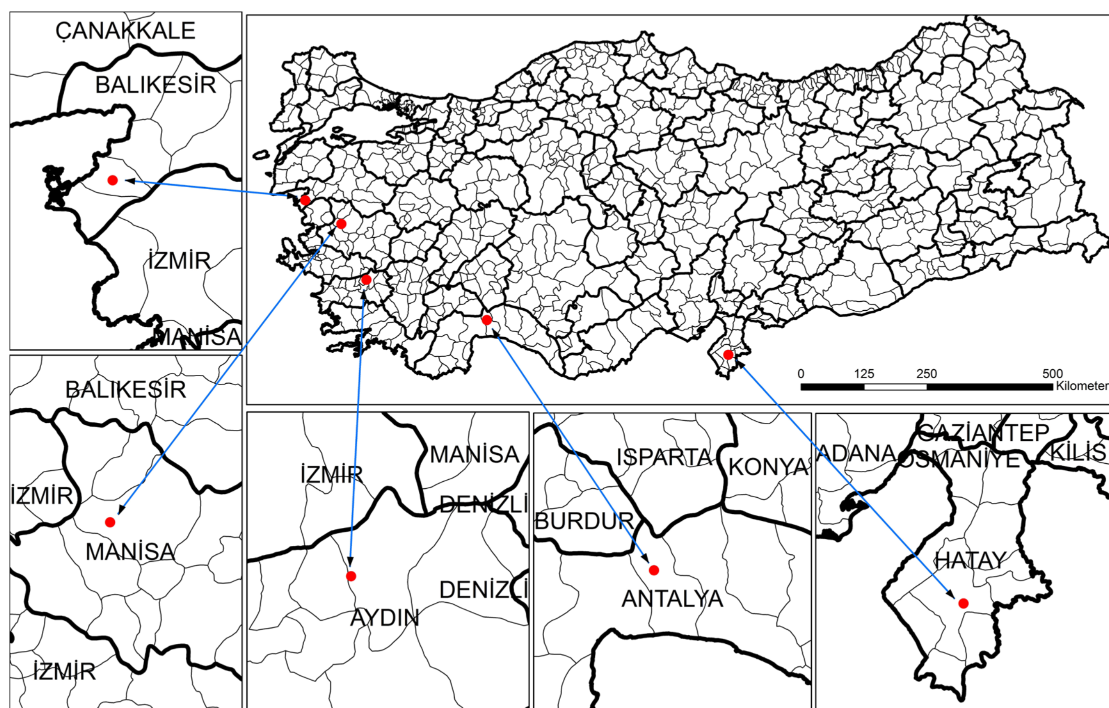

Figure 1. Balıkesir, Aydın, Manisa, Antalya, and Hatay locations, from where *Olea europaea* samples of Gemlik were collected.

2.2. Sample preservation and DNA isolation
Collected leaf samples were immediately placed in a chest cooler containing several ice packs. The leaf samples were transferred to the laboratory within 24 h, immediately frozen in liquid nitrogen, and stored at –20 °C until DNA isolation. Genomic DNA of Gemlik specimens was isolated by CTAB extraction (Maroof et al., 1984). Isolated DNA was resuspended in sterile double distilled water and stored at –20 °C.

2.3. Microsatellite analysis
Microsatellite primers DCA1, DCA3, DCA7, DCA8, DCA9, DCA11, DCA15, DCA16, DCA17, and DCA18 identified in Italian olive cultivars (Sefc et al., 2000); Oe149 identified in *O. europaea* cv. Ayvalik (Dundar and Suakar, 2010); and PAGA2, PAGA5, PAGA9, and PAATT2 identified in *Oleaceae* member *Phillyrea angustifolia* L. (Saumitou-Laprade et al., 1998) were used in the microsatellite analysis. All of the 15 primers were synthesized by IDT Technologies, USA.

PCR reactions were performed in 50-µL reaction volume including 5 µL of 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 2 µM primer, 1 U of *Taq* DNA polymerase, and 0.5 µg of template DNA. All reactions were performed in a BioRad MyCycler thermocycler with 5 min 95 °C initial denaturation, followed by 35 cycles of 1 min at 95 °C, 1 min at the annealing temperature of the particular primer indicated in Table 1, 1 min at 72 °C for extension, and finally 10 min at 72 °C for final extension. Only for the

Table 1. Microsatellite loci used in genome screening.

