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RESEARCH ARTICLE

Genetic Structure and Phylogenetic Analysis of *Liquidambar orientalis* Mill. (Altingiaceae) Populations Based on Non-Coded *psaA/ycf3* Intergenic Region in The Chloroplast Genome in Türkiye

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Abstract

Objective: The genus *Liquidambar*, is one of many woody genera with morphologically similar species in America, Southeast Europe, and Asia and is thought to have existed on Earth for about 65 million years. *Liquidambar orientalis* (Anatolian sweetgum tree) is distributed in southwestern Türkiye. With this study, it was aimed to reveal the sequence differences of the *psaA/ycf3* inter-gene space region of chloroplast DNA (cpDNA) in *L. orientalis* populations for the first time in the scientific literature.

Materials and Methods: In addition, the polymorphism levels, and the molecular evolution of the studied gene region of the *L. orientalis* populations were investigated. 154 samples were collected from 12 different populations belonging to four provinces, Muğla, Burdur, Antalya, and Aydın, and were studied and the *psaA/ycf3* gene encoded by chloroplast DNA was analysed by partial base sequence analysis.

Results: Haplotype diversity (h) and the average nucleotide diversity (π) values for all populations were found to be 0.52933 ± 0.011, and 0.00086 ± 0.0001, respectively. According to the results of our research, the gene flow level (N_m) among the populations was 1.40. F_{ST} values revealed statistically significant genetic differences between the Fethiye-Yanıklar population of Muğla province and other studied populations.

Conclusion: According to the results of the study, Fethiye-Yanıklar, Marmaris-National Park, and Fethiye-İnlice locations, where the highest genetic diversities detected among the studied populations, were found to be important in terms of conservation studies.

Keywords: *Liquidambar*, Sweetgum tree, cpDNA, Conservation genetics

Introduction

Global climate change can have significant impacts on populations in multiple ways. Climate change can lead to habitat loss, fragmentation, and alteration, which can reduce the size and connectivity of populations. This can lead to a reduction in genetic diversity and an increased risk of inbreeding and genetic drift. As temperatures and precipitation patterns change, plant, and animal species may shift their ranges to track suitable habitats. This can result in the loss of genetic diversity in populations that are left behind and the establishment of new populations with reduced genetic diversity. Climate change can also affect the ability of species to adapt to changing environmental

conditions. Genetic diversity is essential for adaptation, and reductions in genetic diversity can limit the ability of species to respond to changing environmental conditions. As species shift their ranges, they may encounter other species and hybridize. This can result in the loss of genetic diversity in the parent species and the creation of new genetic combinations (Pauls *et al.,* 2013).

Conservation genetics is a field of study that uses genetic data to inform conservation efforts for threatened and endangered species. It involves the application of molecular genetic techniques to understand the genetic diversity, population structure, and evolutionary history of species, as well as to develop strategies for their conservation and management (Frankham, 2019). Conservation genetics is important because genetic diversity is essential for the long-term survival of populations and ecosystems. Genetic diversity provides the raw material for adaptation and evolution, and loss of genetic diversity can reduce the ability of populations to adapt to changing environmental conditions and increase the risk of extinction (Doğaç, 2008). Genetic diversity decreases from generation to generation due to factors such as genetic drift, migration, and selection. The effect of genetic drift is dependent on population size, and as genetic drift becomes dominant in small populations, genetic diversity is lost over time and the survival of a population is compromised (Beaumont & Wang, 2019). Wright (1931) proposed a parameter called effective population size (*Ne*) to measure the effect of many factors, such as population size, number of breeding individuals, sex ratio, variation in reproductive success, and non-random mating, on genetic drift. Low *Ne* means high loss of genetic variation, high inbreeding, low fitness, and low adaptation potential. Therefore, the conservation of populations with low *Ne* should be considered. Although demographic data can be used in *Ne* calculations, it is nearly impossible to obtain parameters describing the pedigree and demographics of wild populations. However, genetic drift and inbreeding effects can be determined using genetic marker data and converted into *Ne* estimates (Beaumont & Wang, 2019).

Undoubtedly, the first step in developing appropriate population conservation strategies is to determine the amount and structure of genetic diversity in populations. In the last decades, our knowledge of the genetic diversity of forest trees has improved through the use of molecular markers as alternatives and supplements to classical methods (Tong *et al.,* 2020). It has been noted that DNA sequence data can provide a more precise prediction of separation from allozymes, and studies have reported that both nuclear and chloroplast DNA sequence data are informative in analysing the phylogenetic relationships of discrete taxa (Crawford *et al.,* 1992). The *psaA/ycf3* gene region is a useful marker for studying the genetic diversity of plants because it exhibits a high degree of variation between species, which can be used to distinguish between closely related taxa. This region has been used in molecular phylogenetic studies to infer the evolutionary relationships among plant species and to reconstruct the Tree of Life (Shi *et al.,* 2001; Ickert-Bond & Wen, 2006).

Conservation genetics involves a variety of techniques, including DNA sequencing, microsatellite analysis, and population genetics modelling. These techniques can be used to identify genetically distinct populations, estimate effective population size, and assess the genetic health of populations (Hedrick, 2001). Conservation genetics has many applications, including the development of genetic management plans for endangered species, the identification of priority areas for conservation, and the monitoring of populations over time. It can also be used to identify threats to genetic diversity, such as habitat loss, fragmentation, and climate change, and to develop strategies to mitigate these threats (Willi *et al.,* 2022). Overall, conservation genetics is a critical tool for preserving biodiversity and ensuring the long-term survival of endangered species and ecosystems.

