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Genetic variation among date plum (*Diospyros lotus*) genotypes in Turkey

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ABSTRACT. Turkey is one of the most important genetic resources of the date plum, *Diospyros lotus*, especially in the northeastern part of the country. Authenticating the identity of germplasm resources of *D. lotus* would be of great value for breeding. We examined the genetic variability of 11 *D. lotus* genotypes sampled from Coruh Valley in Turkey. One hundred and twenty-eight DNA markers were generated by 12 random primers. The highest polymorphism ratio was observed with the primer OPA-01 (71%) while the lowest was with OPY-01 (36%). The band size was between 350 and 2500 bp for these primers. The percentage of polymorphic bands was 58%, which demonstrated the efficiency of these primers. The similarity between genotypes ranged from 0.48 to 0.76. The RAPD markers permitted us to distinguish all the genotypes.

Key words: Persimmon; *Diospyros lotus*; RAPD; Genetic diversity

INTRODUCTION

Intensive agriculture has resulted in the loss of much of the genetic diversity that local and traditional genotypes possessed. The new varieties developed through modern techniques are becoming genetically more homogenous than ever before and are, thus, more vulnerable to pathogens and adverse environmental conditions (Asins and Carbonell, 1989). This has prompted scientists to look for new sources of variations to widen the genetic base of the highly resistant genotypes, which can be more adaptable to the local environment. Local genotypes found within the same agro-climatic conditions offer the advantage of being more easily utilized for breeding purposes than those found in far away places. Thus, the precious genetic resources of the local genotypes present should be conserved for their proper utilization. In order to achieve this, it is imperative to assess the genetic variability that exists among local genotypes (Ercisli, 2004; Vijayan et al., 2006).

Diospyros lotus L. is a deciduous tree of up to 15 m in height, from the family Ebenaceae. In Turkey, it grows naturally in the Northeast Anatolia region. In this region, it has been cultivated for edible fruits and has also been used as rootstock for *Diospyros kaki* (Onur, 1990; Onur and Onur, 1995; Ercisli and Akbulut, 2009). Its fruits are globose, 1.5-2.0 cm in diameter and yellow or bluish-black in color at maturity (Yaltirik, 1978).

The fruits are preferably not eaten in the immature form because of their astringent taste. Fresh and mature fruits or dried ones are eaten and sold in the markets, particularly in the northeast part of the country where *D. lotus* trees are abundant. The fruits of this species are also used in folk medicine in Turkey as a treatment for constipation (Baytop, 1984).

In Turkey, few studies have been conducted on *D. lotus*, and also, these studies are concentrated on the physical and biochemical characteristics of fruits (Ayaz et al., 1995; Glew et al., 2005). However, no studies have previously been done on the genetic characteristics of *D. lotus*.

Polymerase chain reaction (PCR)-based molecular markers, including random amplified polymorphic DNA (RAPD), have provided a powerful new tool for breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited traits.

The molecular approach for the identification of plant varieties/genotypes seems to be more effective than the use of traditional morphological markers, because it allows direct access to the hereditary material and makes it possible to understand the relationships between plants (Williams et al., 1990; Paterson et al. 1991).

The purpose of the present study was to assess the genetic diversity within *D. lotus* genotypes using RAPD markers and to compare the genotypes with each other.

MATERIAL AND METHODS

Leaf samples from 11 wild-grown *D. lotus* genotypes were collected in Coruh Valley, which is located in Northeast Anatolia. The leaves were stored immediately at -80°C for later DNA extraction.

