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Full Length Research Paper

Determination of similarity among Turkish sweet gum (*Liquidambar orientalis* Mill.) populations using RAPD markers

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Random amplified polymorphic DNA (RAPD) markers were used to determine genetic relationships among five native populations of *Liquidambar orientalis* Mill. Eighty-four oligonucleotide primers were used in PCR and 782 RAPD markers were evaluated for statistical analysis. Generated Nei-Li's coefficient indicated that the highest similarity was 83.1% between samples from Koycegiz and Cine populations, while the lowest as 68.3% between samples from Gunluklu and Karacaoren populations.

Key words: *Liquidambar orientalis*, RAPD analysis, similarity, sweet gum.

INTRODUCTION

Altingiaceae Horan. (sweet gums) is a small subfamily in the family Hamamelidaceae including about eighteen species belonging to three genera as *Altingia*, *Liquidambar* and *Semiliquidambar*. Genus *Liquidambar* is represented by five species (Shi et al., 2001; Ickert-Bond and Wen, 2006). The genus was much more widespread in the Tertiary than today. This genus has disappeared from Europe, West and North America, Russian and Far East due to extensive glaciations, climate change, unglaciation, respectively. *Liquidambar* is monophyl according to morphological cladistic analysis. It has spread in same latitude as groups after ice age. But today *L. formosana* Hance is distributed in China, *L. edentata* Merr., *L. styraciflua*, *L. macrophylla* Oerst. in North America and *L. orientalis* Mill. in Turkey and Rhodos (Pigg et al., 2004; Ickert-Bond and Wen, 2006).

Liquidambar orientalis Mill. is one of the endemic forest tree species in our country. It is economically important in terms of balsam producing ability and medicine, as well as in the chemical and cosmetic industry. Unfortunately *L. orientalis* is under the risk of extinction because of illegal cutting for obtaining wood material and unconsciously wounding for balsam production. Vegetations of this species are in Mugla province and four restricted areas in Southwest Anatolia. They are large trees, 25 - 40 m in height, deciduous, with palmately lobed leaves arranged spirally on the stems. The flowers are small and formed by male inflorescence knobs and female inflorescence. Fruits are 2 - 4 cm in diameter and form woody multiple capsules containing numerous seeds (Efe, 1987; Ickert-Bond et al., 2005).

Adaptation of plant populations to certain environment and evolution of plant species are directly associated with variation among species. Turkey is a gene centre for perennial, many wild, transitional and cultivated plants of annual and herbaceous and woody plants due to the diversity in its geomorphology, topography and climate (Kaya et al., 1997). Continuity of variation genetically is very important in terms of conservation of biodiversity.

Various biochemical and molecular marker techniques have been used in order to determine the variation in nature. Random amplified polymorphic DNA (RAPD) is one of the PCR based tools in genetic studies (Williams et al., 1990). RAPD markers have been used for many types of genetic analysis, including genome mapping,

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geotyping, phylogeny reconstruction, and measuring genetic similarities (Lee et al., 2002; Lamboy, 2007). Although RAPD markers have limitations, they are used for evaluation of whole genome, including non-coding and linked to structural gene regions (Isabel et al., 1995). Compared to the other molecular marker techniques such as RFLP, AFLP; RAPD is quick, the simplest to apply and has advantage of requiring little plant material. This method has been used effectively in the investigation of genetic structures of forest tree populations, such as *Hippophae rhamnoides*, *Picea abies*, *Pinus longaeva* (Bartish et al., 1999; Collignon and Favre, 2000; Lee et al., 2002). But, there are only a few studies and genetic knowledge on *L. orientalis*. In this work, the genetic diversity of five *L. orientalis* populations was investigated by using RAPD analysis. Findings obtained from RAPD analysis will not only determine variation and genetic structure among *L. orientalis* populations but will also contribute to conservation of this species.

MATERIALS AND METHODS

Plant material

Young leaflet branches were sampled from adult reproductive trees in five populations from different vegetations, including 128 m altitude at Kavakarası/Koycegiz (37''52.204' N, 0.28''42.269' E), 47 m altitude at Fethiye/Gunluklu (36''53.954' N, 0.28''42.860' E), 192 m altitude at Aydin/old bridge location of Cine stream, (37''27.879' N, 0.28''0.402' E), 151 m altitude at Aydin/Ilica location of Imamlar Village, (37''52.263' N, 0.27''54.458' E) and Isparta/Karacaoren Dam (36''43.139' N, 0.29''01.376' E). After plant material had been collected, they were preserved at -70°C.