Conservation genetics can be used to protect endangered plant species in several ways. Conservation genetics can help identify genetically distinct populations of endangered plant species. These populations may have unique adaptations or genetic diversity that should be conserved. By identifying these populations, conservationists can develop targeted conservation strategies that focus on protecting these unique populations (Hedrick & Hurt, 2012). Conservation genetics can be used to assess the genetic diversity of populations of endangered plant species. This information can be used to determine the genetic health of populations and to identify populations that are at risk of extinction due to low genetic diversity. By identifying populations with low genetic diversity, conservationists can develop strategies to increase genetic diversity, such as habitat restoration or crossbreeding with genetically diverse populations (Doğaç, 2008). Conservation genetics can inform the development of breeding programs for endangered plant species. For example, genetic analysis can identify populations or individuals that are genetically diverse or have traits that are important for survival in a changing environment. This information can be used to maximize genetic diversity and adaptive potential in captive breeding programs (Witzenberger & Hochkirch, 2011).

Conservation genetics can help understand the structure of populations of endangered plant species, including their relationships to other populations. This information can be used to determine the best locations for translocations, which involve moving individuals to new locations to establish or augment populations (Moritz, 1999). Conservation genetics can be used to monitor changes in genetic diversity over time in endangered plant species. This information can be used to assess the success of conservation actions and to modify management strategies as needed (Willi *et al.,* 2022). Overall, conservation genetics can provide valuable information for developing effective conservation strategies for endangered plant species. By identifying key populations, assessing genetic health, and guiding conservation actions, conservation genetics can help ensure the long-term survival of endangered plant species and the ecosystems they inhabit.

The genus *Liquidambar* belongs to the family Altingiaceae, although previously species of this genus were generally considered within the *Hamamelidaceae*. The *Liquidambar* genus is the only genus in the Altingiaceae family with discrete distributions seen in Turkey, East Asia, and North America (Li *et al.,* 1997a). The distribution of closely related plant species across different geographies has long attracted the attention of both plant systematists and biogeographers (Wen & Zimmer, 1996). The origin and development of the Asian and North American divides have been extensively discussed by various researchers (Wen, 1999; Milne, 2004; Beatty & Pro van, 2010; Schmickl *et al.,* 2010).

The genus *Liquidambar* is distributed at approximately the same latitudes on Earth. They spread across America, Southeast Europe, and Asia. The species has four different species; *L. acalycina* (Chang sweetgum tree) located in Central and Southern China, *L. formosana* (Chinese frankincense) located in South China, North Korea, South Korea, Taiwan, Laos, and North Vietnam, *L. styraciflua* (American sweetgum tree) located at Southeast and Central America and Mexico and *L. orientalis* (Anatolian sweetgum tree) located at southwestern Turkey (Ickert-Bond *et al.,* 2005). Genetic divergence studies conducted on *Liquidambar* species using isozymes (Hoey & Parks, 1991, 1994) and molecular techniques (Li *et al.,* 1997a, b; Li & Donoghue, 1999; Shi *et al.,* 2001; Ichert-Bond & Wen, 2006; Özdilek *et al.,* 2012) showed that *L. orientalis* and *L. styraciflua* are phylogenetically closer than the others.

The genus *Liquidambar* is one of many woody genera with morphologically similar species on different continents and is thought to have existed on Earth for about 65 million years. Therefore, species of the genus *Liquidambar* are called relict species (Hoey, 1990; Hoey & Parks, 1991; Akman *et al.,* 1992). The fragmentation and spread of species in the genus *Liquidambar* can be attributed to a combination of geological events, climate change, seed dispersal mechanisms, and human influence. These factors have caused the isolation and divergence of sweetgum species, ultimately leading to the current distribution observed today (Sun *et al.,* 2019; Hoey & Parks, 1991; Özdilek *et al.,* 2012; Joannin *et al.,* 2007; Wang *et al.,* 2020; Ďurkovič & Lux, 2010).

Although some researchers studying *Liquidambar* argue that *L. orientalis* cannot be endemic to Turkey as *L. orientalis* is found outside the country, in northern Syria, the 12 Islands, and Rhodes Island, some researchers argue that this is not true and that *L. orientalis* species have been transported to distribution areas outside the country through culture. Despite these different views, it is generally accepted that *L. orientalis* species is an endemic species to Turkey (Acatay, 1963; Atay, 1985; Efe, 1987; Günal, 1994; İstek & Hafızoğlu, 1998; Alan & Kaya, 2003; Velioğlu *et al.,* 2008). *Liquidambar orientalis* species has two different varieties in Turkey. These varieties are *L. orientalis* var. *orientalis* and *L. orientalis* var. *integriloba* Fiori (Doğaç, 2008). Relict endemic *L. orientalis* species spreads in Western Anatolia. Although it mainly spreads in Köyceğiz, Marmaris, Fethiye, Ula, and Dalaman districts in Muğla province, there are *Liquidambar* trees in certain regions in Aydın, Denizli, Antalya, Burdur and Isparta provinces (Acatay, 1963; Atay, 1985; Efe, 1987; Günal, 1994; İstek & Hafızoğlu, 1998; Alan & Kaya, 2003; Velioğlu *et al.,* 2008; Aydıngöz & Bulut, 2014).

Liquidambar orientalis is a long-lasting tree and can live for about 200-300 years. It has a shallow root structure. The tree has a thin and long body structure when it is young. A thicker trunk structure is observed in older trees (Günal, 2006; Doğaç, 2008). In addition to its ecological value, *Liquidambar* is an important species with its everyday use and economic value. There are many different areas where *Liquidambar* trees have been used from past to present.

