THE PHYLOGENETIC ANALYSIS OF *Liquidambar orientalis* Mill. VARIETIES BY COMPARING THE NON-CODING "*trn*" REGIONS OF THE CHLOROPLAST GENOME

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MELİS OR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

AUGUST 2007

Approval of the Thesis

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ABSTRACT

THE PHYLOGENETIC ANALYSIS OF *Liquidambar orientalis* Mill. VARIETIES BY COMPARING THE NON-CODING "*trn*" REGIONS OF THE CHLOROPLAST GENOME

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August 2007, 89 Pages

Liquidambar L. genus are represented with four species in the world and one of these species, Turkish sweet gum *(Liquidambar orientalis* Miller*)* is naturally found only in southwestern Turkey with limited distribution in Muğla Province. The presence of increasing threats to its genetic resources signifies the importance of studying the phylogenetic relationships and genetic diversity in this relict endemic species.

In this study, 18 different populations were sampled throughout the species range and noncoding transfer ribonucleic acid (*trn*) region of chloroplast DNA was studied to asses the phylogenetic relationships and genetic diversity. Experimental studies included the extraction of DNA, amplification and sequencing of the *trn* region of the chloroplast DNA. Molecular evolutionary

analysis was done by using MEGA version 3.1 and Arlequin 2.000 softwares. Sequences from six other species of *Liquidambar* (*L. styraciflua* from USA, *L. macrophylla* from Mexico, *L. formosana* from *V*ietnam, *L. acalycina* from China, *L. formosana* from China and *L. acalycina* from USA) in the database were also included in the analysis.

Moleculer diversity results show that population located in Muğla-Yatağan district has the highest number of polymorphic sites among the other populations of Turkish sweet gum. Population located in Marmaris-Günnücek has an average genetic distance value of 0.0032 within population, being the highest within the studied populations of Turkish sweet gum. The average genetic distance within variety *orientalis* (0.0011) was the greatest among all the varieties, but the most separated or divergent populations were members of variety *integriloba*. For both varieties and geographic groups, average diversity within was found to be the greatest portion (greater than 80%) of the total sequence diversity. The geographic groups located in Denizli and Muğla-Yatağan showed the highest average genetic distances within location, with a value of 0.0014. The genetic distance between the closest neighbor of Turkish sweet gum, American *L. styraciflua* was 0.0002, whereas the genetic distance between the most distant neighbors (Vietnamese *L. formosana,* Chinese *L. acalycina* and *L. formosana*) was 0.0051.

Based on the molecular diversity analysis, seven populations were found to be important for conservation issues and two of them located in Marmaris have the highest priority. The most variant geographic groups are located in Denizli and Muğla-Yatağan districts. These populations could be considered as good candidates for future *in-situ* or *ex-situ* conservation programs

Key Words: *Liquidambar orientalis*, *trn*, cpDNA, genetic variance, phylogeny

ÖZ

KLOROPLAST GENOMUNDAKİ KODLANMAYAN "*trn*" BÖLGELERİNİN KARŞILAŞTIRILMASI YAPILARAK *Liquidambar orientalis* Mill. VARYETELERİNİN FİLOGENETİK ANALİZİ

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Ağustos 2007, 89 sayfa

Liquidambar L. cinsi dünya çapında 4 türe sahip bir cinstir. Bu türlerden biri olan Sığla ya da Günlük Ağacı, doğal olarak sadece Güneybatı Türkiye' de Muğla civarında sınırlı bir alana yayılmıştır. Relik endemik olan bu türün genetik kaynaklarına yönelik tehditler, bu türde filogenetik ve genetik çeşitlilik çalışmalarının önemini arttırmıştır.

Bu çalışmada, türün doğal yayılış alanlarında mevcut olan 18 adet populasyondan örnek alınmış ve kloroplast genomundaki kodlanmayan transfer ribonükleik asit (*trn)* bölgesine dayalı dizi analiziyle genetik çeşitliliği ve filogenetik ilişkileri saptanmıştır. Bu amaçla, DNA izolasyonu, kloroplast genomundaki *trn* bölgesinin çoğaltılması ve dizi analizi gerçekleştirilmiştir. Filogenetik ilişkilendirmeler ve genetik çeşitlilik verileri dizi analizi sonuçları kullanılarak, MEGA 3.1 ve Arlequin 2.000 yazılımları ile yapılmıştır. Veri

tabanından alınan *Liquidambar* cinsine ait 6 türün (ABD'den *L. Styraciflua* ve *L. acalycina*, Meksika'dan *L. macrophylla*, Vietnam'dan *L. formosana*, Çin'den *L. Acalycina* ve *L. formosana*) dizileri de analize dahil edilmiştir.

Moleküler çeşitlilik sonuçları, Muğla-Yatağan bölgesinde bulunan populasyonun çalışılan diğer populasyonlara göre daha fazla polimorfik bölgeye sahip olduğunu göstermiştir. Marmaris-Günnücek'te bulunan populasyonun, 0.0032 ortalama genetik mesafe değeriyle, populasyon içi en yüksek farklılaşmaya sahip olduğu görülmüştür. Varyete *orientalis*'in 0.0011 ortalama genetik mesafe değeriyle en yüksek farklılaşmaya sahip olan varyete olduğu belirlenmiş, fakat en farklılaşmış ya da çeşitlilik gösteren populasyonların varyete *integriloba*'ya dahil olduğu gözlenmiştir. Hem coğrafi konumlara hemde varyetelere göre toplam çeşitliliğin büyük bir kısmının (>%80) gruplar arası değil, grup içi farklılıktan kaynaklandığı saptanmıştır. Denizli ve Muğla-Yatağan'da bulunan populasyonların 0.0014 genetik mesafe değeriyle en yüksek genetik çeşitliliğe sahip olduğu görülmüştür. Sığla ağacının en yakın komşusu Amerika Birleşik Devletleri'den *L. styraciflua* ile arasındaki genetik mesafe 0.0002 iken, en uzak komşuları Vietnam'dan *L. formosana,* Çin'den *L. acalycina* ve *L. formosana* ile aralaraındaki genetik mesafe 0.0051 olarak saptanmıştır.

Çalışmanın sonuçlarına dayanılarak, Marmaris bölgesinde bulunan yedi populasyonun gen kaynaklarının koruma kapsamına alınması ve sürekliliklerinin sağlanması önerilmiştir. Populasyonlar coğrafi konumlarına göre incelendiklerinde, en çok çeşitliliği gösteren bölgelerin Denizli ve Muğla-Yatağan-Yılanlı olduğu ortaya çıkmıştır. Bu populasyonların *in-situ* (yerinde) koruma programları ya da *ex-situ* (yapay) olarak gen kaynaklarının korunması için, olası projelerde göz önünde bulundurulması önerilmiştir.

Anahtar Kelimeler: *Liquidambar orientalis*, *trn*, cpDNA, genetik çeşitlilik, filogenetik

To Mom, Dad, my lovely sister and my love Oytun

ACKNOWLEDGMENTS

I would like to express my gratitude and deep appreciation to my supervisor Prof. Dr. Zeki Kaya for his guidance, positive suggestions and also for the great research environment he had provided. Next, I would like to express my thanks to all the jury members for their comments and criticism.

I would like thank TUBITAK TOVAG for supporting this study, Ercan Velioğlu, Süleyman Işık Derilgen, Murat Nur, Belkıs Korkmaz from Forest Tree Seeds and Tree breeding Research Directorate. I would like to thank Osman Efe and Rumi Sabuncu. I wish to express my deep appreciation to Dr. Yasemin İçgen, Dr. Burcu Çengel and Dr. Gaye Kandemir for their support.

I want to thank Özge Tozkar, H. Elif Güren, Ayten Dizkırıcı, Zeynep Özkurt, Evrim Yıldırım, Kubilay Yıldırım, Aysun Güvendiren and Aslı Özdilek for their great friendship and assistance.

I would like to thank my friends in my work place; Oğuz Balcı, Müge Mısırlıoğlu Tırman and my boss Dr. Serdar Tuncer for such a friendly research environment they had provided.

I would like to express my appreciation to my best friends Buket Başmanav and Nilüfer Afşar for their endless love and support who made me know that they are always there when I needed.

Finally, I would like to thank my family; My mom, dad, my crazy sister, my love Oytun Akman and Akman Family (which I am a part of, now) for their love, support and patience over the years. This thesis is dedicated to them.

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LIST OF ABBREVIATIONS

AMOVA	Analysis of Molecular variance
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
ETOH	Ethanol
ITS	Internal Transcribed Spacer Region
MEGA	Molecular Evolutionary Genetic Analysis
MST	Minimum Spanning Tree
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
OTU	Operational Taxonomical Units
PCR	Polymerase Chain Reaction
SDS	Sodiumdodecylsulphate
IUCN	The World Conservation Union
IGS	Intergenic Spacer
t-RNA	Transfer Ribonucleic Acid
CTAB	Cetyl Trimethyl Ammonium Bromide

CHAPTER 1

INTRODUCTION

1.1. Literature review over genus *Liquidambar* L.

The family Altingiaceae Horan. (1843), consists of approximately 15 tree species which includes the most commonly known genus, *Liquidambar* L., containing four to five intercontinentally disjunct species in the temperate zone of the northern hemisphere (Ickert *et al*., 2005). These species are distributed in eastern Asia (*Liquidambar acalycina* and *Liquidambar formosana*), eastern north America to central America (*Liquidambar styraciflua*) and western Asia including Turkey and Rhodes Island (*Liquidambar orientalis*) (Meyer, 1993) (Figure 1.1).

