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Full Length Research Paper

Genetic analysis on wild olives by using RAPD markers

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The aim of this study was to detect genetic similarities and distances among wild olive trees by RAPD-PCR technique. Olives are raised at a high range from Artvin on the north, up to Hatay on the south and up to Mardin on the Southeast part of Turkey. The Aegean, Mediterranean, Marmara and Black Sea regions of Turkey have suitable climatic conditions for olive breeding. Olive breeding had a rapid increase in the Aegean Region during recent years, among the agricultural products. Therefore, it is required to know the genetic relationships between wild olives and their cultural relatives in order to improve genetic resources and our knowledge of their evolutionary background. In this study, samples were mainly collected from Manisa province and additionally, samples were taken from Izmir and Mugla provinces for comparison. Genomic DNA's were extracted from young leaves and PCR was used to generate RAPD bands. 60 random primers obtained from Operon Tech. were tested by RAPD- PCR. Total of 38 primers were given highly polymorphic and continuous scorable bands. 167 bands were found and each primer was screened at an average of 4.4 polymorphic bands.

Key words: RAPD, PCR, wild olive.

INTRODUCTION

The olives (*Olea europaea,* L.) are 1 of the oldest cultivated crop trees of mankind. The cultivation of olive tree started in ancient era in the Mediterranean Basin thousand of years ago. Olives are raised in Turkey at a highly wide range from Artvin, on the north, up to Hatay on the south and up to Mardin on the Southeast part of Turkey. The Aegean, Mediterranean, Marmara and Black Sea regions of Turkey have suitable climatic conditions for olive breeding. One of the regions having the most suitable breeding conditions among the places which olives are raised is the Aegean Region. The *O. europaea* spp*. europaea* has 2 sub-types; these are wild olives, or *O. europaea oleaster*, which is also named as oleasters and the cultured varieties named as *O. europaea sativa* (Mendilcioglu, 1999). Wild olives or oleasters are abundantly found in the Aegean Region. In view of these provinces where olives are one of the commonly cultivated agricultural products, Izmir owns a share of 12.57%; Mugla a share of 11.64% and Manisa 11.59% (Sesli et al., 2007).

Genetic analysis on the variation within and among olive trees based on molecular techniques is an important part for the suitable cultivar sampling and also, it is helpful in determining the genetic relationships among wild oleasters and their cultural type relatives, therefore, it is required to know the genetic relationships between wild olives and their cultural relatives in order to improve genetic resources and our knowledge of their evolutionary background (Kockar and Ilikci, 2003; Baldoni et al., 2006).

Rapid identification of the economically important plants, their clones, varieties and types is essential for plant breeding and genetics. For this purpose, morphological characters such as leaves, flowers or seeds have previously been used as markers but the drastic effect of environmental conditions on genotype makes it difficult to perform a healthy evaluation on the basis of phenotype. Together with the discovery of DNA-based techniques, the need of fast and reliable methods for plant identifycation has found a new way. DNA – based markers are not influenced by environment condi-tions such as agronomic situation or phenologic phases of the plants. Presently, DNA-based markers give us a chance for directly comparing the organisms at molecular level. The use of DNA-based molecular markers have become popular during the recent years in plant breeding as well as olives

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along with the other economically important plants (Gonzalo et al., 2000). Owing to the molecular techniques, molecular markers are used for olive characterization, including Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR) and Sequence Characterized Amplified Regions (SCAR) (Wu et al., 2004). RAPD is a fast and commonly used molecular technique for screening the genome of living organisms by arbitrary primers (Welsh and McClelland, 1990; Williams et al., 1990).

RAPD was initially used for determining the genetic structure of plants such as wheat, corn, barley and grapes (D'ovidio et al., 1990; Shattuck et al., 1990; Weining and Landridge, 1991; Gogorcena et al., 1993; Collins and Symons, 1993). RAPD is used successfully in detecting genetic polymorphism and similarity of both oleasters and cultured varieties of olives and studies indicate that DNA-based markers are suitable for determining polymorphism as the genetic variety is high in olive trees. (Gemas et al., 2000; Gonzalo et al., 2000; Wu et al., 2004, Wiesman et al., 1998; Mekuria et al., 2004).