Microsatellit primer	Primer sequence	Annealing temp.	Reference
DCA1	5’- CCTCTGAAAATCTACACTCACATCC -3’ 5’- ATGAACAGAAAGAAGTGAACAATGC -3’	50 °C	Sefc et al. 2000
DCA3	5’- CCCAAGCGGAGGTGTATATTGTTAC -3’ 5’- TGCTTTTGTCGTGTTTGAGATGTTG -3’	50 °C	Sefc et al. 2000
DCA7	5’- GGACATAAAACATAGAGTGCTGGGG -3’ 5’- AGGGTAGTCCAACTGCTAATAGACG -3’	60 °C	Sefc et al. 2000
DCA8	5’- ACAATTCAACCTCACCCCCATACCC -3’ 5’- TCACGTCAACTGTGCCACTGAACTG -3’	55 °C	Sefc et al. 2000
DCA9	5’- AATCAAAGTCTTCCTTCTCATTTCG -3’ 5’- GATCCTTCCAAAAGTATAACCTCTC -3’	55 °C	Sefc et al. 2000
DCA11	5’- GATCAAACTACTGCACGAGAGAG -3’ 5’- TTGTCTCAGTGAACCCTTAAACC -3’	50 °C	Sefc et al. 2000
DCA15	5’- GATCTTGTCTGTATATCCACAC -3’ 5’- TATACCTTTTCCATCTTGACGC -3’	50 °C	Sefc et al. 2000
DCA16	5’- TTAGGTGGGATTCTGTAGATGGTTG -3’ 5’- TTTTAGGTGAGTTCATAGAATTAGC -3’	50 °C	Sefc et al. 2000
DCA17	5’- GATCAAATTCTACCAAAAATATA -3’ 5’- TAATTTTTGGCACGTAGTATTGG -3’	50 °C	Sefc et al. 2000
DCA18	5’- AAGAAAGAAAAAGGCAGAATTAAGC -3’ 5’- GTTTTCGTCTCTCTACATAAGTGAC -3’	50 °C	Sefc et al. 2000
Oe149	5’-AGTAGTCAAATGGTCCCTCTATCTAATG-3’ 5’-AAGGTAATATTCTTCTCTTAATCCCAGA-3’	50 °C	Dundar and Suakar, 2010
PAGA2	5’- AAGGAAGATGGGTCTTTGGG -3’ 5’- ATTCAGCTCCTCCACCTTCA -3’	60 °C	Saumitou-Laprade et al. 1999
PAGA5	5’- TCTTTGCTTCGTTGCTTTTG -3’ 5’- TCTTGCTCCCTCGACATTTT -3’	56 °C	Saumitou-Laprade et al. 1999
PAGA9	5’- CAACACTCAACAGCCACCAC -3’ 5’- GGACCGTCATTATGTGAGGC -3’	61–56 °C (touchdown)	Saumitou-Laprade et al. 1999
PAATT2	5’- CACCTCCCGGTTAACAAGAA -3’ 5’- TGACGCGGTTATTTTGTGAA -3’	60 °C	Saumitou-Laprade et al. 1999

PAGA2 primer, the reactions were performed by the use of touchdown PCR, starting with the annealing temperature of 61 °C ending up with 56 °C after 5 cycles and continuing with 56 °C for the next 30 cycles.

2.4. RAPD analysis

Twenty RAPD primers were obtained from Operon Technologies (CA, USA) (Table 2). PCR reactions were performed in 50-µL reaction volume including 5 µL of 10X PCR buffer, 2.5 mM $MgCl_2$, 400 µM dNTP, 2 µM primer, 1 U of *Taq* DNA polymerase, and 0.5 µg of template DNA. All reactions were performed in a BioRad MyCycler thermocycler with the same reaction conditions described for microsatellite analysis with the specific annealing temperatures indicated in Table 2.