Liquidambar is used in the construction of furniture, houses, and ornaments, and as a landscape material. As a wood structure, it is highly resistant to rotting that may be caused by water (Acatay, 1963; Atay, 1985; Bozkurt *et al.,* 1989). Today, sweetgum oil is still used in diseases such as asthma, bronchitis, lung disease, ulcer, and gastritis. In addition to these, it is widely used among people to relieve rheumatic pain due to its analgesic properties. Besides, it is

believed to have an antibacterial effect and allows wounds to heal quickly without leaving scars (Huş, 1949; Örtel, 1988; Acar, 1989; Bozkurt *et al.,* 1989; İstek & Hafızoğlu, 1998; Aydıngöz & Akbulut, 2014). Sweetgum oil is used in many natural fragrance perfumes and is known to effectively remove the smell of sweat. Sweetgum oil is used as a fixative in perfumery and prevents volatile fragrances from flying for a long time. For this reason, it is a highly preferred raw material in the perfume industry (Hus, 1949; Örtel, 1988; Acar, 1989; Bozkurt *et al.,* 1989; İstek & Hafızoğlu, 1998; Aydıngöz & Akbulut, 2014).

In its native range, *L. orientalis* is an important component of many forest ecosystems, providing habitat and food for a variety of wildlife species. However, like many tree species, it is threatened by habitat loss and overexploitation, and conservation efforts are needed to ensure its survival. The area of *Liquidambar* forests has been greatly reduced due to fires in recent years, the cutting down of trees for agricultural and tourism purposes, and the unconscious destruction of trees to produce sweetgum oil (Ürker *et al.,* 2014). While the range of distribution of *Liquidambar* forests was 7,000 hectares in 1947, these areas have shrunk over the years. These areas were recorded as 6,312 hectares in 1949, 4,316 hectares in 1955, 1,337 hectares in 1980, 1,215 hectares in 1988, and 3,200 hectares in 2002. Today, the distribution area of *Liquidambar* is thought to be in the range of 1,500-2,000 hectares (Ürker *et al*., 2014; Arslan & Şahin, 2016). In recent years, many decisions have been made to protect the sweetgum forest lands, and steps have been taken to increase the distribution areas of sweetgum forests (Ürker *et al.,* 2014).

The general purpose of this study is to determine the level and structure of genetic diversity in 12 populations of *L. orientalis* by using the polymorphic *psaA/ycf3* region of chloroplast DNA (cpDNA) for the first time in Turkey. Since the *L. orientalis* populations are a good example of populations that have been fragmented recently and very quickly, the findings to be obtained are increasing rapidly in nature as a result of intense human activities and are important in terms of their contribution to the literature in the fields of population and conservation genetics regarding the genetics of such populations. Given the suggestions presented following the findings, it is believed that the development of conservation programs suitable for the characteristics of the region for the species and their immediate implementation are important in terms of the last goal for implementation. We also investigate the molecular diversity pattern of *L. orientalis* populations by cultivar and geographic region to provide additional data to address cultivar and species-level taxonomy problems of *L. orientalis*.

Materials and methods

Plant material

To obtain a complete representation of the natural distribution of Eastern Sweetgum, 12 natural populations were identified from different regions in southwestern Turkey in collaboration with the Turkish Ministry of Environment and Forestry, Forest Tree Seeds, and Tree Growth Research Directorate (Fig. 1, Table 1). Transects

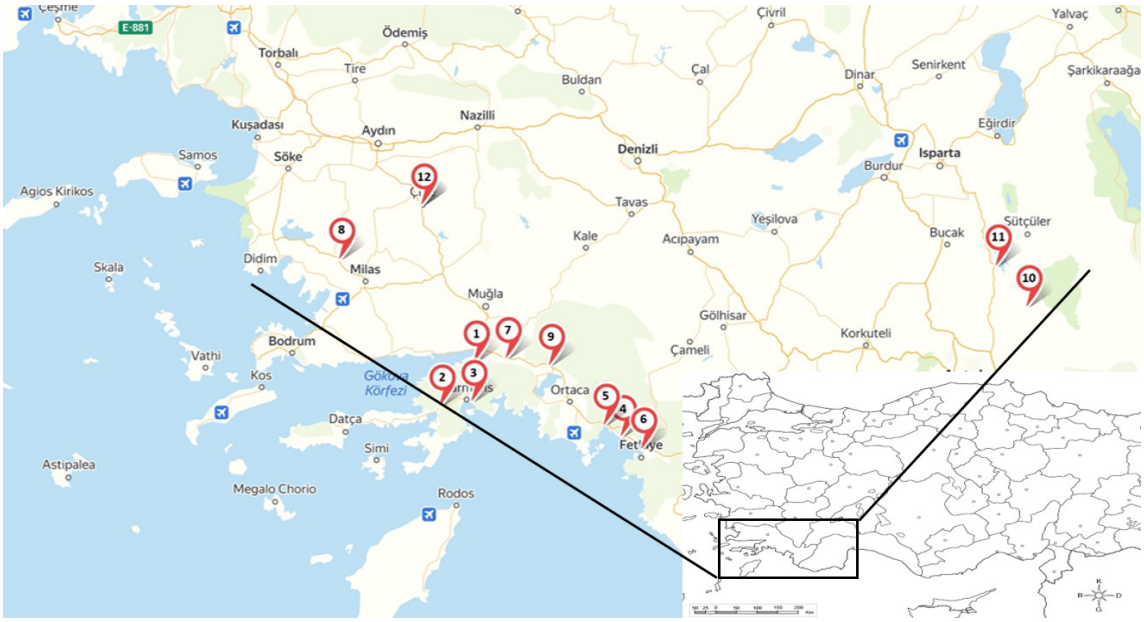

Figure 1. *Liquidambar orientalis* populations that were studied in the field.

(sampling units extending between two different points in the study area) were taken at different points so that the most homogeneous sampling within the determined population would be achieved. These transects were collected from young leaves, preferably on older trees, at intervals of 50-80 meters depending on population size. The samples were placed in ice boxes with ice molds, and the samples were brought to Muğla Sıtkı Koçman University, Faculty of Science, Plant Biotechnology laboratory and stored at -80 °C until DNA extraction.