Another species of *Liquidambar* is defined by Sosa (1978) with the southern populations in Mexico and central America and is formerly designated as a separate species, *Liquidambar macrophylla* Oersted. However, most of the researchers just recognize one species from USA that is *L. styraciflua* (Meyer, 1993) due to some controversies concerning the elimination of *Liquidambar macrophylla* (Ickert-Bond and Wen, 2006) and including it with *Liquidambar styraciflua.*

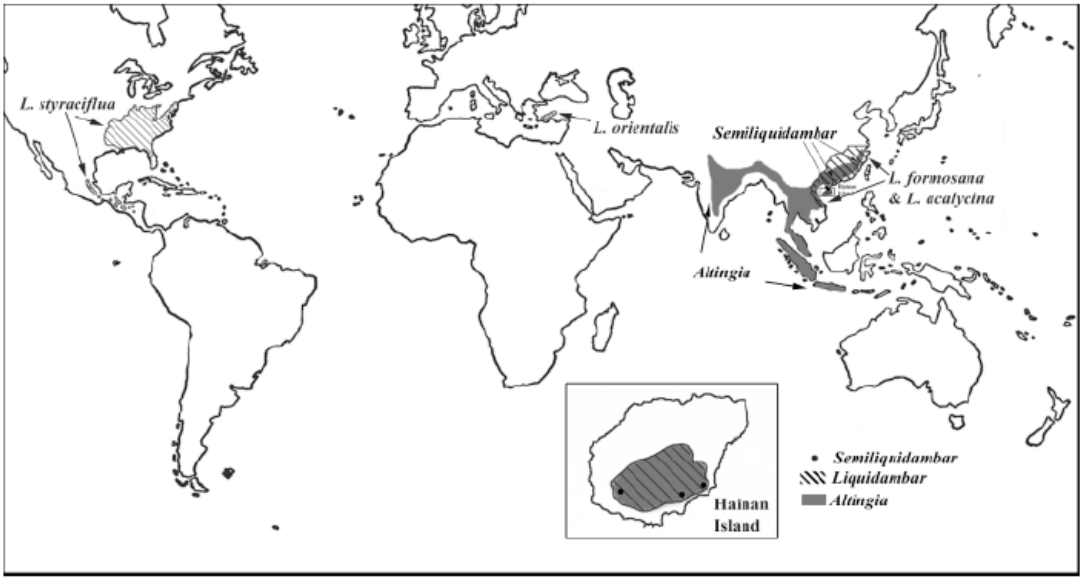

Figure 1. 1 Distribution map for genera Altingiaceae (Ickert-Bond et al., 2005)

Generally, *Liquidambar*, *Altingia Noronha*, and *Semiliquidambar* have been accepted as a subfamily of the Hamamelidaceae, as either the Altingioideae (Endress, 1989; Li and Donoghue, 1999) or (incorrectly) Liquidambaroideae (Bogle, 1986). The subfamily has been elevated to family status as the Altingiaceae by some authors (Rao, 1974), and currently this taxonomic level is widely accepted (Judd *et al*., 2002; APG II, 2003).

All members of these arborescent taxa are dicotyledonous, flowering trees which have unisexual, capitate, globose, woody infructescences composed of 25–50 helically arranged, bilocular capsules that bear several viable seeds per fruit and many abortive seeds (Ickert-Bond *et al*., 2005). *Liquidambar* has a trunk which is normally straight and does not divide into double or multiple leaders and has side branches which are small in diameter on young trees, creating a pyramidal form (Gilman and Watson, 1993). The glossy, star-shaped leaves turn bright red, purple, yellow or orange in the fall and in early winter (Figure 1.2).

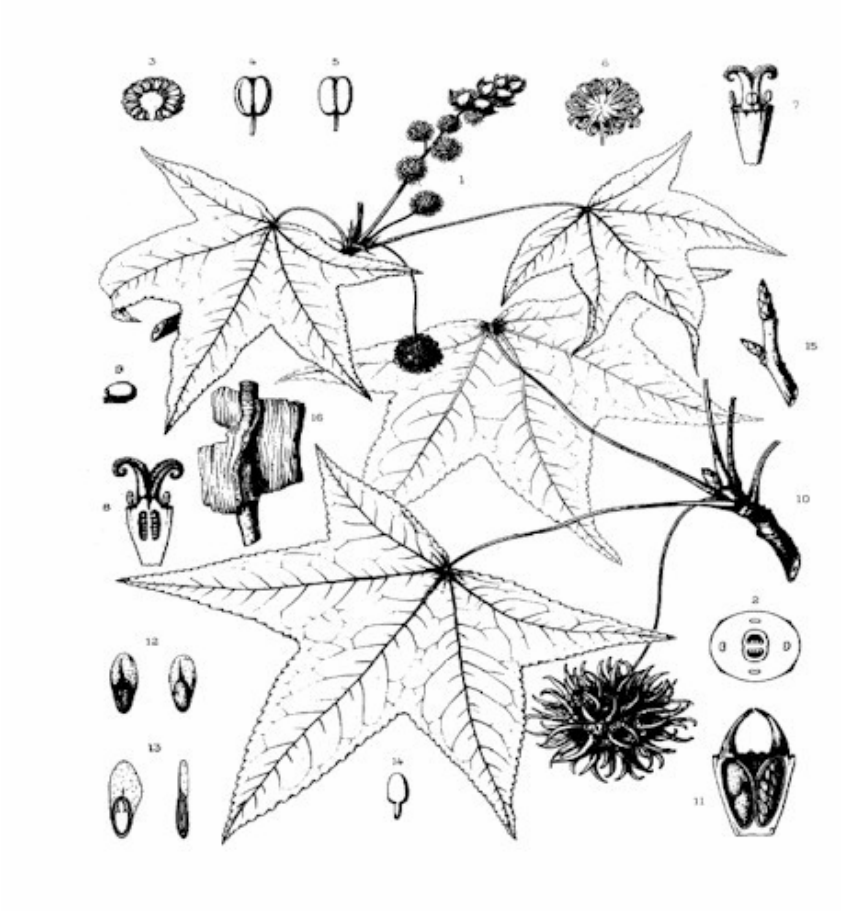

Figure 1. 2 Five lobed leaves, flowers and seeds of *Liquidambar* (Sprague, 2007)

Liquidambar genus was much more widespread in the Tertiary, but has disappeared from Europe due to extensive glaciation in the north and the Alps, which has served as a blockade against southward migration (Svenning, 2003). It was also present in western North America and the unglaciated Russian Far East, but disappeared later due to climate change (Svenning, 2003). The presence of *Liquidambar* fossils in the Pliocene of Italy (Martinetto, 1998) is consistent with the widespread occurrence of *Liquidambar* in Europe in the Tertiary and indicates that the present-day intercontinental disjunctions are due to glaciation events including the impact of the Pleistocene cooling in Europe (Wen, 1999).

1.2. Literature review over *Liquidambar orientalis*

Turkey is a species-rich country with a wide range of endemic species. There are natural habitats of 10500 taxa in Turkey, 3500 of which are endemic (Kaya and Raynal, 2001). *Liquidambar orientalis* is one of these endemic species with a high economical and ecological value (Efe, 1987).

L. orientalis has unisexual flowers that bloom from March to April (Alan and Kaya, 2003). The flowers are small, produced in a dense globular inflorescence 1-2 cm in diameter, pendulous on a 3-7 cm stalk (Figure 1.3). The fruit is a woody multiple capsule 2-4 cm in diameter (popularly called a 'gumball'), containing numerous seeds. The height of the tree varies between 35-40 m with a straight trunk of 100 cm in diameter (Alan and Kaya, 2003). The seeds are produced each year, but abundant seed crops occur in every three years. The optimum altitude for the species to live is between 0-400 m with a rainfall of 1000-1200 mm and a temperature of 18°C (Alan and Kaya, 2003).

The distribution is restricted to a very small area mainly in Southwest Turkey and Rhodes Island (Figure 1.4) within 0-1000 m elevational range. The forests of *L. orientalis* are located within a Special Environment Protection Area between Dalyan and Köyceğiz in Muğla Province, In this area, a 286 hectares zone is a nature reserve and arboretum for the conservation of the species. A large population is also found in Marmaris. Because of the tourist potential of these two areas, they are better known by their oriental sweet gum forests, although a large population covering nearly 100 hectares is also found in an inland region within Aydın Province extending between Çine, Köşk and Umurlu districts. There is another sweet gum forest area (88.5 hectares) under protection in Bucak district of Burdur along Karacaören dam reservoir on the road to Antalya. The scattered trees are also found locally in

Beyağaç and Tavas districts of Denizli Province. The present-day distribution of the species decreased significantly since the 1940s level of 6000-7000 hectares, although the protective measures and infrastructure in place helped to stop the loss of stands and led to slight improvements (Çelik *et al*., 1997).