2.5. AFLP analysis

Pre-amplification and selective amplification of AFLP analysis were performed according to the AFLP Analysis System I instruction manual of Invitrogen (CA, USA). The analysis was performed on 50 olive samples selected among 60 collected samples that were previously analyzed by the use of RAPD and microsatellite primers. Genomic DNA samples were digested with *Eco*RI and *Mse*I restriction enzymes and selective amplifications were performed by the use of the following 3 primer combinations by touchdown PCR: E-AGG/M-CAG, E-ACC/M-CTC, and E-ACG/M-CTG.

2.6. Visualization of amplification products and data analysis

PCR amplification products of microsatellite, RAPD and AFLP analyses were separated on 5% polyacrylamide gels prepared by mixing 29:1 acrylamide/bisacrylamide solution in 10% (v/v), 10X TBE electrophoresis buffer. Electrical power was applied as 5 V/cm gel for 3 h. Silver staining was performed according to Caetano-Anolles and Gresshoff (1994). The gels were preserved in 7.5% acetic acid solution and visualized in TIF format by the use of Biolab gel documentation system (UV Tech., USA). Band analyses were performed by Scion Image (Scion Corporation, MD, USA) digital gel analysis software.

Table 2. RAPD primers used in genome screening.

RAPD primer	Primer sequence	Annealing temperature
OPC-04	5’- CCGCATCTAC -3’	36 °C
OPC-07	5’- GTCCCGACGA -3’	34 °C
OPC-08	5’- TGGACCGGTG -3’	34 °C
OPC-10	5’- TGTCTGGGTG -3’	36 °C
OPC-13	5’- AAGCCTCGTC -3’	36 °C
OPC-15	5’- GACGGATCAG -3’	36 °C
OPC-16	5’- CACACTCCAG -3’	36 °C
OPZ-02	5’- CCTACGGGGA -3’	34 °C
OPZ-05	5’- TCCCATGCTG -3’	36 °C
OPZ-06	5’- GTGCCGTTCA -3’	36 °C
OPZ-07	5’- CCAGGAGGAC -3’	34 °C
OPZ-11	5’- CTCAGTCGCA -3’	36 °C
OPZ-13	5’- GACTAAGCCC -3’	36 °C
OPZ-16	5’- TCCCCATCAC -3’	36 °C
OPI-12	5’- AGAGGGCACA -3’	36 °C
OPX-03	5’- TGGCGCAGTG -3’	34 °C
OPX-09	5’- GGTCTGGTTG -3’	36 °C
OPA-01	5’- CAGGCCCTTC -3’	34 °C
OPA-19	5’- CAAACGTCGG -3’	36 °C
OPK-16	5’- GAGCGTCGAA -3’	36 °C

2.7. Statistical analysis

Similarity coefficients were calculated according to Nei and Li (1979) as F = 2 × (number of common fragments)/(number of fragments in the first sample) + (number of fragments in the second sample). Since the same number of monomorphic fragments was obtained for all analyses, except a few random differences in several individuals, cluster analysis could not be performed.

3. Results

Throughout the study, genomic DNA samples from 60 olive specimens were screened by the use of 20 RAPD, 15 microsatellite, and 3 AFLP primers, together producing 2250 amplification products.

3.1. Microsatellite analysis

Microsatellite primers amplified 6.2 loci on average in the genomes of Gemlik specimens. All 15 primer pairs were successful in the amplification of the particular loci. Four primer pairs, PAGA2, PAGA5, PAGA9, and PAATT2, which were previously identified and characterized in Oleaceae member *Phillyrea angustifolia* L., were proven to be present in the olive genome as well. Only 4 individuals produced polymorphic bands when different primers were used (Figure 2). Analyses with the Scion Image Gel Analysis System also confirmed the presence of polymorphic bands. Similarity coefficients for these particular individuals varied between 0.14 and 0.6 (Table 3).

3.2. RAPD analysis

For 20 different primers used in RAPD analysis, on average 11.4 bands were obtained. Only OPZ-02 primer was not able to produce bands for any of the 60 individuals. Polymorphic bands were obtained only for Aydın 3, Antalya 4, and Hatay 7 (Figure 3), which exhibited similarity coefficients of 0.98, 0.97, and 0.4, respectively (Table 4).