Table 1. Information on the samples collected and the locations where the samples were collected.

LOCATION NUMBERS	LOCATION INFORMATION	Variety information	Number of Sequenced Individuals
1	Marmaris - Çetibeli	*L. orientalis* var. *integriloba* Fiori	10
2	Marmaris - Değirmenyanı	*L. orientalis* var. *integriloba* Fiori	21
3	Marmaris - Milli Park	*L. orientalis* var. *integriloba* Fiori	10
4	Fethiye - Günlüklü	*L. orientalis* var. *orientalis*	10
5	Fethiye - İnlice	*L. orientalis* var. *orientalis*	10
6	Fethiye - Yanıklar	*L. orientalis* var. *orientalis*	9
7	Ula - Kızılyaka	*L. orientalis* var. *integriloba* Fiori	22
8	Milas - Selimiye	*L. orientalis* var. *orientalis*	10
9	Köyceğiz - Toparlar	*L. orientalis* var. *integriloba* Fiori	22
10	Antalya- Gebiz	*L. orientalis* var. *integriloba* Fiori	10
11	Burdur- Bucak	*L. orientalis* var. *integriloba* Fiori	11
12	Aydın- Çine	*L. orientalis* var. *orientalis*	9
Total			**154**

DNA Extraction, PCR amplification, and DNA sequencing

Total genomic DNA was isolated from leaf tissues by using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). Within the scope of the study, a total of 154 sequences were obtained from 12 locations, and the *psaA/ycf3* gene encoded by chloroplast DNA was analysed by partial base sequence analysis. The amplification of the relevant region and sequencing was performed by amplifying with PG1 (5'-CATTCCTCGAACGAAGTTTTTACGGGATCC-3') and PG2R (5'-TCCCGGTAATTATATTGAAGCGCATAATTG -3') primers (Ickert-Bond & Wen, 2006). PCR reactions were performed by the PCR conditions (Shi *et al.,* 2001; Ickert-Bond & Wen, 2006) for the relevant region; the first denaturation step was at 94°C for 5 min, then at 94°C for 1 min, at 54°C for 1 min, at 72°C for 1 min and the last step was carried out at 72°C for 10 minutes, with a total of 35 cycles. Sequencing was conducted on an ABI 310 genetic analyser automatic sequencer (Applied Biosystems) by BM Labosis.

Data Analyses

The 720-bp portion of *psaA/ycf3* cpDNA sequences from 154 *L. orientalis* samples were acquired in the present study and previously published chloroplast *psaA/ycf3* gene sequences (GenBank accession numbers DQ352230-DQ352257) were aligned using CLUSTALW in MEGA 7 software (Kumar *et al.,* 2016). The sequences were analyzed by grouping them into two datasets. The first dataset contained only the sequences of *L. orientalis* from the current study. The second dataset included both studied *L. orientalis psaA/ycf3 s*equences and sequences from *Liquidambar* species available from the GenBank. The basic molecular diversity statistics (number of variable sites, average number of nucleotide differences between haplotypes, number of haplotypes, number of parsimony-informative sites, haplotype diversity) were performed with Dnasp (ver. 5.0) (Librado & Rozas, 2009). Descriptive statistics, namely divergence within species, genetic distance, transition, and transversion, were performed for *Liquidambar* species as well as among varieties and geographical regions for *L. orientalis* using MEGA 7 software (Kumar *et al.,* 2016). The Analysis of Molecular Variance (AMOVA) test was carried out with Arlequin version 3.5 (Excoffier *et al.,* 2007). Median-joining networks of haplotypes, with the inclusion of previously defined haplotypes, were constituted by using NETWORK (ver. 4.6) (Bandelt *et al.,* 1999; Polzin & Daneschmand, 2003).

Results

The *psaA/ycf3* sequences from natural *L. orientalis* populations from Turkey and other *Liquidambar* species

find out that the number of variable and conserved regions differentiated between the species, meanwhile the GC contents did not change significantly (32.1% to 32.8%). Among the analyzed species, *L. orientalis* has the most variable and least conserved sites. The parsimony-informative sites were found as 4 in *Altingia*, 2 in *L. orientalis,* and 0 in *L. macrophylla*, *L. styraciflua*, *L. acalycina*, *L. formosana,* and *Semiliquidambar*. The transition to transversion ratio was highest in *Semiliquidambar* and lowest in *L. orientalis* (Table 2a). The highest number of parsimony-informative sites was found in *Altingia* and *L. orientalis* (4 and 2 respectively). Within the varieties *L. orientalis* var. *orientalis* had the least conserved sites and highest variable sites, parsimony-informative sites, and transition rates, while *L. orientalis* var. *integriloba* had the lowest variable and parsimony-informative sites and highest transversions (Table 2b).

Six haplotypes were obtained in a variable sequence form in a total of 154 individuals studied. New haplotypes were deposited in GenBank under accession numbers OR365068- OR365073 (available after August 01, 2023). It was found that 4 of these haplotypes were unique to Turkey, and 1 of them was common with the haplotype in the previous study (Ickert-Bond & Wen, 2006). A 720 bp sequence analysis of the *psaA/ycf3* intergenic spacer region of 12 populations of *L. orientalis* (a total of 154 individuals) was performed. 6 haplotypes were found in a total of 154 samples belonging to the 12 populations studied (Fig. 2).