Determination of molecular markers peculiar to the varieties of olive plant ensures to discover phylogenetic connection between the plants and in addition to ensure accurate and easy designation of varieties. Since olive is a plant growing in a long period of time, molecular markers would also help developing further production planning as well as selecting the best variants.

This study aims at examining the genetic structure of wild olive samples obtained from different sites in the Akhisar, Manisa of Aegean Region in Turkey, as well as samples from Izmir and Mugla for the purpose of comparison. These are preliminary results derived from the first phase of study and it is planned to work with more arbitrary primers for wild olives and to search olive genome more deeply and thoroughly by developing SCAR markers in the next phase.

MATERIAL AND METHODS

Plant material

Saplings of oleasters were obtained from villages of Manisa, Izmir and Mugla where olive breeding is very common. Saplings were transferred to the greenhouse, for the purpose of DNA extraction; total of 15 samples were chosen between saplings from healthy and uniform young trees. Fresh leaves were collected and stored in liquid nitrogen until DNA extraction.

DNA extraction

Genomic DNA was extracted from young leaves by using Doyle and Doyle method (1987). For DNA extraction, plant tissues were crushed with liquid nitrogen in mortar. Ground tissues were immediately transferred to 1.5 ml Eppendorf tubes, 700 µl preheated CTAB extraction buffer (65°C, CTAB 2%, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl, 2% - mercaptoethanol) added onto leaf powder, mixed several times by gentle inversions. Samples with CTAB buffer were incubated for 30 min in 65°C hot water bath; tubes were mixed by inversions in every 5 min. Tubes were removed from water bath and waited to be cooled down and then added to 700 µl of cold 24:1 Chloroform: Isoamyl Alcohol. Tubes were spun for 10 min at 10.000 rpm/min in a refrigerated centrifuge. Supernatants were poured into new tubes; 600 µl of cold 24:1 Chloroform: Isoamyl Alcohol was added and mixed by gentle inversions for 5 min. Samples were spun for 10 min at 10.000 rpm/min in a refrigerated centrifuge again and supernatants were transferred to fresh tubes including 10 M Ammonium Acetate and 3 M Sodium Acetate; 500 µl cold Isopropanol was added and mixed by shaking very gently for DNA precipitation. Precipitated DNA was removed with pipette and washed with 70% Ethanol. DNA's were dried and re-suspended in 50 µl EDTA.

Following steps of DNA extraction, for the determination of DNA quality and concentration of DNA samples, samples were subjected both to spectrophotometric analysis and run in 0.8% agarose gels. In spectrophotometric analysis, each sample of DNA was calculated by their optical density values at 230, 260 and 280 nm. Optical density ratios were evaluated and only good quality DNA samples were used in PCR (Wu et al., 2004).

RAPD-PCR analysis

A total of 60 primers from Kits A, Q, I (Operon Technologies, Alameda, CA, USA) were used for RAPD-PCR analysis. PCR amplifications were carried out in a total volume of 25 l. PCR mix included 25 ng. template DNA, 2.42 l. 10 X PCR reaction buffer (with $MgCl_2$, Sigma), 0.44 l. dNTP and dTTP (Sigma), 1 M primer, 0.13 l Taq DNA polymerase (Sigma). PCR cycles were performed with initial denaturation of 94°C for 60 s and 35 cycles of 20 s 94°C; 20 s 35°C; 30 s 72°C. Final extension was performed at 72°C for 5 min. PCR amplifications were carried out in 96 - well Thermal cycler (Eppendorf Master Cycler) and all amplifications were carried out twice. A PCR mixture without template DNA was put in each analysis as a control.

PCR products were separated 1.5% agarose gels (Sigma) in 0.5 X TBE buffer with 0.5 g/ml ethidium bromide at 100 V constant voltage. For evaluating the base pair length of bands, DNA ladder (Fermentas) was loaded on first lane of each gel.

Data analysis

Gels were visualized with Photo Print (Vilber Lourmat, France) imaging system and analysis of RAPD bands were performed by BioOne D++ software (Vilber Lourmat, France). The RAPD bands (markers) were scored as 1 if present and 0 if absent. Only clear and reproducible bands were used for binary data matrix and dendrogram was constructed by POPGEN32 program according to Nei's coefficient and then UPGMA algorithm (Unweighted Pair-Group Method Using Arithmetic Averages) was chosen for hierarchical clustering analysis method (Sneath and Sokal, 1973; Nei, 1972; Yeh et al., 1997).