3.3. AFLP analysis

AFLP analysis was performed on 50 individuals, with 10 representing each particular region. None of the 3 AFLP primer couples produced any polymorphic bands for the investigated individuals (Figure 4), revealing that the 50 different individuals representing the 5 different regions were genetically identical.

4. Discussion

Screening of 60 different Gemlik specimens (collected from 5 different olive growing regions of Turkey) by the use of 20 different RAPD and 15 different microsatellite primers and additional screening of 50 individuals by the use of 3 different AFLP primers produced 2250 amplification products in total. Polymorphic bands were obtained only for 7 individuals, while 3 polymorphisms were detected by RAPD and 4 polymorphisms by microsatellite analysis. AFLP analysis alone could not detect any polymorphism. Primers that produced polymorphic band profiles were different from each other and the bands were obtained for different individuals. Therefore, the observed differences could most likely be explained by rare somatic chromosomal mutations including deletions, duplications, inversions, and translocations in meristematic somatic cells that were inherited into clones.

For seed propagated crops like cereals or grain legumes, mutation, genetic recombination, and gene flows between cultivated plants and between the crop and its wild relatives are the sources of new allelic combinations and variations. On the other hand, none of these factors, except mutation, are assumed to have affected the diversity

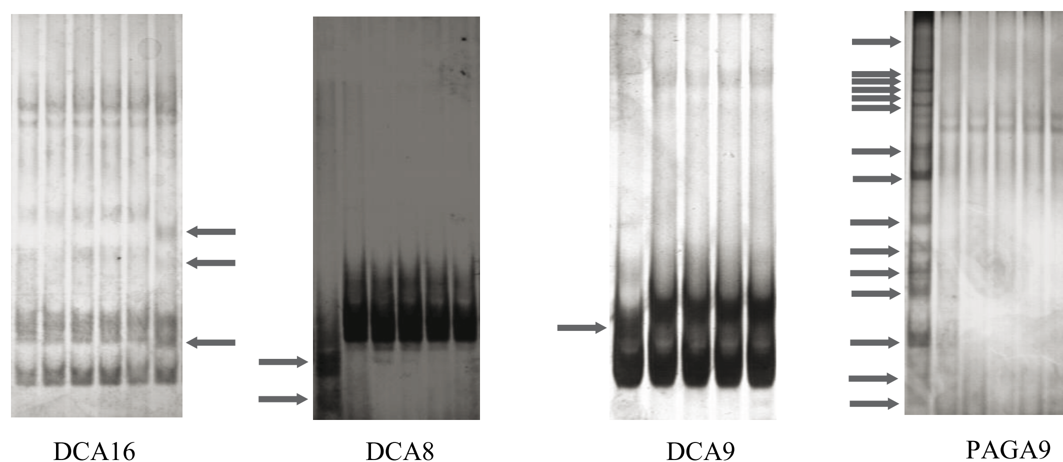

Figure 2. *Olea europaea* cv. Gemlik individuals, Antalya 2, Hatay 12, Antalya 9, and Balıkesir 3, that showed polymorphism for DCA16, DCA8, DCA9, and PAGA9 microsatellite primers, respectively. Arrows indicate polymorphisms for the particular individual in comparison to the monomorphic individuals.

Table 3. Microsatelitte fingerprinting profiles of Gemlik cultivar by use of 14 microsatellite loci.

Microsatellite primer	Total band number*	Polymorphic band number	Percentage of polymorphic bands	Polymorphic olive individual	Similarity coefficient
DCA1	300	-	-	-	-
DCA3	420	-	-	-	-
DCA7	240	-	-	-	-
DCA8	480	2	0.4%	Hatay 12	0.4
DCA9	120	1	0.8%	Antalya 9	0.5
DCA11	540	-	-	-	-
DCA15	540	-	-	-	-
DCA16	420	3	0.7%	Antalya 2	0.6
DCA17	300	-	-	-	-
DCA18	360	-	-	-	-
PAGA2	360	-	-	-	-
PAGA5	300	-	-	-	-
PAGA9	255	14	5%	Balıkesir 3	0.14
PAATT2	240	-	-	-	-
Total	5235	20			
Average (per individual)	6.2	0.02			

* "Total band number" is the number of bands that primers produced for 60 individuals collected from 5 different regions.