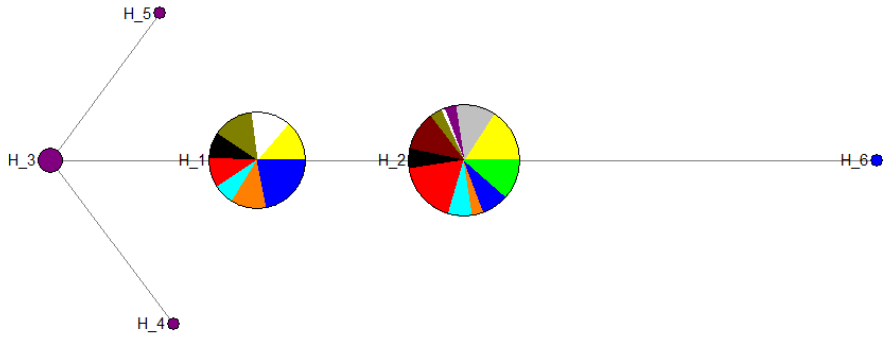

Figure 2. Network analysis result of the haplotypes of the *psaA/ycf3* inter-gene space region. (Toparlar: Yellow, Kızılyaka: Red, Çetibeli: Green, National Park: Black, Değirmenyanı: Blue, Daily: Orange, İnlice: Ice blue, Selimiye: Grey, Burns: Purple, Gebiz: Brown, District: Olive green and Çine: Shown in white).

Table 2. cpDNA *psaA/ycf3* gene molecular diversity values; a) *Liquidambar*, *Semiliquidambar*, and *Altingia* species (data obtained from this work and Genbank), b) *Liquidambar orientalis* (data contained only in this study)

(a) Characteristics of cp DNA *Psa-Ycf* gene region	***L. orientalis***	***L. macrophylla***	***L. styraciflua***	***L. acalycina***	***L. formosana***	***Semiliquidambar* sp.**	***Altingia* sp.**	**Overall**
Sequence length	720	720	720	720	720	720	720	720
Number of singleton sites	4	0	0	0	0	1	1	5
Number of parsimony-informative sites	2	0	0	0	0	0	4	12
Number of conserved sites	714	720	720	720	720	719	715	703
Number of variable sites	6	0	0	0	0	1	5	17
GC content range (%)	32.1	32.6	32.6	32.6	32.8	32.7	32.6	32.3
Transitions (%)	16.95	33.33	33.33	33.33	33.33	99.58	49.99	26.02
Transversions (%)	83.05	66.67	66.67	66.67	66.67	0.42	50.01	73.98
Transition/transversion bias (R)	0.193	0.45	0.45	0.45	0.45	187.95	0.96	0.33

(b) Characteristics of cp DNA *Psa-Ycf* gene region	***L. orientalis* var. *orientalis***	***L. orientalis* var. *integriloba***	**Overall**
Sequence length	720	720	720
Number of singleton sites	2	2	4
Number of parsimony-informative sites	2	1	2
Number of conserved sites	716	717	714
Number of variable sites	4	3	6
GC content range (%)	32.1	32.1	32.1
Transitions (%)	27.23	4.65	17.13
Transversions (%)	72.77	95.35	82.87
Transition/transversion bias (R)	0.35	0.05	0.20

Table 3. Distribution and frequencies of haplotypes of the *psaA/ycf*3 intergene space region of *L. orientalis* populations.

Haplotype/ Populations	Köyceğiz Toparlar	Ula Kızılyaka	Marmaris Çetibeli	Marmaris Millipark	Marmaris Değirmenyanı	Fethiye Günlüklü	Fethiye İnlice	Fethiye Yanıklar	Milas Selimiye	Antalya Gebiz	Burdur Bucak	Aydın Çine
H1	8 (0.364)	6 (0.273)		5 (0.5)	13 (0.619)	7 (0.7)	4 (0.4)				8 (0.727)	8 (0.889)
H2	14 (0.636)	16 (0.727)	10 (1)	5 (0.5)	7 (0.333)	3 (0.3)	6 (0.6)	3 (0.333)	10 (1)	10 (1)	3 (0.273)	1 (0.111)
H3								4 (0.444)				
H4								1 (0.111)				
H5								1 (0.111)				
H6					1 (0.0476)							

Haplotype 2, which contains 57.14% of the total 720 base sequences, was found to be common to all populations and is considered the inherited haplotype for our country. It was observed in all populations except 4 populations in the other high-frequency Haplotype 1 (38.31%). The other haplotypes had a low frequency. The frequency of Haplotype 3 was 2.59%, and it was observed to be specific to the Muğla Fethiye Yanıklar population. The frequencies of Haplotype 4 and Haplotype 5 are 0.65 and are specific to the Muğla Fethiye Yanıklar population. The frequency of haplotype 6 was also 0.65, and it was observed to be specific to the Muğla Marmaris Değirmenyanı population (Table 3).

Haplotypes are similar to each other, with base changes ranging from 1 to 6 nucleotides (no deletion or insertion observed). Genetic diversity data for all populations are presented in Table 4. Haplotype diversity (*h*) ranges from 0.0000 (Selimiye, Çetibeli, and Çine) to 0.75000 (Yanıklar). The haplotype diversity (*h*) value for all populations is 0.52933 ± 0.011. The nucleotide diversity (π) between haplotypes ranges from 0.0000 (Selimiye, Çetibeli, and Cine) to 0.0021 (Yanıklar) and the average nucleotide diversity (π) for all populations is 0.00086 0.0001. Although more samples were taken from Toparlar, Değirmenyanı, and Kızılyaka populations, the haplotype numbers in the populations ranged from 2 (Toparlar and Kızılyaka) to 3 (Değirmenyanı). All populations except Selimiye, Çetibeli, and Cine populations showed relatively high levels of genetic variation. There are 6 polymorphic regions in 720 base pairs (bp) (0.83% of the total length); 4 of these 6 regions are singleton, that is, the mutation occurs in a single haplotype base sequence, and 2 of them are determined as parsimony-informative regions. The G+C content was 0.321 and Tajima's D value was -0.8877.