Genomic DNA extraction and amplification

Total genomic DNAs were extracted from frozen young leaflet according to modifying Walbot (1988) method along with high pure PCR template preparation kit (Roche). In order to homogenize, 1 g of young frozen leaves was mashed in mortar with liquid nitrogen. And then, homogenisation was carried out by mashing in extraction buffer (15% sucrose (w/v), 50 mM Tris HCl (pH 8.0), 50 mM EDTA, 250 mM NaCl). Homogenate was transferred to microtube, and then microtube centrifuged at 4°C for 5 min. Pellet was suspended in suspension buffer (20 mM Tris HCl (pH 8.0), 10 mM EDTA). Microtubes were incubated at 70°C for 15 min after adding 20% (w/v) SDS and in ice for 30 min after adding 7.5 M ammonium acetate. Binding buffer and proteinase K were added to supernatant after centrifugation. The microtubes were well shaken and then incubated at 72°C for 10 min and suspension was transferred to filter tube. Before centrifugation, isopropanol was added to the filter tubes. Pellet was suspended with inhibitor removal buffer and centrifugation was performed for 1 min. Washing buffer was added to the pellet. After centrifugation, the pellet was suspended with elution buffer and later the tubes were centrifuged for 1 min. Finally, DNA was suspended in double distilled sterile water.

A total of 84 oligonucleotide 10mer OPERON primers were used in the RAPD analysis. Table 1 includes all of primers and sequences used in this study. Random sites in the genome were amplified in final concentrations of 50 ng of template, 15 - 30 ng of arbitrary primer, 300 µM of each dNTP, 2.5 mM $MgCl_2$ and 0.05 U of Taq DNA polymerase. Polymerase chain reactions were performed in a total volume of 25 µl with in a Rea com T-C-Y thermocycler programmed for initial denaturation of 94°C for 5 min, followed by 40 cycles of 1 min at 94°C for denaturation of dsDNAs, 1 min at 36°C for annealing of primer to ssDNA and 2 min at 72°C for extension of new DNA chain. Final extension was completed at 72°C for 10 min. Finally, reactions in the tubes were halted at 4°C.

Amplification products were analysed on 1.7% agarose gels dyed with ethidium bromide in 1x TAE buffer. Gels were run for 2 h at 60 V. Genomic DNA fingerprinting belonging to samples collected from five vegetation areas was visualized under UV light and photographed on Polaroid 667 film with Polaroid MP-4 Land Camera (CAMAG REPROSTAR)

Data analysis

A total of 782 RAPD markers were generated by PCR and all of them evaluated for statistical analysis. The Multi Variate Statistical Package (MVSP) 3.1 computer programme was used to determine the diversity. Pair-wise comparisons of unique and shared polymorphic amplification products were used to generate Nei-Li's coefficient among these populations (Nei and Li, 1979). A dendogram of the five populations was constructed using an unweighted pair-group method with arithmetical averages (UPGMA).

RESULTS AND DISCUSSION

A total of 84 10-mer primers were used in the RAPD analysis. Thirty nine of these primers had 70% G+C content and 45 had 60%. Random amplified polymorphic DNA findings showed 782 DNA markers were amplified in different populations of the same species (Figure 1). These 782 markers generated range from 900 to 2500 bp. Two hundred eighty six were monomorphic and 496 were polymorphic.

The 782 markers were evaluated by MVSP 3.1 computer programme for calculation of similarity among the *L. orientalis* populations according to Nei-Li's coefficient (Table 2). The most similar populations were Cine and Koycegiz (83.1%) whereas the most distant populations between Karacaoren and Gunluklu (68.3%).

The dendogram showed that five populations coming from common ancestry are separated to four brunches (Figure 2). Gunluklu was equidistant from all other populations, Karacaoren from Aydin, Cine and Koycegiz. Aydin population is closely related to Cine and Koycegiz cluster.

Turkey has extensive biodiversity in forest trees because of diversity in its geomorphology, topography and climate conditions (Kaya et al., 1997). General situation of Turkey was discussed in the 3rd meeting of European Forest Genetic Resources Programme (EUFORGEN) and five species (*Liquidambar orientalis*, *Alnus orientalis*, *Platanus orientalis*, *Pterocarya fraxifolia*, *Ostrya carpinifolia*) were added to the list for conservation,

Table 1. 10-mer RAPD primers and their sequences used in the RAPD analysis.