Genomic DNA was extracted from powdered (ground in liquid nitrogen) leaf materials using the modified method described by Lin et al. (2001). Approximately 10-15 mg tissue from each plant sample was snap-frozen in liquid nitrogen in 2-mL Eppendorf tubes. A volume of 1000 µL DNA extraction buffer [100 mM Tris-HCl, pH 8.0;

50 mM EDTA, pH 8.0; 500 mM NaCl; 2% SDS (w/v); 2% 2-mercaptoethanol (v/v); 1% PVP (w/v)] was added and well mixed. The mixture was incubated at 65°C in a water bath for 40 min with intermittent shaking at 5-min intervals. The mixture was centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was transferred to a clean 1.5-mL tube, mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and then centrifuged. The supernatant was collected and mixed with 1/10 volume 10% CTAB-0.7 M NaCl in a clean tube. After centrifugation, the supernatant was collected and mixed gently with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of 0.6 volume freezer-chilled isopropanol, for 10 min at -20°C. DNA was pelleted by centrifugation at 12,000 *g* for 10 min and the isopropanol was poured off; the DNA recovered was allowed to air-dry before being dissolved in 100 µL TE buffer.

The samples were screened for RAPD variation using the standard 10-base primers supplied by Operon. A 30-µL reaction cocktail was prepared as follows: 3.0 µL 10X buffer, 1.2 µL dNTPs (10 mM), 1.2 µL magnesium chloride (25 mM), 2.0 µL primer (5 µM), 0.4 µL *Taq* polymerase (5 U), 19.2 µL water, and 3.0 µL sample DNA (100 ng/µL). A total of 70 RAPD primers were tested in this study, and the polymorphisms obtained with the primers are shown in Table 1.

The thermocycler (Eppendorf Company) was programmed as follows: 2 min at 95°C; 2 cycles of 30 s at 95°C, 1 min at 37°C, and 2 min at 72°C; 2 cycles of 30 s at 95°C, 1 min at 35°C, and 2 min at 72°C; 41 cycles of 30 s at 94°C, 1 min at 35°C, and 2 min at 72°C, and a final 5-min extension at 72°C, followed by cooling down to 4°C. The markers were checked twice for their reproducibility.

The PCR products (27 µL) were mixed with 6X gel loading buffer (3 µL) and loaded onto an agarose (1.5%, w/v) gel in 0.5X TBE (Tris-borate-EDTA) buffer, and electrophoresis was at 70 V for 150 min. The gel was stained in an ethidium bromide solution (2 µL/100 mL 1X TBE buffer) for 40 min, and the bands were visualized under UV in a Bio Doc Image Analysis System with the Uvisoft analysis package (Cambridge, UK).

The positions of scorable RAPD bands were transformed into a binary character matrix (1 for the presence and 0 for the absence of a band at a particular position), which was entered in the RAPDistance computer program (Armstrong et al., 1994). These data were used for the calculation of pairwise genetic distances between cultivars using the Jaccard coefficient (JC). The computer program calculated the degree of genetic dissimilarity between each pair of the 21 genotypes using the simple equation: $JC = 1 - a / (a + b + c)$, where a is the number of bands shared by plant x and plant y, b is the number of bands in plant x, and c is the number of bands in plant y. The JC ignores the absence of matches. The distance matrix was used for cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA).

RESULTS

A total of 70 decamer oligonucleotide primers were used to investigate 11 *D. lotus* genotypes. The results of RAPD analysis are summarized in Table 1. The 12 random primers generated a total of 128 RAPD bands. The highest number of polymorphic band (9) was obtained with OPH-14 (Table 1). The size of the amplicons varied from 350 to 2500 bp. Primer OPY-01 gave the lowest number of RAPD products (Table 1).

Table 1. Total number of amplified fragments and number of polymorphic fragments generated by polymerase chain reaction using selected random decamers.

Primer code	Sequence 5'→3'	Size (bp)	Polymorphic bands	Monomorphic bands	Total	P (%)
OPA-01	CAGGCCCTTC	850-2250	5	2	7	71
OPA-04	AATCGGGCTG	600-1300	6	3	9	50
OPH-14	ACCAGGTTGG	600-1400	9	5	14	64
OPH-18	GAATCGGCCA	700-2500	6	4	10	60
OPH-19	CTGACCAGCC	600-2000	7	4	11	64
OPH-20	GGGAGACATC	500-1000	5	7	12	42
OPW-13	CACAGCGACA	350-1100	5	5	10	50
OPW-20	TGTGGCAGCA	400-900	5	6	11	46
OPY-01	GTGGCATCTC	600-1600	4	7	11	36
OPY-08	AGGCAGAGCA	500-2400	6	6	12	50
OPY-15	AGTCGCCCTT	350-1400	5	6	11	46
OPY-17	GACGTGGTGA	400-1300	6	4	10	60
Total		350-2500	69	59	128	58