Genetic relationships between populations were determined by comparing F_{ST} (genetic differentiation coefficient between species) values. F_{ST} values showed that the Yanıklar population differed significantly from other populations genetically. While among the *L. orientalis* populations, the Cine population, and the populations with the highest genetic differentiation were Gebiz, Çetibeli, and Selimiye populations (0.88166), the populations with the lowest genetic differentiation, although not statistically significant, were observed between the Bucak and Günlüklü populations with a value of -0.10351 (Table 5). The gene flow level *L. populations* (*Nm*) was observed as 1.40. The main group was formed based on the presence of two varieties of the *L. orientalis* species. Accordingly, Group 1 (*integriloba* variety) was formed as Muğla Toparlar, Muğla Değirmenyanı, Burdur Bucak, Antalya Gebiz, Muğla National Park, Muğla Kızılyaka and Muğla Çetibeli, Group 2 (*orientalis* variety) was formed as Muğla Selimiye, Muğla Yanıklar, Muğla İnlice, Muğla Günlüklü and Aydın Çine. As a result of the AMOVA analysis of the species, it was concluded that the main contribution to the genetic variation was caused by the variation among individuals with 71.52%. The genetic variation between the groups was observed to be low with -0.46% (Table 6).

Table 4. Haplotype diversity in *L. orientalis* populations. N: Sample number, Hp: Haplotype number, *h*: Haplotype diversity, π: nucleotide diversity.

Populations	N	Hp	*h*	π
Muğla Toparlar	22	2	0.48485	0.00067
Muğla Selimiye	10	1	0.00000	0.00000
Muğla Yanıklar	9	4	0.75000	0.00201
Muğla Değirmenyanı	21	3	0.52857	0.00095
Muğla Mill Park	10	2	0.55556	0.00077
Muğla Kızılyaka	22	2	0.41558	0.00058
Muğla İnlice	10	2	0.53333	0.00074
Muğla Günlüklü	10	2	0.46667	0.00065
Muğla Çetibeli	10	1	0.00000	0.00000
Antalya Gebiz	9	2	0.22222	0.00031
Burdur Bucak	11	2	0.43636	0.00061
Aydın Çine	10	1	0.00000	0.00000
Average	**12.83**	**2**	**0.52933**	**0.00086**

The relationships between the previously published base sequences and the haplotypes obtained from the study (GenBank access numbers DQ352230-DQ352257) were determined using the Network program (Fig. 3). Network analysis based on haplotype provides a better understanding

Table 5. The F_{ST} values of the *psaA/ycf3* intergenic gap region of *L. orientalis* populations and the significance of differentiation according to these values (*P < 0.05)

Populations	Toparlar	Değirmenyanı	Bucak	Gebiz	Millipark	Kızılyaka	Çetibeli	Selimiye	Yanıklar	Çine	İnlice	Günlüklü
Toparlar	****											
Değirmenyanı	0.06818	****										
Bucak	0.17603	-0.04417	****									
Gebiz	0.24063*	0.42731*	0.68824*	****								
Millipark	-0.03733	-0.04692	0.00927	0.44444*	****							
Kızılyaka	-0.02785	0.15317*	0.29631*	0.15466	0.03861	****						
Çetibeli	0.24063	0.42731*	0.68824*	0.00000	0.44444*	0.15466	****					
Selimiye	0.24063	0.42731*	0.68824*	0.00000	0.44444*	0.15466	0.00000	****				
Yanıklar	0.40123*	0.30939*	0.30246*	0.55326*	0.28810*	0.44884*	0.55326*	0.55326*	****			
Çine	0.37135*	0.05699	-0.02507	0.88166*	0.21493	0.49233*	0.88166*	0.88166*	0.33824*	****		
İnlice	-0.07536	0.00838	0.11239	0.33333	-0.08889	-0.03821	0.33333	0.33333*	0.31318*	0.33870*	****	
Günlüklü	0.14036	-0.05916	-0.10351	0.66667*	-0.02222	0.25977	0.66667*	0.66667*	0.28665*	-0.00411	0.07407	****

Table 6. Molecular analysis of variance (AMOVA) of the *psaA/ycf3* intergene gap region.

Structure	Source of Variation	% of total variance	Fixation index
Two Variety	Between groups	-0.46	*FCT*= -0.00458
	Between populations / within groups	28.94	*FSC* = 0.28808
	Within individuals	71.52	*FST*=0.28482

of the phylogenetic relationships between *Liquidambar*, *Semiliquidambar*, and *Altingia* species in Turkey and the world, and the status of the genetic structure. As a result of the network analysis, *Semiliquidambar* and *Altingia* samples exhibited a strong phylogeographic structure by showing a very good structure and differed quite well from *L. orientalis* haplotypes. However, while *L. acalycina* haplotypes were clustered with *Semiliquidambar* and *Altingia* haplotypes, and *L. formosana* haplotypes were clustered with *Altingia* haplotypes. While *L. orientalis* samples were clustered closely with *L. macrophylla* and *L. styraciflua* samples, both the gene bank and the haplotypes obtained from the study showed a strong structure.

Discussion

While there is not much information available specifically on the genetic diversity of this species, studies of related species in the family Altingiaceae have shown that there can be significant genetic variation within and between populations. This genetic diversity is important for maintaining the long-term viability of populations and ecosystems, and efforts to conserve *L. orientalis* through habitat protection and restoration are essential for ensuring its survival. As a result of literature reviews, it has been determined that polymorphism determination studies for *L. orientalis* were carried out with various methods. However, a limited number of samples (Ickert-Bond & Wen, 2006) has been detected among these methods investigating the polymorphic *psaA/ycf3* region of the chloroplast genome. Since the chloroplast genome has been studied for the first time in *L. orientalis* populations in terms of this region, it is essential to compare the results obtained from these different methods with the results of our study in terms of revealing the genetic variation and polymorphism within and between populations. According to the accepted mutation rate and base substitution in plant chloroplasts, the evolutionary divergence time of the species of the *Liquidambar* genus was predicted to be 8.6 MYA. This divergence time corresponds to the Late Miocene of the Tertiary period. The divergence time of *L. styraciflua* and *L. orientalis* was estimated about a million years later. The results support the probability of a more extended linkage