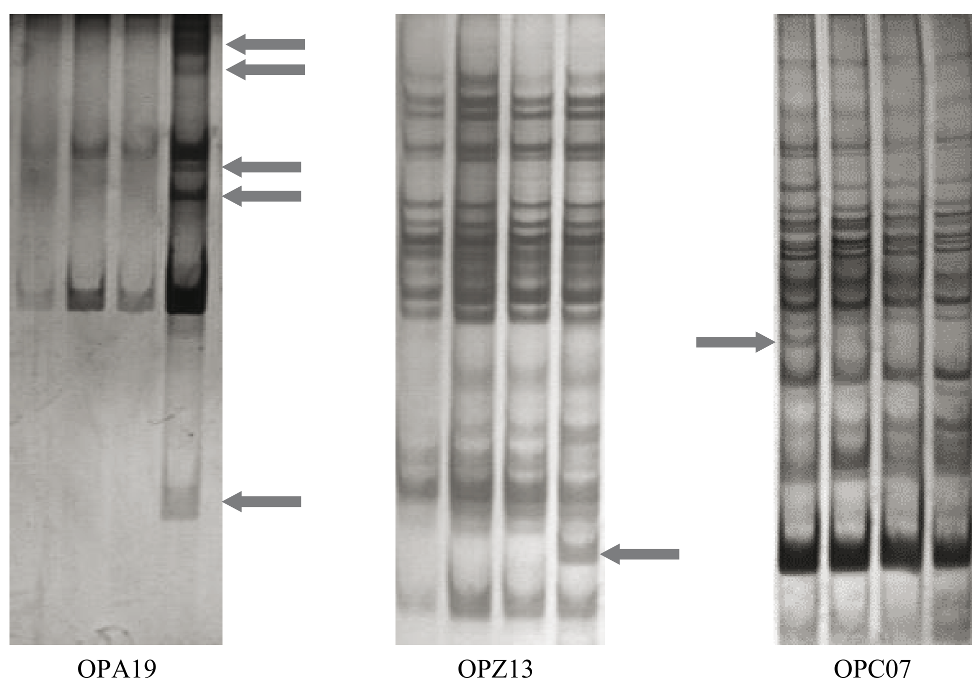

Figure 3. *Olea europaea* cv. Gemlik individuals, Hatay 7, Antalya 4, and Aydın 3, that showed polymorphism for OPA19, OPZ13, and OPC07 RAPD primers, respectively. Arrows indicate polymorphisms for the particular individual in comparison to the monomorphic individuals.

Table 4. RAPD fingerprinting profiles of Gemlik by use of 20 primers.

RAPD Primer	Total band number*	Polymorphic band number	Percentage of polymorphic bands	Polymorphic olive individual	Similarity Coefficient
OPC-04	1020	-	-	-	-
OPC-07	1201	1	0.08%	Aydın 3 (A3)	0.98
OPC-08	1140	-	-	-	-
OPC-10	360	-	-	-	-
OPC-13	900	-	-	-	-
OPC-15	720	-	-	-	-
OPC-16	720	-	-	-	-
OPZ-02	0	-	-	-	-
OPZ-05	600	-	-	-	-
OPZ-06	900	-	-	-	-
OPZ-07	1020	-	-	-	-
OPZ-11	720	-	-	-	-
OPZ-13	901	1	0.11%	Antalya 4 (A4)	0.97
OPZ-16	240	-	-	-	-
OPI-12	660	-	-	-	-
OPX-03	960	-	-	-	-
OPX-09	420	-	-	-	-
OPA-01	300	-	-	-	-
OPA-19	125	5	4%	Hatay 7 (H7)	0.4
OPK-16	780	-	-	-	-
Total	13687	7			
Average (per individual)	11.4	0.0058			

* "Total band number" is the number of bands that primers produced for 60 individuals collected from 5 different regions.

of vegetatively propagated crops (Zohary, 2004; Scarcelli, 2011). Rare and random polymorphisms determined through screening of Gemlik genomes by the use of microsatellite and RAPD markers are therefore likely to be produced by chromosomal mutations on repeated and nonrepeated regions of genomic DNA, respectively.