L. orientalis trees have varying properties that differ according to the elevation; trees that grow in altitudes of 0-400 m are named as plain sweet gum whereas the ones that grow at higher altitudes are called as mountain sweet gum. Mountain sweet gums occur in small groups and tolerate frost better (Alan and Kaya, 2003).

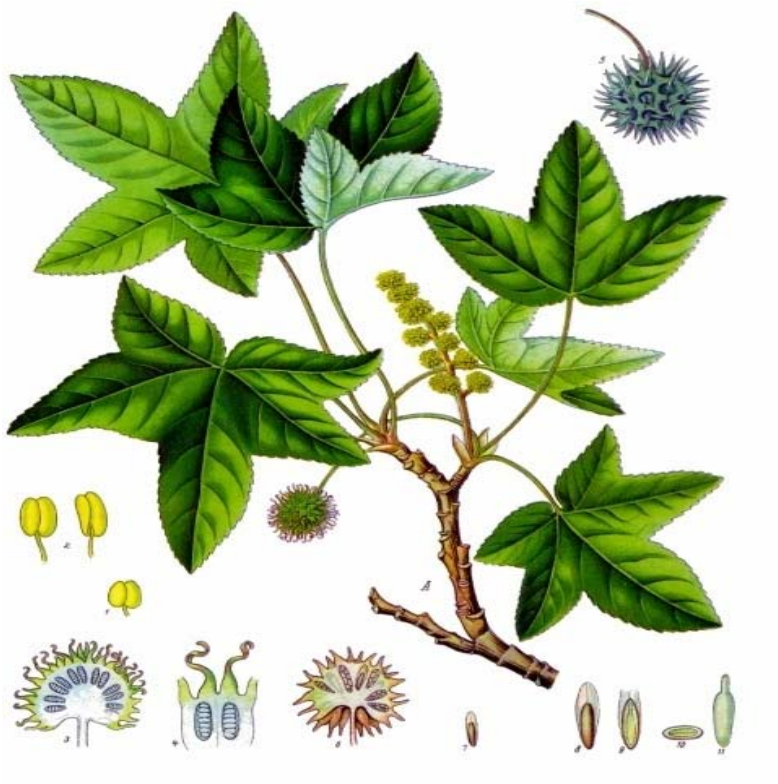

Figure 1. 3 Five lobed leaves, flowers and seeds of *L. orientalis* (Köhler, 1887)

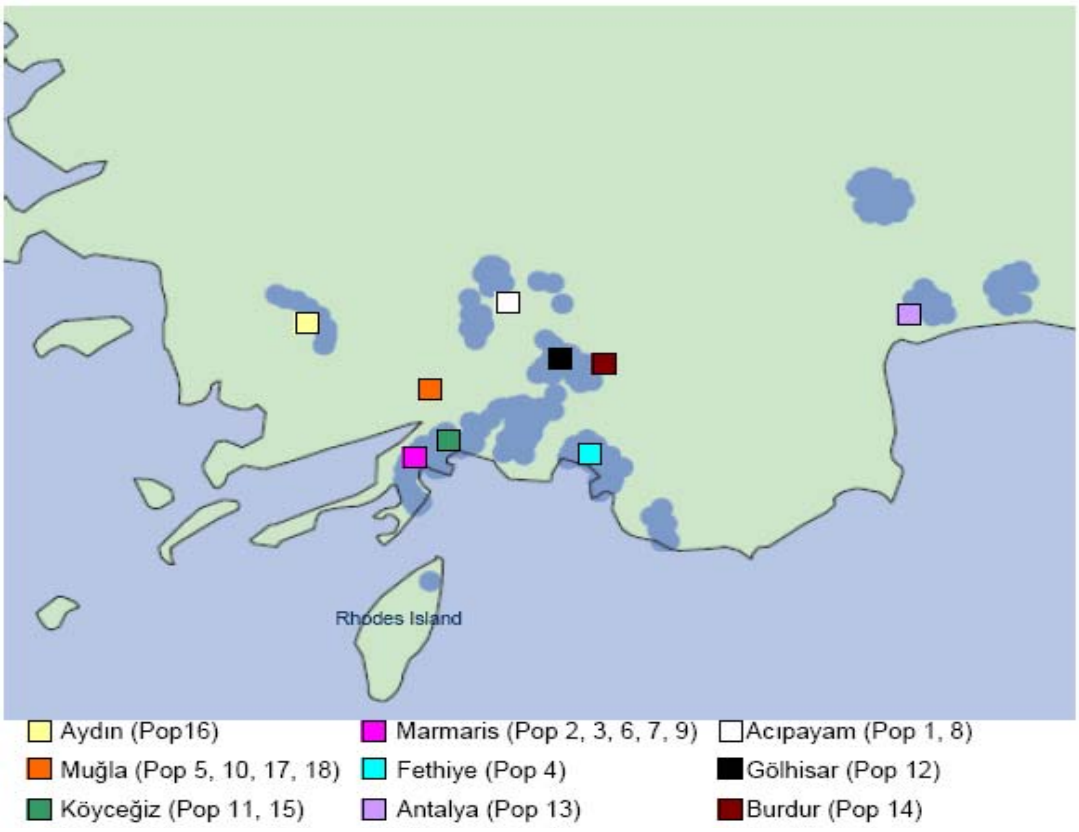

Figure 1. 4 Distribution of *Liquidambar orientalis* in Turkey and Rhodes Island (Alan and Kaya, 2003). Blue spots indicate the natural distribution of Turkish sweet gum.

Figure 1. 5 Soap produced by Sweet gum balsam (Otacı A.Ş., 2007)

The economical value arises from the balsam which is naturally produced by the species. This balsam is used mainly in cosmetics (Figure 1.5) and pharmaceutical industry. It is shown that sweet gum storax has antibacterial activity against many bacteria and therefore is an effective antiseptic (Sağdıç *et al*., 2005).

There are three defined varieties of *L. orientalis* in Turkey (Efe, 1987), which are

1. *L. orientalis* var. *orientalis,*
2. *L. orientalis* var. *integribola* and
3. *L. orientalis* var. *suber.*

However, there are no genetic or strong systematic data that would prove these proposed varieties to be valid. Field studies support the findings on two varieties (var. *orientalis* and var. *integriloba* Flori) in the revision study done for 'Flora of Turkey' by Peşmen (1972), who classified them by the presence or absence of secondary lobes on leaves. In this study, only 2 of the varieties are used to study the phylogenetic relations of the populations; *L. orientalis* var. *orientalis* and *L. orientalis* var. *integribola*.

The range of *L. orientalis* in Turkey decreased dramatically in the previous years from 6312 ha (Huş, 1949) to 3000 ha (Alan and Kaya, 2003) because of habitat destruction, grazing and poor balsam extraction methods which harm trees seriously. Because of this decrease in the habitat size, the species is defined as vulnerable in the nature and critically endangered in the future by IUCN (Walter and Gillet, 1998).

Conservation programs for the establishment of this species include two gene conservation forests located in Isparta and Muğla Provinces, one seed orchard in Fethiye-Göcek, and finally two seed stands in Fethiye and Marmaris districts (Forest Tree Seeds and Tree breeding Research

Directorate, 2007). The other protection regions that include *L. orientalis* populations are Datça-Bozburun Special Environment Protection Region, Fethiye-Göcek Special Environment Protection Region and Gökova Special Environment Protection Region (The Presidency of Special Environment Protection Institution, 2007). One of the gene conservation forests of *L. orientalis*, Isparta Sweet Gum Forest Nature Conservation Area, is located in Sütçüler Forest Management Directorate in a 88.5 ha area. This site includes the natural populations of *L. orientalis* as well as species of 50 plant families, 70 taxa, 4 of which are endemic (Fakir and Doğanoğlu, 2003). There are efforts for the conservation of this species in these areas, but there is a lack of genetic knowledge and research on Turkish sweet gum which is needed for the further establishments.

1.3. Transfer Ribonucleic Acid region of the chloroplast DNA (cpDNA)

Chloroplast DNA sequence variations are being widely used in phylogenetic studies (Palmer, *et al*., 1988; Learn, *et al*., 1992). The non-coding regions display the highest frequency of mutations and can be efficiently used for evolutionary-relationship analysis (Taberlet *et al*., 1991). One of these non-coding regions; the t-RNA (*trn*T-*trn*F) region is the most extensively examined cpDNA fragment due to its wide use in addressing phylogenetic relationships at the levels below family (Taberlet *et al*.,1991; Kelchner 2000). This region is composed of the *trn*L (UAA) gene and two flanking intergenic spacers (IGS), i.e. *trn*T-L and *trn*LF. The *trn*L gene consists of two highly conserved exons that are split by a group I intron, in which both flanks are also quite conservative whereas the central part is highly variable (Bakker *et al*., 2000). Although the *trn*T-*trn*F regions (Figure 1.6) exhibit a quite high substitution rate in many plant groups (Bayer and Starr 1998; Bakker *et al*.

