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RAPD assay of wild-type olives in Turkey

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ABSTRACT. Genetic similarities and distances between wild-type olives in Turkey were studied using an RAPD-PCR assay. Seven wild olive tree samples were collected from villages in Manisa and Izmir provinces. Genomic DNA was extracted from young leaves and the RAPD-PCR assay was used to generate RAPD markers. Sixty-five random primers obtained from Operon Technologies were tested for the assay (OP-A 1-20, OP-I 1-20, OP-Q 1-20, and OP-J 1-5). Thirty-two of these primers yielded 115 highly polymorphic bands. The mean number of usable bands per primer for all the samples was 3.59. The genetic distance values ranged from 0.1498 to 0.6845, and genetic similarity values varied from 0.8609 to 0.5043. We found that the closest samples based on their genetic distance and similarity values were from Harlak and Sabancilar; the most distant samples were from Bornova and Bademli.

Key words: RAPD; Wild olives; Genetic analysis

INTRODUCTION

The olive tree is one of the oldest agriculturally important trees of humankind. It is believed that the cultivated type olives derived from *oleasters* or wild olives of the Mediterranean Basin and that they are spread throughout the world through human activities (Besnard et al., 2001). *Olea europaea* spp *europaea* consists of two subspecies; these are wild olives (*Olea europaea oleaster*) and the cultivated olives named *Olea europaea sativa* (Mendilcioglu, 1999). Wild-type olives are important for improvement studies because of their high degree of resistance against unfavorable environmental conditions and diseases. For this purpose, wild olives are a good source for understanding the mechanism of disease resistance/susceptibility or selection of disease-resistant plants.

Identification between and within olive types could be determined by morphological or phenotypic traits, but these traits are severely affected by environmental conditions and difficult to evaluate (Claros et al., 2000). However, DNA-based markers are not influenced by environmental conditions, and they allow direct scanning of the plant genome (Martins-Lopes et al., 2007; Doveri et al., 2008). Genetic polymorphism of the plants can be detected by many different DNA-based marker methods such as RFLPs (Besnard and Berville, 2000), AFLPs (Sanz-Cortés et al., 2003; Owen et al., 2005), SSRs (Rallo et al., 2000; Cipriani et al., 2002; Belaj et al., 2004), ISSRs (Gemas et al., 2004), and random amplified polymorphic DNAs (RAPDs) (Belaj et al., 2001; Besnard et al., 2001; La Rosa et al., 2003; Wu et al., 2004; Ganino et al., 2007). Additionally, chloroplast and mitochondrial DNA analyses are helpful in examining the genetic structure of plants (Besnard et al., 2002).

DNA-based markers used in breeding and genetic studies of agriculturally important plants have specific advantages in terms of saving time compared to classical breeding methods, where they are not affected by environmental factors and produce results in a short time. RAPD markers, being one of the DNA-based markers, enable random sections of DNA to be rapidly reproduced using short polynucleotide primers. The polymerase chain reaction (PCR)-based RAPD assay was developed by Welsh and McClelland (1990) and Williams et al. (1990), and the RAPD markers are commonly used for genetic classification and determination of genetic diversity among both wild and cultivated olive types (Belaj et al., 2001; Besnard et al., 2001; La Rosa et al., 2003; Wu et al., 2004; Ganino et al., 2007).

Olives are raised in Turkey in a very wide range from the Aegean and Mediterranean and Marmara to Black Sea regions because these regions have favorable climatic conditions for olive breeding. The Aegean region has the most suitable breeding conditions among these regions, and olives are one of the commonly cultivated agricultural products in Manisa and Izmir provinces of the Aegean region; Izmir has a production share of 12.57% and Manisa 11.59% (MOARA, 2006; Sesli and Tokmakoglu, 2006).

Establishing genetic variation within and between olive types is a considerable part of cultivar sampling and is a support in determining the genetic relationships among wild types and their cultivated type relatives. Besides, knowledge of the genetic relationships between wild olives and their cultivated relatives is necessary to improve genetic resources and our understanding of their evolutionary background (Kockar and Ilikci, 2003; Baldoni et al., 2006). Presently, DNA-based markers give us a chance to make direct comparison of the organisms at the molecular level, as the use of DNA-based molecular markers has become popular in plant breeding as well as olives along with the other agriculturally important plants (Claros et al., 2000).

The primary objective of this study was to examine the genetic similarity and distance

between wild olive samples from Izmir and Manisa provinces of the Aegean region. The wild olive samples were obtained from different villages of Manisa and Izmir. The other objective was to gain any information on the present genetic state of wild olives in these provinces.

MATERIAL AND METHODS

Plant material

Samplings of *Olea europaea oleasters* were obtained from villages of Manisa and Izmir. A total of 7 samples were chosen among saplings from healthy and uniform young trees. Fresh leaves were collected and stored in liquid nitrogen until DNA extraction. Table 1 shows the wild olives used in this study and the provinces supplying them.

Table 1. Provinces supplying wild olives.