Primer code	Sequences	Primer code	Sequences	Primer code	Sequences
OPA02	5'-TGCCGAGCTG-3'	OPC08	5'-TGGACCGGTG-3'	OPF02	5'-GAGGATCCCT-3'
OPA03	5'-AGTCAGCCAC-3'	OPC17	5'-TTCCCCCCAG-3'	OPF03	5'-CCTGATCACC-3'
OPA04	5'-AATCGGGCTG-3'	OPC19	5'-GTTGCCAGCC-3'	OPF04	5'-GGTGATCAGG-3'
OPA05	5'-AGGGGTCTTG-3'	OPD01	5'-ACCGCGAAGG-3'	OPF05	5'-CCGAATTCCC-3'
OPA06	5'-GGTCCCTGAC-3'	OPD02	5'-GGACCCAACC-3'	OPF06	5'-GGGAATTCGG-3'
OPA08	5'-GTGACGTAGG-3'	OPD03	5'-GTCGCCGTCA-3'	OPG01	5'-CTACGGAGGA-3'
OPA09	5'-GGGTAACGCC-3'	OPD04	5'-TCTGGTGAGG-3'	OPG02	5'-GGCACTGAGG-3'
OPA11	5'-CAATCGCCGT-3'	OPD05	5'-TGAGCGGACA-3'	OPG03	5'-GAGCCCTCCA-3'
OPA12	5'-TCGGCGATAG-3'	OPD06	5'-ACCTGAACGG-3'	OPG07	5'-GAACCTGCGG-3'
OPA13	5'-CAGCACCCAC-3'	OPD07	5'-TTGGCACGGG-3'	OPG10	5'-AGGGCCGTCT-3'
OPA15	5'-TTCCGAACCC-3'	OPD08	5'-GTGTGCCCCA-3'	OPG11	5'-TGCCCGTCGT-3'
OPA16	5'-AGCCAGCGAA-3'	OPD13	5'-GGGGTGACGA-3'	OPG13	5'-CTCTCCGCCA-3'
OPA18	5'-AGGTGACCGT-3'	OPD14	5'-CTTCCCCAAG-3'	OPG16	5'-AGCGTCCTCC-3'
OPB06	5'-TGCTCTGCCC-3'	OPD17	5'-TTTCCCACGG-3'	OPG17	5'-ACGACCGACA-3'
OPB07	5'-GGTGACGCAG-3'	OPD20	5'-ACCCGGTCAC-3'	OPG18	5'-GGCTCATGTG-3'
OPB09	5'-TGGGGGACTC-3'	OPE01	5'-CCCAAGGTCC-3'	OPG20	5'-TCTCCCTCAG-3'
OPB10	5'-CTGCTGGGAC-3'	OPE02	5'-GGTGCGGGAA-3'	OPZ02	5'-CCTACGGGGA-3'
OPB11	5'-GTAGACCCGT-3'	OPE03	5'-CCAGATGCAC-3'	OPZ03	5'-CAGCACCGCA-3'
OPB13	5'-TTCCCCCGCT-3'	OPE04	5'-GTGACATGCC-3'	OPZ04	5'-AGGCTGTGCT-3'
OPB14	5'-TCCGCTCTGG-3'	OPE05	5'-TCAGGGAGGT-3'	OPZ07	5'-CCAGGAGGAC-3'
OPB15	5'-GGAGGGTGTT-3'	OPE06	5'-AAGACCCCTC-3'	OPZ08	5'-GGGTGGGTAA-3'
OPB16	5'-TTTGCCCGGA-3'	OPE08	5'-TCACCACGGT-3'	OPZ09	5'-CACCCCAGTC-3'
OPB17	5'-AGGGAACGAG-3'	OPE10	5'-CACCAGGTGA-3'	OPZ10	5'-CCGACAAACC-3'
OPB18	5'-CCACAGCAGT-3'	OPE12	5'-TTATCGCCCC-3'	OPZ12	5'-TCAACGGGAC-3'
OPB19	5'-ACCCCCGAAG-3'	OPE13	5'-CCCGATTCGG-3'	OPZ15	5'-CAGGGCTTTC-3'
OPC02	5'-GTGAGGCGTC-3'	OPE14	5'-TGCGGCTGAG-3'	OPZ16	5'-TCCCCATCAC-3'
OPC05	5'-GATGACCGCC-3'	OPE20	5'-AACGGTGACC-3'	OPZ19	5'-GTGCGAGCAA-3'
OPC07	5'-GTCCCGACGA-3'	OPF01	5'-ACGGATCCTG-3'	OPZ20	5'-ACTTTGGCGG-3'

identification, determination of diversity, etc (Turok ve ark, 1999). Vegetation of *L. orientalis* is mainly in Mugla province. Other vegetations are in Aydin, Fethiye and Isparta as little populations. Population from old bridge location of Cine stream will vanish in the near future due to construction of new dam. Thus one vegetation area is under big threat.