2000; Mansion and Struwe, 2004), single length variation of the region larger than 100 base pairs has not been frequently found among congeneric species of angiosperms except in some special lineages (Mes and Hart, 1994; van der Bank *et al*., 2002; Drabkova *et al*., 2004). In some holoparasitic plants, however, the *trn*L intron, even the whole *trn*L gene, has been lost, and *trn*T-L and *trn*L-F IGS regions, if they exist, show great sequence divergence and large deletions (dePamphilis *et al*., 1997; Freyer *et al*., 1995; Lohan and Wolfe, 1998). The region between the *trn*T (UGU) and *trn*F (GAA) is particularly suitable for evolutionary studies because of ;

i. The succession of the conserved *trn* genes and several hundred base pairs of non-coding regions,
ii. The higher rate of mutations in the single-copy regions,
iii. And the absence of gene rearrangements among many species (Wolfe *et al*., 1987).

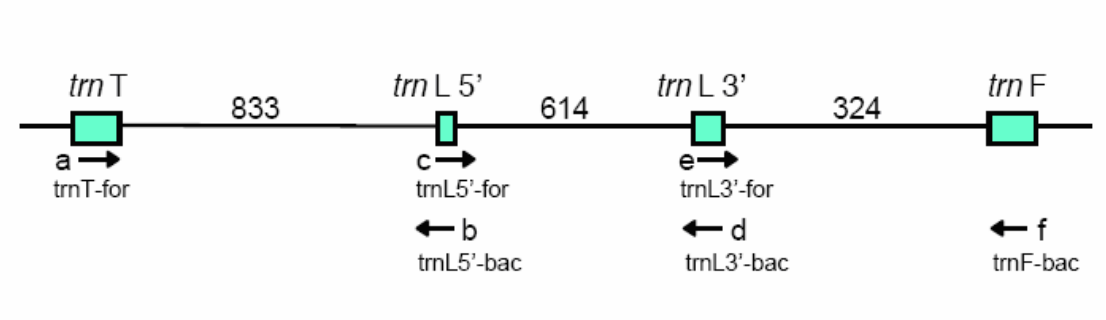

Figure 1. 6 *trn* region of the cpDNA (Taberlet, et al., 1991)

In this study, the evolutionary relations and the diversity indices of sweet gum populations of Turkey were explored by using a molecular approach that includes the phylogenetic analysis, by comparing the non-

coding t-RNA (*trn*) regions of the chloroplast DNA. Three regions within *trn* sequences were used, which are indicated by arrows in the Figure 1.6. The first region is between *trn*T and *trn*L5' amplified by *trn*a and *trn*b primer set, the second one is between *trn*L5' and *trn*L3' that is amplified by *trn*c and *trn*d primers. The last region that lies between *trn*L3' and *trn*F is amplified by *trn*e and *trn*f primer set.

1.4. Rationale and significance of the study

Turkish sweet gum is one of the symbols of Turkey's diversity-rich nature as it is both economically and ecologically important. Although *L. orientalis*, being a relict-endemic species, is an important species of Turkey, the size of population decreases dramatically because of habitat destruction, grazing and poor balsam extraction methods. There is little known about the genetic structure and phylogenetic relations of Turkish sweet gum varieties. With the current study, those gaps in the literature will be filled in an extent in the light of this study. Data obtained on the diversity and variance in the populations representing whole range of Turkey would be used to construct better conservation programs for sustainability of the species. In accordance with the balsam producing activity, selected varieties or populations would be chosen for planting of the species for economical purposes. Although there are previous studies dealing with taxonomy of the species, there is little known about the genotypic basis of taxonomy in the variety and species level of Turkish sweet gum which could be solved in an extent by the completion of this study.

1.5. Goal and objectives of the study

The main goal of this study was to detect the phylogenetic relationships among Turkish sweet gum varieties as well as *Liquidambar* genus and to determine the genetic diversity of sweet gum populations in Turkey, with respect to geographic distribution.

CHAPTER 2

MATERIALS and METHODS

2.1. Plant material

In 2005, Turkish sweet gum leaves were collected from 18 distinct populations representing the natural range of species with 25 individuals in each population (Table 2.1), in cooperation with the Forest Tree Seeds and Tree Breeding Research Directorate, Ministry of Environment and Forest.

The sampled individuals of a population were selected to be 100 m apart from each other. But the distance between the individuals of a population was not a subject of matter considering sampling, when the population is located in a narrow area, like along a river (For example, Serik and Günlük Populations).

The altitude between sampled individuals of a population was also selected to be less than 300 m. The sample leaves were preserved in -70°C refrigerators until DNA isolation.

The populations are also grouped according to four geographic regions (Table 2.1) which are;

Group 1: Denizli

- Population 1 (Acıpayam-Alcı)
- Population 8 (Acıpayam-Bozdağ)

Group 2: Muğla (Köyceğiz, Marmaris and Fethiye)

- Population 2 (Marmaris-Çetibeli)
- Population 3 (Marmaris-Değirmenyanı)
- Population 4 (Fethiye-Günlükbaşı)
- Population 5 (Muğla-Kızılyaka)
- Population 6 (Marmaris-Günnücek)
- Population 7 (Marmaris-Günnücek)
- Population 9 (Marmaris-Hisarönü)
- Population 10 (Muğla-Kıyra)
- Population 11 (Köyceğiz-Köyceğiz)
- Population 15 (Köyceğiz-Köyceğiz-Tb)
- Population 16 (Aydın-Umurlu)

Group 3: Antalya-Burdur

- Population 12 (Gölhisar-Pamucak)
- Population 13 (Antalya-Serik)
- Population 14 (Burdur-Söğütdağ)

Group 4: Muğla-Yatağan-Yılanlı

- Population 17 (Muğla-Yatağan)
- Population 18 (Muğla-Yılanlı)

Table 2. 1 Turkish sweet gum population names and codes, altitude, variety and groupings of populations

Population names and locations	Codes	Varieties	Altitude (m)	Geographic groups	Stand type
1. Acıpayam-Alcı	AK	unknown(U)	1100	1	Pure Population
2. Marmaris-Çetibeli	CE	*integriloba(I)*	30	2	Vegatation mixed within stream
3. Marmaris-Değirmenyanı	DE	*integriloba*	5	2	Pure Population
4. Fethiye-Günlükbaşı	FE	*integriloba*	5	2	Pure Population
5. Muğla-Kızılkaya	FI	*integriloba*	50	2	Pure Population
6. Marmaris-Günnücek	GC	*integriloba*	5	2	Pure Population
7. Marmaris-Günnücek	GN	*integriloba*	5	2	Pure Population
8. Acıpayam-Bozdağ	GU	unknown	1100	1	Pure Population
9. Marmaris-Hisarönü	HO	*integriloba*	10	2	Vegatation mixed within stream
10. Muğla-Kıyra	KI	*integriloba*	50	2	Scattered trees
11. Köyceğiz-Köyceğiz	KO	*integriloba*	10	2	Pure Population
12. Gölhisar-Pamucak	PA	unknown	250	3	Near stream
13. Antalya-Serik	SE	unknown	30	3	Near stream
14. Burdur-Söğütdağ	SO	unknown	550	3	Pure Population
15. Köyceğiz-Köyceğiz	TB	*integriloba*	10	2	Clonal
16. Aydın-Umurlu	UM	*orientalis(O)*	250	2	Vegatation mixed within stream
17. Muğla-Yatağan	YA	*orientalis*	250	4	Vegatation mixed within stream
18. Muğla-Yılanlı	YL	*orientalis*	250	4	Vegatation mixed within stream

(Group 1: Denizli, Group 2: Muğla, Group 3: Antalya and Burdur, Group 4: Muğla-Yatağan-Yılanlı)

2.2. DNA isolation from leaf tissues

The leaves were ground by liquid nitrogen in mortors to form a fine powder until a whitish color was observed. DNA was isolated with the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). In 1.5 ml centrifuge tubes, 0.2 grams of ground leaves were subjected to 750 µl of preheated CTAB to 60 °C. The samples were incubated with this solution in a water bath at 60°C for 30 minutes. After incubation, 750 µl of chloroform:isoamyl alcohol (24:1) was added. The tubes were mixed gently and centrifuged at 10 000 rpm for 10 minutes. After this step, two phases were formed; one aqueous phase which contains the DNA and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. There is also an interface between these two phases containing most of the cell debris, many degraded proteins and etc. The aqueous phase, which is a clear and colorless phase, is taken to a new centrifuge tube with a wide bore pipette tip. About 2/3 volumes of cold isopropanol is added (that is nearly 600 µl) and the tubes were mixed gently. The samples were put into -70 °C refrigerators for at least for 1 hour and than centrifuged at 13 000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 750 µl of 70% EtOH. The tubes were centrifuged at 13 000 rpm for 5 minutes and the supernatant was discarded. The pellets were left to dry at room temperature for 1 hour and then 50 µl of PCR grade water was used to re-suspend the DNA. The DNA presence was detected by 0.8% agarose gel electrophoresis (Figure 2.1).