Three microsatellite loci, DCA9, DCA16, and DCA17, in particular were considered very effective for olive cultivar discrimination previously by Sarri et al. (2006) since the loci were able to distinguish nearly 100 genotypes, excluding some identical ones. The same loci were also used in this study and were unable to distinguish 60 different individuals, except DCA9 and DCA16, detecting polymorphisms on the genomes of individuals Antalya 9 and Antalya 2, respectively. Except for these rare and random differences, all Gemlik individuals sampled from the same region and from distant localities exhibited 100% identity.

Some unexpected results regarding the identity of cultivars were obtained in various studies. For 118 olive cultivars collected from the Mediterranean Basin, 20 different microsatellite markers confirmed genetic differences in the great majority, while some cultivars collected from different regions and referred to as different

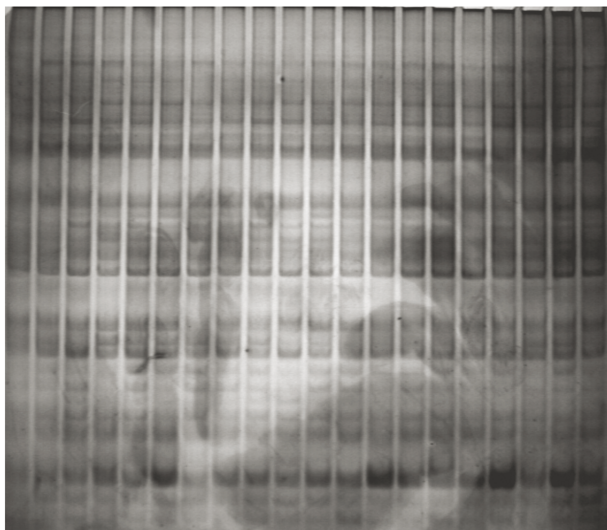

Figure 4. AFLP profiles produced by E-AGG/M-CAG primer pair for 4 individuals from each of the Hatay, Balıkesir, Aydın, Antalya, and Manisa regions, respectively. AFLP analysis could not detect any polymorphic loci among specimens.

cultivars were determined to be genetically identical (Sarri et al., 2006). In a similar study, RAPD analysis were performed on 56 olive cultivars collected from Malaga Province in Spain and only 22 different band profiles were obtained for a particular RAPD marker (Claros et al., 2000). In addition to the identification of specific cultivars that were originally thought to have different genotypes, there are reports of cultivars consisting of different genotypes that were formerly thought to be genetically stable. For example, RAPD analysis revealed 11 different Italian varieties morphologically indistinct from each other (Vergari et al., 1996). Israeli cultivar Nabali was also reported to be composed of genetically different varieties (Wiesman et al., 1998). In a similar study, an ancient Portuguese cultivar, Galega, was determined to be composed of different genotypes and cannot be considered a cultivar (Gemas et al., 2004). In a preliminary study on Gemlik genetic identification by the use of SSR markers, about 8% of the olive samples collected from the southern Marmara region that were identified as Gemlik by the growers did not share the same SSR alleles with the Gemlik cultivar present at the Atatürk Central Horticultural Research Institute, Yalova (Ipek et al., 2009, 2012). These studies confirm the necessity of investigating the genetic stability of cultivars, thereby preventing potential homonymy and synonymy problems, especially if they systematically dominate orchards where many local cultivars have existed and been used traditionally for long periods of time.

5. Conclusions

The existence of intracultivar genetic variation within Gemlik collected from old orchards was reported previously (Ipek et al., 2009, 2012). However, the findings of the present study showed that genetic variation does not exist among recently propagated Gemlik trees found in young orchards located throughout the county. Lack of genetic variation in new plantations of Gemlik can facilitate the certification of Gemlik products. However, crop yields and the quality of fruits and olive oil may not be the same for the genetically stable cultivars grown in geographically and climatically different locations. Further studies are required to correlate genetic stability and product quality standardization of table olives and olive oil of Gemlik.

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