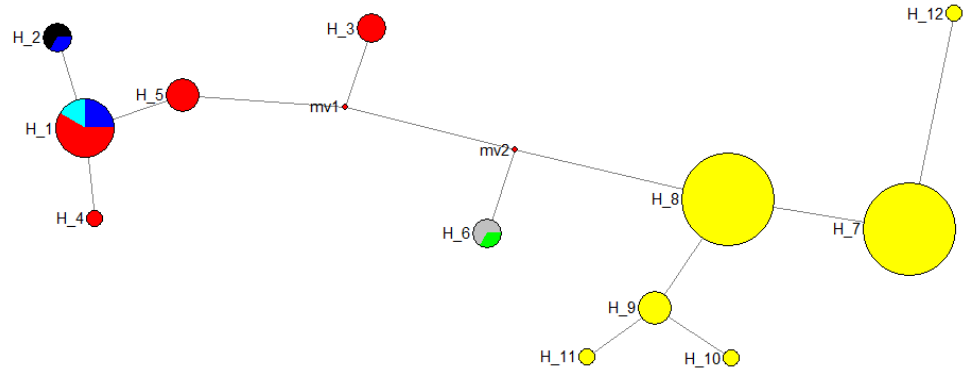

Figure 3. Network graph obtained from analysis of the cpDNA *psaA/ycf3* intergene gap region. (*Semiliquidambar* sp.: Blue, *Altingia* sp.: Red, *L. orientalis*: Yellow, *L. acalycina*: Ice blue, *L. formosana*: Black, *L. macrophylla*: Grey, *L. styraciflua*: Green).

between *L. styraciflua* and *L. orientalis* by way of the North Atlantic Land Bridge (Hoey & Parks, 1991; Özdilek *et al.,* 2012). For floristic swaps between eastern Asia and eastern North America through the North Atlantic Land Bridge, two ways were recommended. One of them is between Europe and east Asia, and the other one is between eastern North America and Europe (Tiffney, 1985, 2000; Wen, 1999). In the early Tertiary, a land connection between Europe and eastern North America took place with Greenland, and this connection seems like a more acceptable North Atlantic Land Bridge connection. This situation may have enabled the continuousness of *Liquidambar* species (Wen, 1999; Jiao & Li, 2009). The pollen and fossil studies showed that the *L. orientalis* was widespread in Europe (Joannin *et al.,* 2007; Sadori *et al.,* 2010; Worobic & Gedl, 2010; Hristova & Ivanov, 2009; Sakala & Prive-Gill, 2004) and Turkey (Kasapligil, 1976) before the Pleistocene. The Miocene cooling may have hindered the continuousness of North American and Turkish species of the *Liquidambar* genus.

The results obtained from the studies support the sister group relationship between the western Asian *L. orientalis* and the eastern North American *L. styraciflua*, in parallel with the results previously obtained with allozyme (Hoey & Parks, 1991, 1994) and DNA sequence data (Li *et al.,* 1997a, Li *et al.,* 1997b; Li & Donoghue, 1999, Shi *et al.,* 1998; Shi *et al.,* 2001; Ickert-Bond & Wen, 2006; Özdilek *et al.,* 2012). *Liquidambar macrophylla* Oersted from Central America is generally considered congener with *L. styraciflua*. Results from sequence analyzes of cpDNA show that North American sweetgum (*L. styraciflua*) and East Asian sweetgum (*L. formosana* and *L. acalycina*) are the most distant intercontinental species. In the obtained network graph, it was observed that *L. orientalis* samples were closely related to *L. styraciflua* and *L. macrophylla* samples, and *L. acalycina* and *L. formosana* were closely related to *Semiliquidambar* and *Altingia* species (Fig. 3). Hoey & Parks (1991) estimated divergence times between *Liquidambar* species based on isozyme data using the methods of Nei (1987) and Thorpe (1982) and *L. styraciflua* differentiated from *L. orientalis* approximately 7 mya (Nei's) or 13 mya (Thorpe's) and 10 mya (Nei's) or 17 mya (Thorpe's) ago from east Asian *L. formosana*. Our results are consistent with the studies carried out with sweetgum species (Hoey & Parks, 1991, Li *et al.,* 1997a; Li & Donoghue, 1999; Shi *et al.,* 2001; Ickert-Bond & Wen, 2006; Özdilek *et al.,* 2012), and this result should be a consequence of longer connection between *L. orientalis* and *L. styraciflua* through the North Atlantic Land Bridge.

Plant genetic sources are one of the basic components of biodiversity. Plant conservation genetics ensures instruments to lead restoration and conservation endeavours, monitor and measure achievement, and eventually reduce extinction hazards by preserving species as living creatures capable of evolving in spite of changing conditions (Kramer & Havens, 2009). Throughout the years, conservation genetics has voluminously developed our understanding of processes that are associated with small population size and habitat fragmentation. Inbreeding, which is admitted a significant phenomenon in conservation genetics, is thought to be the intermediary between the loss of variants owing to genetic drift and the impacts of this loss on the possibility of extinction (Ouborg *et al.,* 2010). Even though conservation genetics has concentrated on sequence variation, much less attention has been paid to variation in gene expression. It is largely unclear how gene expression is altered by changes in regulatory mechanisms as a function of genetic drift and inbreeding. In addition to this, it has been suggested that gene expression may be closer to phenotypic variation compared to the gene sequences. Environmental stress, such as increased temperature, drought, and lack of nutrients, can cause changes in genomic pathways, both in animals and plants (Ouborg *et al.,* 2010).