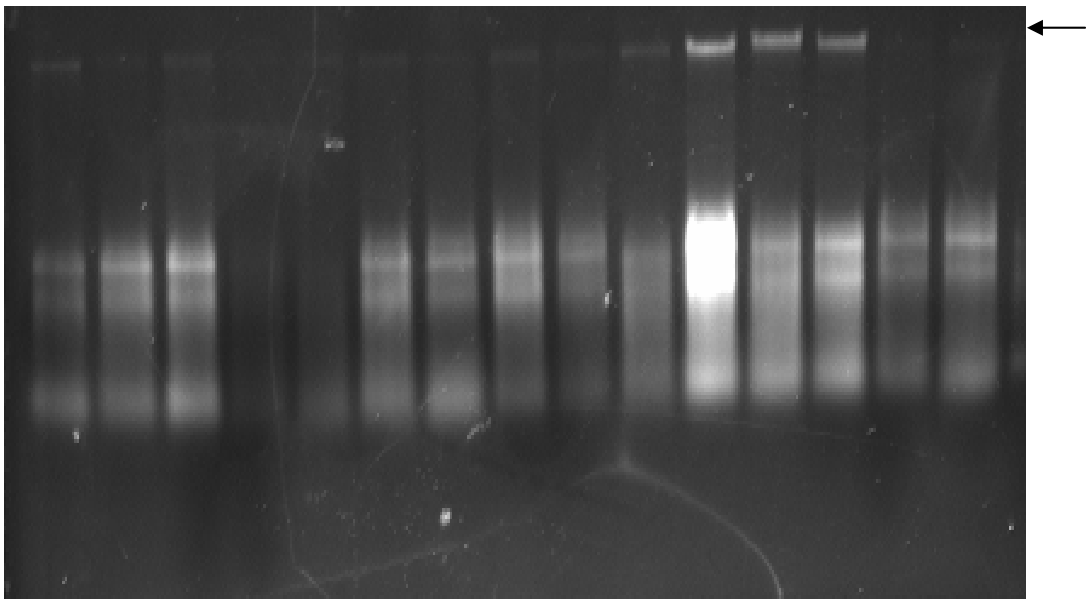

Figure 2. 1 The photo showing 0.8% agarose gel electrophoresis of genomic DNA (indicated with arrow)

2.3. DNA Quantification

DNA concentrations of samples were measured with DYNA QuantTM 200 fluorometer (Hoefer Pharmacia Biotech Inc., Uppsala). Two ml of distilled water was put in the cuvette, and it was dried with tissue paper. After drying, cuvette was inserted into the instrument and it was calibrated as blank with the distilled water. Then, calibration was done with DNA standard solution to a value of 100 ng/μl (Low range). Two microliters of DNA sample was diluted with 2 ml of distilled water and then the DNA measurements were done (Table 2.2). After quantification, the samples suitable for Polymerase Chain Reaction (PCR) were selected.

Table 2. 2 Turkish sweet gum population names and codes, altitude, variety and groupings of populations

Population names and locations	Codes	Varieties	Altitude (m)	Geographic groups	Stand type
1. Acıpayam-Alcı	AK	unknown(U)	1100	1	Pure Population
2. Marmaris-Çetibeli	CE	*integriloba(I)*	30	2	Vegatation mixed within stream
3. Marmaris-Değirmenyanı	DE	*integriloba*	5	2	Pure Population
4. Fethiye-Günlükbaşı	FE	*integriloba*	5	2	Pure Population
5. Muğla-Kızılkaya	FI	*integriloba*	50	2	Pure Population
6. Marmaris-Günnücek	GC	*integriloba*	5	2	Pure Population
7. Marmaris-Günnücek	GN	*integriloba*	5	2	Pure Population
8. Acıpayam-Bozdağ	GU	unknown	1100	1	Pure Population
9. Marmaris-Hisarönü	HO	*integriloba*	10	2	Vegatation mixed within stream
10. Muğla-Kıyra	KI	*integriloba*	50	2	Scattered trees
11. Köyceğiz-Köyceğiz	KO	*integriloba*	10	2	Pure Population
12. Gölhisar-Pamucak	PA	unknown	250	3	Near stream
13. Antalya-Serik	SE	unknown	30	3	Near stream
14. Burdur-Söğütdağ	SO	unknown	550	3	Pure Population
15. Köyceğiz-Köyceğiz	TB	*integriloba*	10	2	Clonal
16. Aydın-Umurlu	UM	*orientalis(O)*	250	2	Vegatation mixed within stream
17. Muğla-Yatağan	YA	*orientalis*	250	4	Vegatation mixed within stream
18. Muğla-Yılanlı	YL	*orientalis*	250	4	Vegatation mixed within stream

(Group 1: Denizli, Group 2: Muğla, Group 3: Antalya and Burdur, Group 4: Muğla-Yatağan-Yılanlı)

Table 2. 3 Mean DNA concentrations obtained from 18 Turkish sweet gum populations and varieties

		DNA Concentration ng/ml		
L. orientalis var. integriloba		**Mean±Standard deviation**	**Minimum**	**Maximum**
Marmaris-Çetibeli (ÇE)		3.30±2.3	0	6
Marmaris-Değirmenyanı (DE)		3.50±2.8	0	9
Fethiye-Günlükbaşı (FE)		9.50±5.0	4	20
Muğla-Kızılyaka (FI)		7.50±6.5	1	25
Marmaris-Günnücek (GC)		8.75±4.4	2	14
Marmaris-Günnücek (GN)		7.60±4.8	1	15
Marmaris-Hisarönü (HÖ)		4.25±2.5	1	8
Muğla-Kıyra (KI)		3.17±3.2	1	10
Köyceğiz- Köyceğiz (KÖ)		7.75±5.6	1	21
Köyceğiz-Köyceğiz (TB)		4.42±3.0	1	10
(n=10)	**Total**	**5.97±2.47**	**3.17**	**9.50**
L. orientalis var. orientalis				
Aydın-Umurlu (UM)		2.25±1.9	0	6
Muğla-Yatağan (YA)		6.25±4.3	2	17
Muğla-Yılanlı (YL)		5.75±5.0	0	15
(n=3)	**Total**	**4.75±2.17**	**2.25**	**6.25**
Unknown				
Gölhisar-Pamucak (PA)		7.91±5.3	2	19
Antalya-Serik (SE)		4.50±2.8	0	11
Burdur-Söğütdağ (SÖ)		4.92±4.3	0	14
Acıpayam-Bozdağ (GÜ)		9.20±6.9	0	21
Acıpayam-Alcı (AK)		12.00±4.8	3	23
(n=5)	**Total**	**7.71±3.11**	**4.50**	**12.00**

2.4. Amplification of t-RNA region of the chloroplast DNA

Three sets of primers (*trn*a and *trn*b, *trn*cand *trn*d, *trn*e and *trn*f) were used initially for PCR amplification. Later two sets were selected to use in sequencing according to the quality and specificity of the bands formed after PCR. The primer sequences are as follows:

For *trn*T- *trn*L5' region:
*trn*a (Forward): 5' CAT TAC AAA TGC GAT GCT CT 3'
*trn*b (Reverse): 5' TCT ACC GAT TTC GCC ATA TC 3'

For *trn*L5'-*trn*L3' region:
*trn*c (Forward): 5' CGA AAT CGG TAG ACG CTA CG 3'
*trn*d (Reverse): 5' GGG GAT AGA GG ACT TGA AC 3'

For *trn*L3'-*trn*F region:
*trn*e (Forward): 5' GGT TCA AGT CCC TCT ATC CC 3'
*trn*f (Reverse): 5' ATT TGA ACT GGT GAC ACG AG 3'

PCR reactions were performed in a total volume of 50 µl, containing 3 mM $MgCl_2$, 50 pmol of each primer, 1 unit of *taq* DNA polymerase (Fermentas, Ontorio, Canada), 0.1 mM of dNTPs mix (Fermentas, Ontorio, Canada) (Table 2.3). The thermal cycler (Eppendorf-Mastercycler, Eppendorf, Canada and Technegenius Thermocycler, Techne, USA) program was set as follows; an initial denaturation step at 95ºC for 5 minutes, 30 cycles of; denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 50 seconds and a final extension step at 72ºC for 5 minutes (Table 2.4). After PCR, the products were analyzed with 2% agarose gel electrophoresis. The sequencing reactions were performed by using only *trn*c-d and *trn*e-f sets because of their good quality when compared to *trn*a-b set in which the

band was either missing or bad in quality. The PCR products were purified for further applications. The purification of the products was done according to the manufacturer's directions (PCR Purification Kit Metis Biotechnologies, Ankara, Turkey). To chech out the quality of the purification, the purified samples were loaded to 2% agorose gel visualized by ethidium bromide staining.