RESULTS

Band evaluation

PCR reactions of initial series were implemented in order to test the primers; to determine suitable PCR conditions and to fit RAPD analysis. 38 primers out of 60 yielded clear and stable bands as a result of first screening. Total of 167 loci were detected and all of them were polymerphic. Each primer showed an average of 4.4 polymorphic bands. Totally 57 bands were yielded from OP-A primers.

Table 1. Primers where most bands are obtained, numbers of bands and band ranges.

Primer	Nucleotide line	Number of loci per primer	Base pair lengths
OP-A 9	GGGTAACGCC	16	5163 - 1343 bp.
OP-A 10	GTGATCGCAG	12	3603 - 780 bp.
OP- Q17	GAAGCCCTTG	6	4882 - 4388 bp.
OP- I 14	TGACGGCGGT	24	1266 - 304 bp.

Table 2. Genetic distance and similarity matrix obtained from OP-A, OP-Q, OP-I primers in wild olives (Nei, 1972).

	Pınarcık	Ça lak	Harlak	Sabancılar	Bornova	Yayakırıldık	Bademli	Karacakas
Pınarcık	****	0.8323	0.9102	0.9102	0.7844	0.8743	0.7665	0.8024
Ça lak	0.1835	****	0.8263	0.8024	0.7126	0.7665	0.7186	0.7066
Harlak	0.0941	0.1907	****	0.9042	0.8024	0.8563	0.7605	0.7964
Sabancılar	0.0941	0.2202	0.1007	****	0.7784	0.8563	0.7485	0.7844
Bornova	0.2428	0.3389	0.2202	0.2505	****	0.7545	0.6587	0.7186
Yayakırıldık	0.1344	0.2660	0.1551	0.1551	0.2817	****	0.7725	0.7844
Bademli	0.2660	0.3305	0.2738	0.2897	0.4175	0.2582	****	0.7246
Karacakas	0.2202	0.3473	0.2276	0.2428	0.3305	0.2428	0.3222	****

**** Values above the diagonal indicate genetic similarity, whereas values below the diagonal indicate genetic distance.

Primers OP-A 9 and OP-A 10 showed the highest number of RAPD bands (16 and 12, respectively) whereas OP-A 8 showed no band. Totally 39 bands were obtained from OP-Q primer set and most bands were yielded by OP-Q17 with a total of 6 bands. Least bands were observed in OP-Q1, OP-Q3, OP-Q4 primers with one band in each. It was determined that molecular sizes of totally 39 bands varied between 6333 bp and 2627 bp. Scorable bands were not obtained from primers OP-Q 5, OP-Q 6, OP-Q 7, OP-Q 8, OP-Q9, OP-Q10. For OP-I primer set, totally 71 bands were determined and maximum loci number was determined as 24 in OP-I 14, whereas minimum number of loci was determined as 3 in OP-I 4. The base length of bands varied between 1568 bp and 304 bp. Table 1 shows the base ranges of primers, where most bands were observed and the pair lengths of bands yielded from these primers.

Hierarchical cluster analysis

The matrix shown in Table 2 was obtained by using Nei's genetic distance coefficient in POPGEN32 software for determining the genetic similarities and distances between wild olives in the study. As a result of cluster analysis conducted by using UPGMA method, the dendrogram of different wild olive types were developed as Figures 1 and 2 illustrates the banding pattern of primer OP-I 17.

The dendrogram of genetic distances conducted by using UPGMA algorithm produced 7 clusters (Figure 1). Data matrix was developed for samples common in all evaluable primers yielding bands while developing the dendrogram and matrix of genetic distances and similarities. Genetic distance values were between 0.0941 (Milas Pınarcık and Akhisar Harlak) (Milas Pınarcık and Akhisar Sabancılar) and 0.4175 (Izmir Bornova and Izmir Dikili Bademli) . Thus, samples closest to each other are (Milas Pınarcık and Akhisar Harlak) (Milas Pınarcık and Akhisar Sabancılar); samples most distant to each other are (Izmir Bornova and Izmir Dikili Bademli) based on their genetic distance values.