In previous studies conducted to determine polymorphism in *L. orientalis* populations, different markers were used (Öztürk, 2008, Doğaç, 2008, Velioğlu *et al.,* 2008, Yüzer, 2019). Eighteen polymorphic loci were found in 320 individuals belonging to 14 populations where polymorphism with 7 different isozymes was investigated (Öztürk, 2008). In another study, a total of 453 loci were determined as a result of the screening performed using 30 RAPD primers on 320 individuals belonging to 14 populations, and an average of 15 polymorphic loci were determined for each primer (Doğaç, 2008). As a result of another study using ISSR primers, an average of 27 polymorphic loci were found (Yüzer, 2019). The high level of genetic diversity determined according to the results of our research indicates that chloroplast DNA is more suitable in polymorphism determination studies for this species.

While the *Nm* value was given as 0.265 for the species that can spread their seeds and pollen over short distances and for the self-pollinated species, the *Nm* value was determined as 4,750 for the species that can spread their seeds and pollen over long distances (Hamrick *et al*., 1990). In addition, while the gene flow level (*Nm*) of 0.50 is considered a critical value, it has been reported that

higher values indicate the opposite results of genetic drift. According to the results of our research, the gene flow level (*Nm*) was determined as 1.40. The fact that this value is above the value of 0.265 indicates that the individuals of the population have pollinated with trees at not very close distances. If the gene flow level (*Nm*) value is less than 4,750, it is an indication that fertilization originating from long distances is not at high levels or does not occur at all.

In Turkey, there are two varieties of *L. orientalis*, namely *L. orientalis* var. *orientalis* and *L. orientalis* var. *integriloba.* Divergence at the intra-species level and relatively low genetic diversity propose that *L. orientalis* populations share ancestral polymorphism from which two varieties may have evolved. Additionally, anthropogenic factors, more recent climatic alterations, and breeding systems may also have conducted to divergence and low genetic diversity. In this study, the three populations with the highest level of genetic diversity were identified as Yanıklar (0.7500), National Park (0.5556), and İnlice (0.53333), respectively. Species must be protected in order to preserve the genetic diversity within the species and to transfer these variations to the next generations. This could be locations evaluated as the important genetic diversity centre and refugiums for the species. Therefore, it is recommended that priority should be given to these three populations in a conservation program to be initiated for the *L. orientalis* species.

In the studies conducted by Doğaç (2008) and Öztürk (2008) using RAPD and isoenzymes, the populations with the highest genetic diversity were reported as Marmaris Çetibeli, Köyceğiz Toparlar and Ula Kızılyaka and Toparlar, Kızılyaka, and İnlice, respectively. However, in the study conducted by Yüzer (2019) using the ISSR marker, it was reported that the three populations with the highest genetic diversity were Fethiye Günlüklü, Fethiye İnlice, and Ula Kızılyaka populations. The studies performed by Özdilek (2007) and Or (2007) using matK and trn regions are the most similar studies in terms of a method to our research using chloroplast DNA regions. Özdilek (2007) reported that the populations with the highest polymorphism are Pamucak, Günlükbaşı, and Toparlar. Yatağan, Serik, and Çetibeli populations were given by Or (2007) as the populations with the highest polymorphism. Considering that some locations and marker systems used in the studies are different and the results obtained from the studies are different, it shows that studying many gene regions by taking samples from all locations in the conservation programs to be planned will yield more successful results.

As a result of the AMOVA analysis of the species in this study, it was concluded that the main contribution to genetic variation was the variation between individuals by 71.52%. This result was similar to the studies conducted by (Özdilek, 2007) and (Or, 2007), using *matK* and *trn* regions; (Özdilek, 2007) reported the variation among individuals as 86.10%, and (Or, 2007) as 91.97%. In addition, no variation was observed between the varieties in AMOVA results, which is the same as the result we obtained in our study.

Currently, *psaA /ycf3* gene sequences from databases for *Liquidambar* species are limited. The more *psaA /ycf3* gene sequences for *Liquidambar* species that become available in databases, will help to better understand the phylogenetic relationship among *Liquidambar* species. As a result, in this study, the *psaA /ycf3* region was used for the first time to determine the genetic diversity of *L. orientalis* species. Since *L. orientalis* species is a relict endemic species in Turkey, it is very important for the species to continue its existence in the future. Therefore, it is recommended to start research on the conservation of *L. orientalis* species as earliest as possible and strengthen the measures taken. Considering the previous studies on the *L. orientalis* species, it is clear that the forest areas of the species continue to decrease, and it is not known exactly how far it will spread. So, first of all, the exact distribution area of *L. orientalis* species, which is one of the important riches of our country, should be determined by authorized persons and institutions, and then these forests should be quickly taken into *in-situ* conservation. After the study, it was recommended that Fethiye-Yanıklar, Marmaris-National Park, and Fethiye-İnlice locations, where the highest genetic diversity was detected in the studied populations, should be taken under protection as a priority. Though some conservation programs are being implemented in the Fethiye Günlüklü and Marmaris National Park locations today, it is recommended to strengthen the measures taken, and it is believed that the population in the Fethiye-Yanıklar area should be taken under protection as soon as possible. It is very important to develop *ex-situ* conservation strategies with or simultaneously with the provision of *in-situ* conservation. Otherwise, we will continue to follow the story of the tragic disappearance of sweetgum forests and sweetgum oil that have lost their value in the last 50-60 years.

Conservation genetics can play an important role in mitigating the impacts of climate change on biodiversity. By identifying genetically distinct populations, assessing genetic diversity and health, and developing breeding

programs or other management strategies, conservation genetics can help ensure the long-term survival of species in a changing environment. However, conservation genetics alone is not sufficient to address the complex and multifaceted challenges of climate change, and broader conservation efforts, including habitat protection and restoration, are also necessary.

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