Table 2. 4 PCR conditions for amplification of *trn* region of chloroplast genome of Turkish sweet gum

PCR contents	Volume used in PCR (µl)	Final Concentration
PCR Grade Water	34.8	NA
$MgCl_2$ (25 mM stock solution)	6	3 mM
10X PCR Buffer	5	1X
dNTP (10 mM of each dNTP)	0.5	0.1 mM
Forward primer (100 µM)	0.5	1 µM
Reverse primer (100 µM)	0.5	1 µM
Taq DNA polymerase (5 u/µl)	0.2	0.02u/µl
DNA	2.5	≈ 0. 5 µg/µl
Total Volume	50	

Table 2. 5 Thermal cycler program for amplification of *trn* region of chloroplast genome of Turkish sweet gum

Temperature (ºC)	Duration	Number of cycles	Purpose
94	5 minutes	1	Initial denaturation
94	30 seconds	30	Internal denaturation
55	30 seconds		Annealing
72	50 seconds		Extension
72	5 minutes	1	Final extension

2.5. Sequencing t-RNA region of the chloroplast DNA

Both forward (*trnc* and *trne*) and reverse (*trnd* and *trnf*) primers were used to sequence the two regions. Amersham dye-terminator sequencing kit (Thermo Sequenase™ II Dye Terminator Cycle Sequencing Kits, Amersham Biosciences, New Jersey, U.S.A) was used for sequencing reactions. Four vials of sequencing reactions were prepared containing ddATP, ddCTP, ddGTP and ddTTP, respectively at a total volume of 10 µl (Table 2.5). The thermal cycler program for sequencing (Table 2.6) was as follows: 5 minutes at 94ºC; for 18 cycles, 30 seconds at 94ºC ; 30 seconds at 55ºC, 45 seconds at 72ºC and for 15 cycles, 30 seconds at 94ºC and 1 minute at 72ºC with a final extention at 72ºC for 5 minutes. Sequencing reactions were purified by ammonium acetate purification as described by the manufacturer (Amersham Biosciences, New Jersey, USA). The purification procedure was as follows;

1. In each tube, 2 µl of 7.5 M ammonium acetate and 30 µl of cold 99% EtOH were added.

2. The tubes were vortexed gently and left on ice for 20 minutes.

3. The tubes were centrifuged at 12 000 rpm for 25 minutes for DNA to precipitate.

4. The Supernatant was discarded and 200 µl of 70% EtOH wash buffer was added.

5. The tubes were again vortexed gently and centrifuged at 13 000 rpm for 5 minutes.

6. The supernatant was discarded and the tubes were left to dry for 20 minutes.

7. Five µl of formamide loading dye was added, the tubes were vortexed and stored at -20 °C refrigerators.

Finally, the products were run on 6% polyacrylamide gel at the automated sequencing system Open Gene (Visible Genetics, Ontorio, Canada) and the data were collected according to the manufacturer's directions.

After the collection of the data, the sequences from the forward primer and the sequences from the reverse primer were aligned and the accuracy of the bases was checked manually. If any inconsistency was present between the two sequences, the sample was neglected from the anaysis.

Table 2. 6 Reaction conditions for sequencing

Sequencing reaction contents	Volume used (µl)	Final concentration
PCR grade water	6.5	NA
Reaction Buffer	0.7	NA
Primer (5µM)	1.0	0.5 µM
ddNTP mix	0.6	0.1 mM
Sequencing enzyme	0.2	0.02u/µl
Purified PCR product	1	NA

Table 2. 7 Thermal cycler program for sequencing

Temperature (ºC)	**Duration**	**Number of cycles**	**Purpose**
94	5 minutes	1	Initial denaturation
94	30 seconds	18	denaturation
55	30 seconds		Annealing
72	45 seconds		Extention
94	30 seconds	15	Denaturation
72	1 minute		Extension
72	5 minutes	1	Final extension

2.6. Data Analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004) and Arlequin (Schneider *et al*., 2000) softwares. The sequences were pre-processed in FASTA format by aligning "-" to the gaps and "N" to the unknown bases between the two regions *trn*cd and *trn*ef of t-RNA. The analyzed sequence data were shorter than the unprocessed sequence data because the beginning and the ends of the data were trimmed. The reason for this shortening was the unreliability of those parts. The quality of sequencing decreases dramatically at the beginning and the end of the sequencing when automatic sequencing systems are used. There are two kinds of data sets that were analyzed. The first data set includes populations grouped according to their respective varieties of *L. orientalis.* In the second set, populations are grouped according to their geographic locations. The geographic locations and the varieties that populations belong were listed in the Table 2.1.

The analysis was done including 6 other species of *Liquidambar* that were available in the database NCBI (National Center for Biotechnology Information, 2007). The names and the gene bank accession numbers of these sequences are as follows:

L. styraciflua from USA (DQ352217)
L. macrophylla from Mexico (DQ352218)
L. formosana from *V*ietnam (DQ352220)
L. acalycina from China (DQ352216)
L. formosana from China (DQ352221)
L. acalycina from USA (DQ352215)

The distances between the populations were computed by using Kimura (1980) two-parameter test in MEGA version 3.1. Kimura (1980)'s two parameter-model corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites. Also when computing distances, pair wise deletion method was used in which gaps and missing data are discarded during analysis when necessary.

The differentiation between species of *Liquidambar* as well as varieties of *L. orientalis* and the genetic structure of *L. orientalis* populations was investigated by an analysis of variance framework, as initially defined by Cockerham (1969, 1973), and extended by others (e.g. Weir and Cockerham, 1984). This is The Analysis of Molecular Variance Approach and carried out by Arlequin Software (AMOVA, Excoffier *et al*., 1992). It is essentially similar to other approaches based on analysis of variance of the gene frequencies, but it takes into account the number of mutations between molecular haplotypes, which first needed to be evaluated.

The covariance components are used to compute fixation indices, as originally defined by Wright (1951, 1965), in terms of inbreeding coefficients, or later in terms of coalescent times by Slatkin (1991).

The transversion, transition and deletion weight were used as "1.0" in the calculations as the polymorphism control level. The minimum spanning network among haplotypes was conducted by pairwise difference method. Molecular diversity and standard diversity indices were also calculated. The mismatch distribution was conducted by pairwise difference method with a bootstrap replication of 1000. In the "Locus by locus" AMOVA analysis and population comparisons, pairwise difference matrix was used with 1000 permutations.

Four kinds of analysis were done that includes firstly the comparisons of the populations generally, second in the geographic level, third in the variety level and the last in the genus *Liquidambar*. The differences among species, among populations and within populations were also determined.

The phylogenetic tree was constructed by using neighbor-joining (NJ) method (Saitou, Nei, 1987) together with bootstrap test analysis. The NJ method uses distance measures to correct for multiple hits at the same sites, and chooses a topology showing the smallest value of the sum of all branches as an estimate of the correct tree. With the NJ method, the S (smallest value of the sum of all branches) value is not computed for all or many topologies. The examination of different topologies is imbedded in the algorithm (Nei and Kumar; 2000), so that only one final tree is produced. If there are *m* sequences, each with *n* nucleotides (or codons or amino acids), a phylogenetic tree can be reconstructed using some tree building method. From each sequence, n nucleotides are randomly chosen with replacements, giving rise to m rows of n columns each. These now constitute a new set of sequences. A tree is then reconstructed with these new sequences using the same tree building method as before. Next, the topology of this tree is compared to that of the original tree. Each interior branch of the original tree that is different from the bootstrap tree it partitions is given a score of 0; all other interior branches are given the value 1. This procedure of resampling the sites and the subsequent tree reconstruction are repeated several hundred times, and the percentage of times each interior branch is given a value of 1 is noted. This is known as the bootstrap value. As a general rule, if the bootstrap value for a given interior branch is 95% or higher, then the topology at that branch is considered "correct". If the value is greater than 50, the topology is considered as informative (Nei and Kumar, 2000). Three phylogenetic trees, those including the analysis in species level, Turkish *L. orientalis* populations and a general phylogenetic tree are constructed by MEGA 3.1 (Figure 3.2, 3.3, 3.4).

CHAPTER 3

RESULTS

3.1. Amplification of the t-RNA region of the chloroplast DNA

Good quality single bands were observed for *trn*c-d and *trn*e-f primer sets but the amplification product for *trn*a-b primer set was either missing or in bad quality (Figure 3.1). Because of the good quality of the bands, the fragments amplified by *trnc*-d and *trn*e-f primers were selected for sequencing.