Genetic similarity values are between 0.9102 (Akhisar Harlak and Milas Pınarcık) (Akhisar Sabancılar and Milas Pınarcık) and 0.6587 (Izmir Dikili Bademli and Izmir Bornova). Thus, samples with closest genetic similarities are (Akhisar Harlak and Milas Pınarcık) and (Akhisar Sabancılar and Milas Pınarcık); samples with most distant genetic similarities are (Izmir Dikili Bademli and Izmir Bornova). Genetic similarity values and genetic distance values had been fully in compliance with each other.

DISCUSSION

Olive tree is a tree approximately 10 - 15 m high, with wide petals and likes the Mediterranean climate. Its fruits are used for consumption and oil production. There are 2 forms in Turkey; one is the cultured form and the other is the wild form. Especially wild forms of olives are commonly available in the Aegean Region (Mendilcio lu, 1999). Environmental factors affect morphologic features of olive plants. Therefore, it is difficult to determine varieties based on the phenotype and thus RAPD-PCR technique was used in defining wild forms in olives as this technique is not affected by environmental conditions.

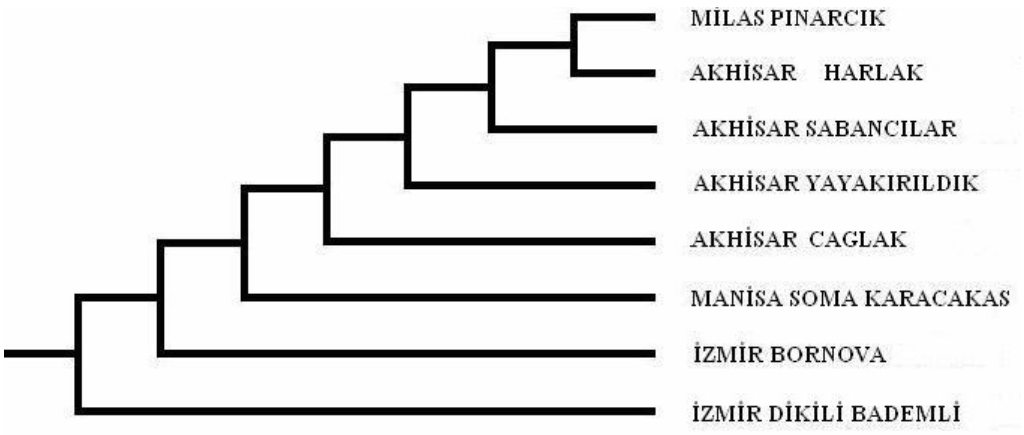

Figure 1. Genetic distance dendrogram produced by evaluable bands as obtained from OP-A, OP-Q, OP-I primers in wild type (Nei, 1972).