Type of olive	Provinces in Turkey
Wild 1	Caglak, Akhisar, Manisa
Wild 2	Harlak, Akhisar, Manisa
Wild 3	Sabancilar, Akhisar, Manisa
Wild 4	Bornova, Izmir, Bornova
Wild 5	Yayakirildik, Akhisar, Manisa
Wild 6	Bademli, Dikili, Izmir
Wild 7	Karacakas, Soma, Manisa

DNA extraction

Genomic DNA was extracted from young leaves using the Doyle and Doyle method (1987). One gram of olive leaves was ground using a mortar and pestle pre-chilled with liquid nitrogen, and 700 µL preheated CTAB extraction buffer (2% CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 2% β-mercaptoethanol) was added to the frozen ground leaves, mixing several times by gentle inversion. Samples with CTAB buffer were incubated for 30 min at 65°C. Tubes were mixed by inversion every 5 min during incubation. After removal from the hot bath, tubes were cooled down and 700 µL cold chloroform:isoamyl alcohol (24:1) was then added followed by vigorous mixing. Tubes were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were transferred to new tubes; 600 µL cold chloroform:isoamyl alcohol (24:1) was added followed by mixing by gentle inversion for 5 min. Samples were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were transferred to fresh tubes containing 10 M ammonium acetate and 3 M sodium acetate. A volume of 500 µL cold isopropanol was added and the contents mixed by shaking very gently for DNA precipitation. Precipitated DNA was removed with a pipette and washed with 70% ethanol. DNA was dried and resuspended in 50 µL EDTA. The crude DNA sample was treated with 1 µL RNAase A for 10 min at 37°C (final concentration 10 µg/µL).

The determination of DNA quality and concentration in samples was performed by both spectrophotometric analysis and running on 0.8% agarose gels. Optical density ratios from spectrophotometric analysis were evaluated and only good-quality DNA samples were used in PCR (Wu et al., 2004).

A total of 65 primers from the kits OP-A 1-20, OP-I 1-20, OP-Q 1-20, and OP-J 1-5

(Operon Technologies, Alameda, CA, USA) were used for RAPD-PCR assay. PCR was performed on an Eppendorf MasterCycler Thermal Cycler in a total volume of 25 µL. The PCR mix included 25 ng template DNA, 2.42 µL 10X PCR buffer with $MgCl_2$ (Sigma), 0.44 µL dNTP (Sigma), 1 µM primer, and 0.13 µL Taq DNA polymerase (Sigma). The amplification reactions were carried out for 60 s at 94°C as an initial denaturation.

The PCR program comprised 35 cycles of 20 s at 94°C for denaturation, 20 s at 35°C for annealing, and 20 s at 72°C for extension, and a final extension was performed at 72°C for 5 min.

Amplification products were loaded onto 1.5% agarose gels (Sigma) in 0.5X TBE buffer with 0.5 µg/mL ethidium bromide at 100 V constant voltage. For evaluating the base pair size of bands, a DNA ladder (Fermentas) was loaded on the first lane of each gel. After the separation of PCR products by agarose gel electrophoresis, gels were visualized with Photo Print (Vilber Lourmat, France) imaging system and analyzed by the BioOne D++ software (Vilber Lourmat, France). The RAPD bands were scored as 1 for present or 0 for absent; only clear bands were scored for the construction of the data matrix. The dendrogram was constructed using the POPGEN32 program according to Nei's coefficient (1972), and the UPGMA algorithm (unweighted pair-group method with arithmetic mean) was chosen for hierarchical clustering analysis (Sneath and Sokal, 1973; Yeh et al., 1999).

RESULTS AND DISCUSSION

A total of 32 primers of 65 yielded clear and stable bands, and a total of 115 loci were detected. The average number of evaluable bands per primer for all the samples was 3.59. A total of 50 bands were obtained with OP-A primers. Primers OP-A 9 and OP-A 10 showed the highest number of RAPD bands (16 and 11, respectively). No bands were obtained using primers OP-A 8, OP-A 17 and OP-A 18. However, 22 bands were obtained with the OP-Q primer set, and the most bands were produced by OP-Q 11 with a total of 11 bands. No bands were obtained with primers OP-Q 3, OP-Q 4, OP-Q 5, OP-Q 6, OP-Q 7, OP-Q 8, OP-Q 9, OP-Q 10. Scorable bands were not obtained with primers OP-I 4, OP-I 14, OP-I 15, OP-I 16, and OP-I 17 for the OP-I primer set; 43 bands were determined and the maximum number of loci was 14 with OP-I 14, whereas the minimum number of loci was found to be 2 with OP-I 15. There were no scorable bands with primers OP-J 1 to OP-J 5. The RAPD profiles obtained with the OP-I 17 primer are shown in Figure 1.