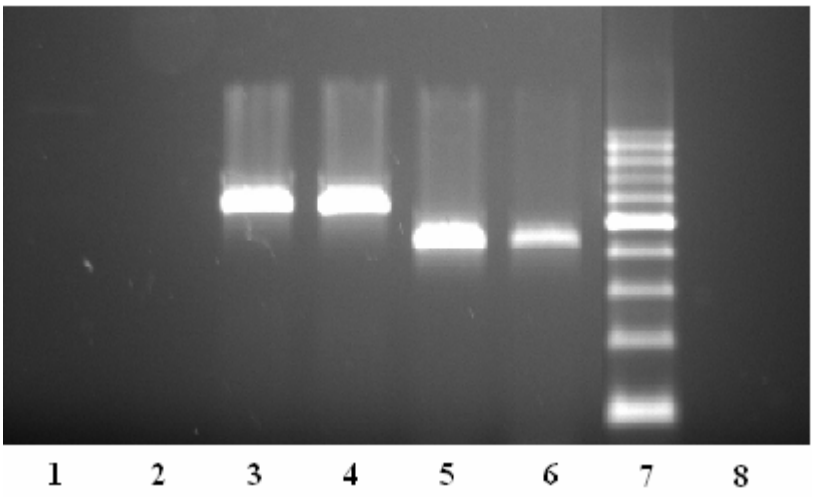

Figure 3. 1 PCR products of *trn* region with primer sets in 2% agarose gel electrophoresis (1-2: *trn* a-b primer set, 3-4: *trn* c-d primer set,, 5-6: *trn* e-f primer set, 7: 100 bp ladder and 8: negative control)

3.2. Sequencing t-RNA region of the chloroplast DNA of Turkish sweet gum

Sequencing reactions were done as described in Chapter 2 and the purified sequence products were run on 6% polyacrylamide gel accordingly (Visible Genetics Automated Sequencing System). Data were collected by automated sequencing system. The sequence data were very good in quality and there were no confusions on the identification of the bases according to the chromatogram data (see Appendix A).

3.3. Data Analysis

3.3.1. Molecular diversity indices of Turkish sweet gum

Moleculer diversity indices were computed by using all the sequenced individuals from 18 Turkish sweet gum populations. For varieties and geographic groupings, polymorphic site means were also computed and stated in Table 3.2 and Table 3.3.

3.3.1.1. Molecular diversity indices of Turkish sweet gum populations

Molecular diversity indices for all the *L. orientalis* populations of Turkey were conducted by Arlequin 2.000 and results are given in Table 3.1. The highest number of transitions was found in Population 1 (Acıpayam-Alcı) and 17

(Muğla-Yatağan). Population 3 (Marmaris-Değirmenyanı) and 7 (Marmaris-Günnücek) had the highest number of transversions and the number of substitutions are highest in Populations 1 (Acıpayam-Alcı), 7 (Marmaris-Günnücek) and 17 (Muğla-Yatağan). There are 7 deletions in Population 13 (Antalya-Serik), being the highest, and after Population 13 (Antalya-Serik), Population 2 (Marmaris-Çetibeli) and 17 (Muğla-Yatağan) has the highest number of deletions. The highest number of polymorphic sites, which is an indication of diversity, was found in Population 17 located in Muğla-Yatağan. Population 13 (Antalya-Serik) had 8 polymorphic sites and after these two populations, population 1 (Acıpayam-Alcı), 2 (Marmaris-Çetibeli), 3 (Marmaris-Değirmenyanı), 7 (Marmaris-Günnücek) and 18 (Muğla-Yılanlı) had high number of polymorphic sites.

Table 3. 1 Molecular diversity indices of Turkish sweet gum populations

Population name	# of transitions	# of transversions	# of substitutions	# of deletions	# of usable loci	# of polymorphic sites
1. Acıpayam-Alcı	3	1	4	2	825	6
2. Marmaris-Çetibeli	0	1	1	5	827	6
3. Marmaris-Değirmenyanı	0	2	2	1	825	3
4. Fethiye- Günlükbaşı	0	0	0	1	825	1
5. Muğla- Kızılkaya	0	0	0	0	825	0
6. Marmaris-Günnücek	0	0	0	1	825	1
7. Marmaris-Günnücek	1	3	4	1	825	5
8. Acıpayam-Bozdağ	0	0	0	1	826	1
9. Marmaris-Hisarönü	0	0	0	1	825	1
10. Muğla-Kıyra	0	0	0	1	825	1
11. Köyceğiz-Köyceğiz	0	0	0	0	825	0
12. Gölhisar-Pamucak	0	0	0	0	825	0
13. Antalya-Serik	1	0	1	7	827	8
14. Burdur-Söğütdağ	0	0	0	0	825	0
15. Köyceğiz-Köyceğiz	0	0	0	1	825	1
16. Aydın-Umurlu	0	0	0	1	825	1
17. Muğla-Yatağan	2	1	3	6	827	9
18. Muğla-Yılanlı	0	1	1	2	826	3

3.3.1.2. Molecular diversity indices of Turkish sweet gum varieties

By using the number of polymorphic sites indicated in Table 3.1, mean values of number of polymorphic sites were computed within varieties. The highest mean number of polymorphic sites was found in var. *orientalis* and the lowest mean number of polymorphic sites was found in var. *integriloba* (Table 3.2).

Table 3. 2 Polymorphic sites of Turkish sweet gum varieties

Variety name	# of polymorphic sites		
	Mean	Minimum	Maximum
orientalis	4.33	1	9
integriloba	1.90	0	6
unknown	3.00	0	8

3.3.1.3. Molecular diversity indices of Turkish sweet gum geographic groupings

By using the number of polymorphic sites indicated in Table 3.1, mean values of number of polymorphic sites were computed within geographic groupings (Table 3.3). The highest mean number of polymorphic sites was found in Group 4 (Muğla-Yatağan-Yılanlı) and the lowest mean number of polymorphic sites was in Group 2 (Muğla).

Table 3. 3 Polymorphic sites of Turkish sweet gum with respect to geographic groupings

Geographic group	# of polymorphic sites		
	Mean	Minimum	Maximum
Denizli	3.50	1	6
Muğla	1.82	0	6
Antalya Burdur	2.67	0	8
Muğla Yatağan Yılanlı	6.00	3	9

3.3.2. Genetic differentiations and sequence diversity of Turkish sweet gum

When computing the genetic distances and sequence diversity, all the individuals with good quality sequence data were included. The parameters were set as stated in section 2.6. The analysis included four kinds of results that are Turkish sweet gum population difference, Turkish sweet gum variety difference, Turkish sweet gum geographic location differences and *Liquidambar* species differences.

3.3.2.1. Genetic distances among Turkish sweet gum populations

As mentioned in the previous sections, there were 4 individuals from each population, that were sequenced and some of the individuals showed no sequence differences with the other individual from the same population as expected. The distances within the populations were computed and indicated in the Table 3.4. When all the individuals were included in the analysis, population number 7 (Marmaris-Günnücek) showed the highest variation within population. Population 1 (Acıpayam-Alcı) and Population 17 (Muğla-Yatağan) have the second highest variation within population. Population 3 (Marmaris-Değirmenyanı) has the third highest variation followed by Population 2 (Marmaris-Çetibeli), 13 (Antalya-Serik) and 18 (Muğla-Yılanlı). Some populations showed no difference within population level with a average genetic distance value of '0'. Those populations having no difference within population level are populations 4, 5, 6, 8, 9, 10, 11, 12, 14, 15, 16 (Table 3.4).

Table 3. 4 Average genetic distances within studied populations of Turkish sweet gum

Population number and name	**Average genetic distance within Turkish populations of *L. orientalis* (±standard error)**
1. Acıpayam-Alcı	0.0024 (±0.0012)
2. Marmaris-Çetibeli	0.0006 (±0.0006)
3. Marmaris-Değirmenyanı	0.0016 (±0.0011)
4. Fethiye- Günlükbaşı	0.0000 (±0.0000)
5. Muğla- Kızılkaya	0.0000 (±0.0000)
6. Marmaris-Günnücek	0.0000 (±0.0000)
7. Marmaris-Günnücek	0.0032 (±0.0016)
8. Acıpayam-Bozdağ	0.0000 (±0.0000)
9. Marmaris-Hisarönü	0.0000 (±0.0000)
10. Muğla-Kıyra	0.0000 (±0.0000)
11. Köyceğiz-Köyceğiz	0.0000 (±0.0000)
12. Gölhisar-Pamucak	0.0000 (±0.0000)
13. Antalya-Serik	0.0006 (±0.0006)
14. Burdur-Söğütdağ	0.0000 (±0.0000)
15. Köyceğiz-Köyceğiz	0.0000 (±0.0000)
16. Aydın-Umurlu	0.0000 (±0.0000)
17. Muğla-Yatağan	0.0024 (±0.0014)
18. Muğla-Yılanlı	0.0006 (±0.0006)

3.3.2.2. Genetic distances and partition of total sequence diversity of Turkish sweet gum varieties

The amount of genetic variation due to populations within variety was computed as 85.71 % of the total variation and 14.29 % of the total variation was attributed to the intervariety diversity (Table 3.5).