There is little knowledge about the genus *Liquidambar* (Alan and Kaya, 2003). Recent studies have focused on phylogenetics of the Hamamelidaceae family. Generally, samples of *L. orientalis* in these studies were obtained from either Washington Arboretum or China Botanical Garden (Li et al., 1999; Shi et al., 2001). There was one research using samples of this species from Turkey (Ickert-Bond and Wen, 2006) and there were two preliminary studies based on research of *Trn* and *matK* regions of cpDNA (Or and Kaya, 2006; Ozdilek ve ark, 2006).

Isoenzyme (Hoey and Parks, 1991) and molecular phylogenetic (Li et al., 1999) studies on *Liquidambar* genus showed that *L. orientalis* and *L. styraciflua* are related more closely than the other extant forms because of their inhabiting same climatic conditions despite geographical distance between them. Shi et al. (2001) supported that *L. styraciflua* and *L. orientalis* were paraphyletic. Also Ickert-Bond et al. (2006) estimated that these two species had the same divergence time according to analyses of five non-coding cpDNA sequence data. They confirmed other researchers' findings.

In this study, five populations of *L. orientalis* were analysed by using RAPD analysis. Significant diversity among different populations of the same species was determined. When Table 2 was analysed, Gunluklu was the most diverse population in comparison to other populations with variable ratios from 31.7 to 27.2%. This made us to speculate that Gunluklu population cannot be

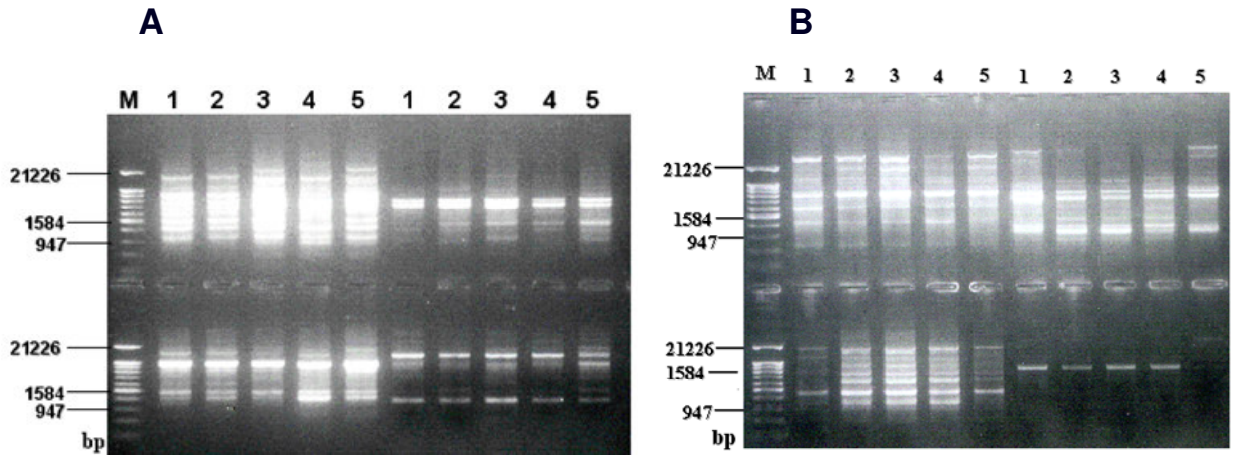

Figure 1. DNA fingerprintings obtained from RAPD analysis by using (**A**) OPE04, OPE05 (upper row of agarose gel), OPE06, OPE08 (lower row of agarose gel) primers (**B**) OPB10, OPB11 (upper row of agarose gel), OPB15, OPB16 (lower row of agarose gel) primers. Lanes (1) Aydin, (2) Cine, (3) Koycegiz, (4) Gunluklu and (5) Karacaoren stations. M indicates λ DNA digested with *Hind*III/*Eco*RI.