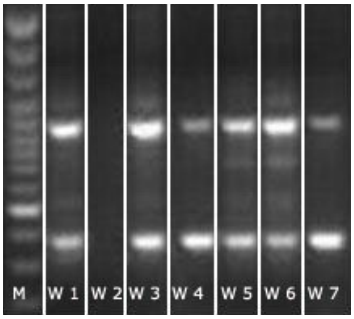

Figure 1. RAPD patterns with the use of primer OP-I 17. M = marker; W 1 (wild 1) = Caglak; W 2 (wild 2) = Harlak; W 3 (wild 3) = Sabancilar; W 4 (wild 4) = Bornova; W 5 (wild 5) = Yayakirildik; W 6 (wild 6) = Bademli; W 7 (wild 7) = Karacakas.

The matrix shown in Table 2 was obtained using Nei's genetic distance coefficient in the POPGEN32 software for determining the genetic similarities and distances between wild olives in the study. As a result of cluster analysis conducted using the UPGMA method, the dendrogram of different wild olive types was constructed as shown in Figure 2.

Table 2. Nei's genetic similarity and distance values obtained for wild-type olives (Nei, 1972).

	Wild 1	Wild 2	Wild 3	Wild 4	Wild 5	Wild 6	Wild 7
Wild 1	****	0.7478	0.7130	0.5826	0.6609	0.5913	0.5739
Wild 2	0.2906	****	0.8609	0.7130	0.7913	0.6522	0.7043
Wild 3	0.3382	0.1498	****	0.6783	0.7913	0.6348	0.6870
Wild 4	0.5402	0.3382	0.3882	****	0.6435	0.5043	0.5913
Wild 5	0.4142	0.2341	0.2341	0.4409	****	0.6696	0.6870
Wild 6	0.5254	0.4274	0.4545	0.6845	0.4011	****	0.6000
Wild 7	0.5553	0.3505	0.3755	0.5254	0.3755	0.5108	****

*Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

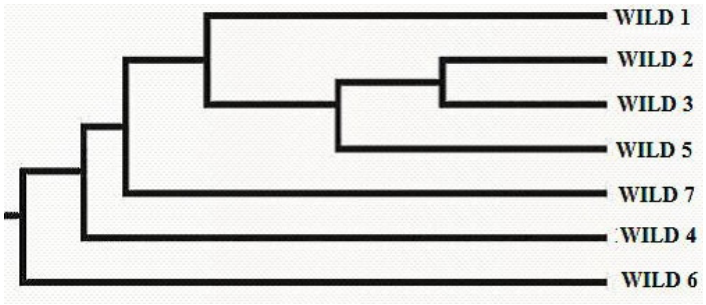

Figure 2. Dendrogram constructed for all the primers studied. For explanations of WILD 1-7, see Table 1.

The data matrix was constructed for samples common in all evaluable primers yielding bands while developing the dendrogram and matrix of genetic distances and similarities. Genetic distance values varied between 0.1498 (wild 2 and wild 3) and 0.6845 (wild 4 and wild 6). Thus, samples closest to each other are wild 2 and wild 3, and samples most distant from each other are wild 4 and wild 6, based on their genetic distance values.

Genetic similarity values varied between 0.8609 (wild 3 and wild 2) and 0.5043 (wild 6 and wild 4). Thus, samples with the closest genetic similarities are wild 3 and wild 2, and samples with the most distant genetic similarities are wild 6 and wild 4. Genetic similarity values and genetic distance values were in full compliance with each other.

It is very important to characterize wild forms of olives because of their intended use in olive improvement studies. In this study, OP-A, OP-Q and OP-I primer sets generated scorable bands in wild olive types, but 5 primers from the OP-J kit (OP-J 1 to OP-J 5) did not generate RAPD bands. The RAPD bands were found to be highly polymorphic as mentioned in other studies. The high degree of polymorphism obtained indicated a potential for selection and availability as a genetic source (Bandelj et al., 2002).

Belaj et al. (2001) reported a high genetic polymorphism rate of olive varieties grown in the Mediterranean basin using RAPD assay with 46 arbitrary primers. The results of this study were promising in our first experiments with OP-A, OP-Q and OP-I primer kits. A more

detailed study with larger numbers of samples using such primer sets could have a significant potential in the determination of genetic markers, due to the number of bands and high polymorphism in wild samples.

It is important to protect wild types of olives particular to regions in terms of breeding; therefore, it is important to study and determine the genetic structures of these olives. Determining genetic markers and supporting such markers with data on phenotypic and morphologic characteristics would have a substantial effect on olive breeding and the identification of olive types (Bernardi et al., 2001). However, changes that may be observed in phenotypic variations despite genetic similarities would probably be associated with environmental conditions and cultivation applications altering genetic expression (Wiesman et al., 1998).

RAPD markers are frequently used in determining the genetic structure of olive trees. RAPD markers are also beneficial as the initial step in displaying a genomic map for plants with unknown or much less known genetic ranges (Wu et al., 2004). The examination of genetic relationships between wild and cultivated types led to the construction of a genetic map of the olive tree and determination of suitable genetic markers for olive improvement studies.

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