The distance matrix showed that the highest genetic similarity (0.76) was between ALS 8 and ALS 10 and the lowest (0.48) was between ALS 3 and ALS 6. This indicates that the genotypes ALS 8 and ALS 10 are genetically closer to each other than to other genotypes. Similarly, the high genetic distance between ALS 3 and ALS 6 points to the possibility of utilizing them for heterosis breeding. The average polymorphism among the genotypes was 58%, which clearly shows significant genetic diversity among the *Diospyros lotus* genotypes. Hence, these genotypes should be preserved as valuable genetic resources for breeding. The dendrogram constructed from the RAPD markers grouped the 11 genotypes into two major clusters (Figure 1). Cluster 1 consisted of four genotypes (ALS 3, ALS 8, ALS 10, and ALS 7), and this cluster further divided 2 subclusters, the first subcluster included ALS 7, ALS 8, and ALS 10 and the second one include only ALS 3. Cluster 2 was divided into 2 subclusters, where ALS 1 and ALS 4 formed a subcluster and ALS 4 clustered alone. In cluster 3, there are also 2 subclusters: ALS 6 and ALS 9 together and ALS 9 and ALS 11 together (Figure 1).

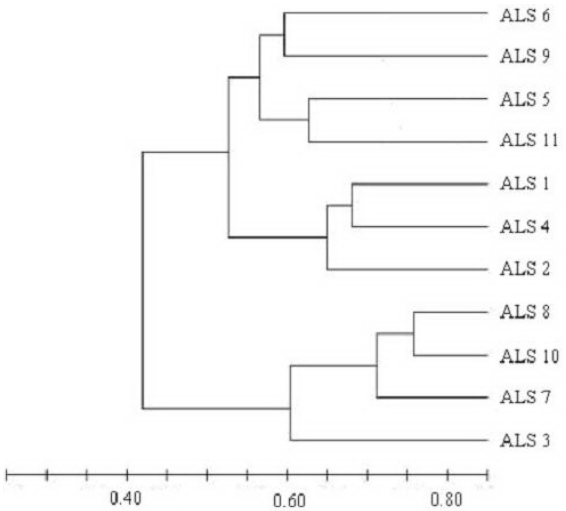

Figure 1. UPGMA dendrogram showing the relationship of *Diospyros lotus* genotypes.

DISCUSSION

The high genetic distances obtained between these genotypes clearly suggest that they must have originated from genetically divergent parents or have a long history of adaptation to their respective micro-climatic regions. Earlier studies using RAPD (Badenes et al., 2003; Gonçalves et al., 2004; Yamagishi et al., 2005) techniques showed large genetic variations present among different *D. kaki* cultivars and genotypes. The distinctness of the *D. lotus* genotypes revealed by RAPD in the present study can be attributed to their seed-propagated nature resulting in high heterozygosity, which has accompanied the long history of cultivation and domestication. Classification of *D. lotus* genotypes based on phenotypic variations or isozyme patterns should be reconsidered in the context of the results obtained from molecular analyses with RAPD. However, the molecular studies with RAPD uncovered considerable differences among genotypes as mentioned above. Thus, RAPD-based molecular markers were able to distinguish different genotypes, which were indistinguishable by isozyme-based markers.

This is the first attempt to use molecular markers to investigate the genetic relationships among *D. lotus* genotypes grown under the same temperate agro-ecological conditions. The determination of genetic relationship based on RAPD markers provides a reliable method for the identification of genotypes, over morphological characters. This investigation provides an understanding of the level and partitioning of genetic variation within *Diospyros lotus* genotypes, which can be an important input into determining efficient management strategies.

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