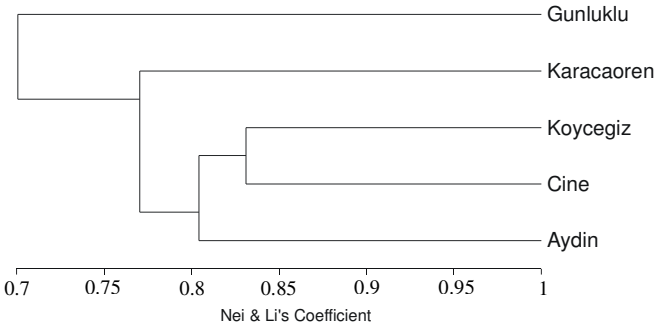

Figure 2. Dendogram from UPGMA clustering of *L. orientalis* populations based on values of Nei and Li similarity coefficients.

native. However Forestry Department in Fethiye declared that this population was native. Forest trees are relatively undomesticated. Unlike other plants they have genetic and phenotypic variation. In neutral forest tree populations, genetic variations exist abundantly (González-Martínez et al., 2006). Therefore, the little population of Gunluklu is more isolated and more varied than other populations.

When Karacaoren is compared to other populations, except Gunluklu, it is clear that Karacaoren is similar to Aydin and Koycegiz (77.4 and 77.5%, respectively) than to Cine population (76.3%).

Although Aydin and Cine populations are the closest populations geographically, Cine is more similar to Koycegiz rather than Aydin. Aydin is on the fault line and there were countless devastating earthquakes in the past (Altunel, 1998). In Ilica location of Imamlar Village, there was a big earthquake in the past and samples used in this study were collected from crushed fault base. Earthquakes might induce geographic isolations between Aydin and Cine populations with time. As a consequence of this, gene flow between fragmented populations might be obstructed. According to Fischer et al. (2000), if formerly larger population was fragmented and gene flow was limited, populations would be genetically differentiated. Populations of plant species are smaller and more isolated from each other today, than they were in the past. This is caused by increase in geographic isolation which decreases gene flow. For these reasons, Aydin population is smaller and more isolated from Cine. Cine and Koycegiz populations are more closely related than Aydin.

RAPD analysis is preferred by many researchers as an effective method to use for identification of genetic variation within and among populations in forest trees (Jordano and Godoy, 2000; Tsuda et al., 2004; Hardy et al., 2006). Also, RAPD markers show levels of polymorphism similar to isoenzyme markers (Lee et al., 2002). There are a lot of reasons for usage of this analysis. One of them is to target amplifying a large number of loci (Bartish et al., 1999; Lee et al., 2002). These loci can be within non-coding DNA and/or regions linked to structural genes (Isabel et al., 1995). Others reasons are the experiment being quick and easy, requiring little amount of plant material. Besides, RAPD is preferred since it is used as selectively neutral genetic marker in population genetic studies (Fischer et al., 2000).

In addition to these advantages this method has significant limitations compared to other molecular marker techniques. RAPDs are dominant markers therefore hete-

Table 2. Similarities among five populations calculated according to Nei and Li (1979) coefficient.

	Aydin	Cine	Koycegiz	Gunluklu	Karacaoren
Aydin	100				
Cine	81.2	100			
Koycegiz	79.7	83.1	100		
Gunluklu	69.0	72.8	70.1	100	
Karacaoren	77.4	76.3	77.5	68.3	100

rozygote genotypes cannot be scored (Isabel et al., 1995). DNA concentration used in PCR is effected by various impurities such as polysaccharides, phenolics etc. These constituents can cause polymorphism. For this reason DNA isolation procedure was modified from Walbot (1988) and used together with commercial kit in this study. Thus PCR procedure was optimised exactly. Also, artifactual DNA fragments can be amplified as false positive (appear in lane but should not) and false negative (do not appear in lane but should) in RAPD analysis. There are two approaches for reduction of effects on analysis of RAPD markers. Either faint or inconsistent bands are not evaluated, only reproducible DNA fragments are accepted as markers, or all visible bands are used for calculation and accepted a certain level of error (Lamboy, 2007). In this study, all visible DNA fragments were evaluated by MVSP 3.1 computer programme for calculation of similarity.

In addition, polymorphism is closely related to G+C content of used primer molecule. A lot of polymorphic markers were amplified by primer with 70% G+C contents while monomorphic markers with 60%, in this study. Findings obtained from this RAPD analysis, not only determine variation and genetic structure among *L. orientalis* populations but will also contribute to conservation of this species.

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