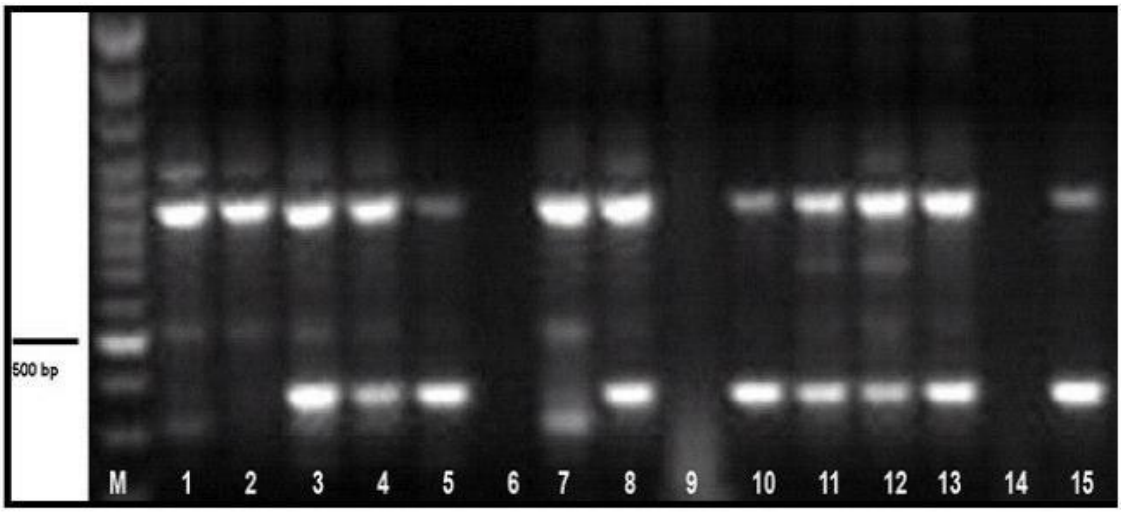

Figure 2. Banding pattern of primer OP-I 17.
M: Fermentas molecular weight marker, 1-2: Milas Pinarcik, 3-4: Akhisar Caglak, 5-6: Akhisar Harlak, 7-8: Akhisar Sabancilar, 9-10: zmir Bornova, 11-12: Akhisar Yayakirildik, 13-14: zmir Dikili Bademli, 15-16: Manisa Soma Karacakas.

Today, olive is an agricultural product having significant economical yields; in this respect, studies on improving the olives are profoundly important. The most significant purpose of olive improvement studies among the others is to obtain strong, healthy fruit-bearing trees. Consequently, it is very important to make characterization of wild forms of olives. DNA fragments were reproduced in different sizes from DNA samples isolated from wild olive leaves with RAPD- PCR method and Operon primers. 60 primers were used in the study. When the products of RAPD were evaluated at the end of study, it was observed that both OP-A, OP-Q and OP-I primer sets generated scorable bands in wild olive types. Present bands were recorded as one and non-present bands were recorded as zero (Banilas et al., 2003).

RAPD-PCR bands derived in our study showed a highly polymorphic structure as mentioned by other researchers. The obtained high polymorphism rate indicates a high genetic diversity in both in wild and culture varieties and consequently a distinct potential in selection and genetic source (Fabrri et al., 1995; Bandelj et al., 2002).

In addition to variety classification, the suitability and efficacy of this technique were examined by researchers (Belaj et al., 1997). Belaj et al. (2004) searched the genetic structure of olive varieties grown in the Mediterranean basin using RAPD technique and determined a high genetic polymorphism rate in the study where 46 random primers were used. The results obtained from OP-A; OP-Q and OP-I primer sets in wild samples were promising in

our first experiments. Examining in more detail and more quantities of samples by using such primer sets indicates that it may have a significant potential in the determination of genetic markers, due to the number of bands and high polymorphism in wild samples. In addition, phenotypic variations observed in site despite of genetic similarities or differences is most probably associated with genetic expression varying with changing environmental conditions and growing applications (Wiesmann et al., 1998). Highly polymorphic bands were also obtained in our study in compliance with the data acquired.

RAPD markers are intensely used in studies towards the determination of genotyping and genetic varieties of olive trees. Wu et al. (2004) indicated that RAPD marker applications are beneficial as the initial step in displaying a genomic map for plants with unknown or much less known genetic ranges.

Genetic similarities and distances between wild forms were characterized biometrically rather than morphologically. The genetic similarities and distances were defined and the genetic diversity between wild forms was put forth in this study. These results will also be used to determine the origins of cultural sub varieties. It is important for olive growing to identify olive types correctly today where their economic values are appreciated better. In case of failure to distribute and identify *O. europea* correctly, problems such as assigning different names to the same olive type grown in different regions result in confusions. It is important for protecting sub-types specific to regions by way of identifying the types to introduce type specific genetic structures and to determine genetic distances and similarities between the types. Finding genetic markers supported by displaying the morphologic characteristics would have a significant effect in identifying olive types (Bernardi et al., 2001).

Continuation of such studies would be an important step to examine genetic relations between especially local sub-types, namely wild types, cultural sub- types and both olive sub-types, to prepare genetic map of olive tree and to determine genetic markers of types by comparing foregoing types. At the same time, examining genetic variety following the RAPD-PCR study by also including SCAR and SSR markers, and discovering the relations between morphologic characteristics and genetic struc-ture by determining morphologic characteristics would be a rapid and effective way in identifying types of olives having economical importance and then determining ge-netic markers specific to types that may be used in improvement studies. In addition, it is aimed by displaying genetic connections between types to assist in perform-ing further production plans and improvement studies for the olive, which is a plant dating back to many years.

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