Table 3. 5 Sequence diversity partitions computed by MEGA 3.1 according to the varieties of Turkish sweet gum

	The amount of genetic variation due to populations within variety	**Amount of genetic variation due to varieties**
Percent of total molecular variation	85.71 %	14.29 %

Average genetic distances within varieties and between varieties of Turkish *L. orientalis* were calculated (Table 3.6). The most divergent group seems to include variety *orientalis* having an average genetic distance value of 0.0011. The unknown and *integriloba* varieties have average genetic distance values of 0.0007 and 0.0006, respectively. The highest genetic distance was found to be between the unknown variety and var. *orientallis* with a value of 0.0009 (±0.0003). The average genetic distance between var. *integriloba* and var. *orientalis* and between unknown variety and var. *orientalis* were found to be 0.0008 (±0.0003) and 0.0006 (±0.0002), respectively.

Table 3. 6 Genetic distances computed among the varieties of Turkish sweet gum

Avarage genetic distance within varieties of *L. orientalis**		**Avarage genetic distance between varieties of *L. orientalis****	
***L. orientalis* varieties**		**unknown**	***integriloba***
unknown	0.0007 (±0.0003)		
integriloba	0.0006 (±0.0002)	0.0006 (±0.0002)	
orientalis	0.0011 (±0.0005)	0.0009 (±0.0003)	0.0008 (±0.0003)

*Genetic distance (±standard error)

3.3.2.3. Genetic distances and partition of total sequence diversity of Turkish sweet gum with respect to geographic groupings

The second data set includes the genetic distances and diversity indices according to the grouping done on geographic locations of the populations. There were 4 main groups and those groups were indicated in Table 2.1. The molecular variation and computed genetic distances were stated in Table 3.7 and Table 3.8, respectively. When the total molecular varation was partitioned, it can be seen that 81.82% of the total variation was within the geographic groups, and 18.18% of the total variation was between the geographic groups (Table 3.7).

The genetic distances were again not so significantly high, but the most divergent groups include the 1st (Denizli) and the 4th (Muğla-Yatağan-Yılanlı) locations. The first and the fourth group consists of the populations 1 (Acıpayam-Alcı), 8 (Acıpayam-Bozdağ), 17 (Muğla-Yatağan) and 18 (Muğla-Yılanlı). The genetic distance between group 1 (Denizli) and group 4 (Muğla-

Yatağan-Yılanlı) was found to be the greatest (0.0014) of between varieties. The lowest genetic distance value of 0.0004 was calculated between group 2 (Muğla) and group 3 (Burdur-Antalya) (Table 3.8).

Table 3. 7 Sequence diversity partitions computed by MEGA 3.1 according to the geographic groupings of Turkish sweet gum

	The amount of genetic variation due to populations within geographic groups	**Amount of genetic variation due to geographic groups**
Percent of total molecular variation	81.82 %	18.18 %

Table 3. 8 Genetic distances computed among geographic groups of Turkish sweet gum

Avarage genetic distance within geographic groups*		**Avarage genetic distance between geographic groups***		
Geographic Groups		**Gp 1 Denizli**	**Gp 2 Muğla**	**Gp 3 Burdur-Antalya**
Gp 1 Denizli	0.0014 (±0.0007)			
Gp 2 Muğla	0.0005 (±0.0002)	0.0010 (±0.0004)		
Gp 3 Burdur-Antalya	0.0002 (±0.0002)	0.0008 (±0.0004)	0.0004 (±0.0002)	
Gp 4 Muğla-Yatağan-Yılanlı	0.0014 (±0.0007)	0.0014 (±0.0005)	0.0010 (±0.0004)	0.0008 (±0.0004)

*Genetic distance (±standard error)

3.3.2.4. Genetic distances between Turkish sweet gum and other species of Liquidambar

The genetic distances between the species of *Liquidambar* was computed including all of the individuals of *L. orientalis* populations sampled from Turkey (Table 3.9). It can be seen that the lowest genetic distance value was between *L. styraciflua* and *L. orientalis*. Again, the greatest genetic distance values were estimated between Turkish sweet gum and Vietnamese *L. formosana*, Chinese *L. acalycina* and *L. formosana* (0.0051). The genetic distance value between Mexican *L. macrophylla* and Turkish sweet gum indicates that Mexican *L. macrophylla* is the second closest neighbor.

Table 3. 9 Genetic distances computed within *Liquidambar* genus

	1	2	3	4	6
1. Turkish *L. orientalis*					
2. Vietnamese *L. formosana*	0.0051 ± (0.0023)				
3. Mexican *L. macrophylla*	0.0012 (±0.0010)	0.0049 (±0.0023)			
4. Chinese *L. acalycina*	0.0051 (±0.0023)	0.0000 (±0.0000)	0.0049 (±0.0023)		
5. Chinese *L. formosana*	0.0051 (±0.0023)	0.0000 (±0.0000)	0.0049 (±0.0023)	0.0000 (±0.0000)	
6. American *L. styraciflua*	0.0002 (±0.0001)	0.0049 (±0.0023)	0.0000 (±0.0000)	0.0049 (±0.0023)	0.0049 (±0.0023)

*Genetic distance (±standard error)

3.3.3. Partition of total molecular variation in Turkish sweet gum populations

The Analysis of Moleculer Variance (AMOVA) was carried out by Arlequin version 2.000. The computation parameters were set as indicated in the section 2.6. Again three kinds of analysis was done; one according to the genus *Liquidambar*, one according to the varieties and one according to the geographic groupings of Turkish sweet gum. These AMOVA results were indicated in Table 3.10 and Table 3.11.

3.3.3.1. Partition of total molecular variation in varieties of Turkish sweet gum

The percentage of variance due to varieties was 2.38% of the total variance while it was 5.65% among populations within varieties. The great potion of total variance (91.97%) was found to be within populations (Table 3.10).

Table 3. 10 AMOVA results for all the Turkish sweet gum populations grouped according to their varieties

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of total variation
Among varieties	2	2.859	0.02267 Va	2.38
Among populations within varieties	15	15.606	0.05372 Vb	5.65
Within populations	38	33.250	0.87500 Vc	91.97
Total	**55**	**51.714**	**0.95139**	**100**

3.3.3.2. Partition of total molecular variation in geographic groupings of Turkish sweet gum

When the AMOVA analysis was carried out according to the geographic distribution of studied populations, the percentage of variation due to geographic groups was found to be 1.97%. About 5.89% of total variation was found to be within geographic groups and again greater portion (92.14%) of total variation was within populations (Table 3.11).

Table 3. 11 AMOVA results for all Turkish sweet gum populations grouped according to their geographic locations

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of total variation
Among geographic groups	3	3.845	0.01875 Va	1.97
Among populations within geographic groups	14	14.620	0.05589 Vb	5.89
Within populations	38	33.250	0.87500 Vc	92.14
Total	**55**	**51.714**	**0.94964**	**100**

3.3.4. Phylogenetic trees constructed by MEGA 3.1

When the distances among all individuals within populations that were sequenced were computed by MEGA 3.1, the diversity within some of the populations was not so high (Table 3.4) and for that reason, phylogenetic tree construction was done by choosing just one representative individual from each population.

Three phylogenetic trees, those including the analysis in genus *Liquidambar*, Turkish *L. orientalis* populations and a general phylogenetic tree including all *L. orientalis* varieties and other *Liquidambar* species are constructed by MEGA 3.1 (Figure 3.2, 3.3, 3.4). Bootstrap values and the branch lengths are also given on the phylogenetic trees above and below the branches, respectively. According to the phylogenetic tree (Figure 3.2), it was seen that 15 of the Turkish populations of *L. orientalis* were grouped together with an exception of 3 populations (Populations 4, 7 and 10) which are

representatives of *L. orientalis integriloba*. The bootstrap values show that those topologies were just informative, but the group that included the other 3 species (*L. formosana* from *V*ietnam, *L. acalycina* from China, *L. formosana* from China) in one main branch and all the others in the second branch was found to be “correct” as it had a bootstrap value greater than 95. The closest neighbors of Turkish *L. orientalis,* were shown to be *L. orientalis* and *L. styraciflua* from USA and *L. macrophylla* from Mexico.

When the phylogecetic tree (Figure 3.3) constructed with the only studied populations of Turkish sweet gum is considered, it is seen that Population 3 located in Marmaris-Değirmenyanı is found in a separate branch from the other populations of Turkey. Populations 4 (Fethiye-Günlükbaşı), 7 (Marmaris-Günnücek) and 10 (Muğla-Kıyra) are the other three populations that are found to be specialized, by forming another branch within the main branch which included 14 remaining populations of Turkish sweet gum.

According to the varieties, the average sequence divergence values were not significantly high for both within and between varieties, so for phylogenetic tree construction just one individual from each population was selected as a representative. In this phylogenetic tree (Figure 3.4), the varieties did not show any observable variance and are also grouped in the same branch with American *L. orientalis*. Again American *L. styraciflua* is the closest neighbor followed by Mexican *L. macrophylla* and the remaining species are grouped together in another branch.

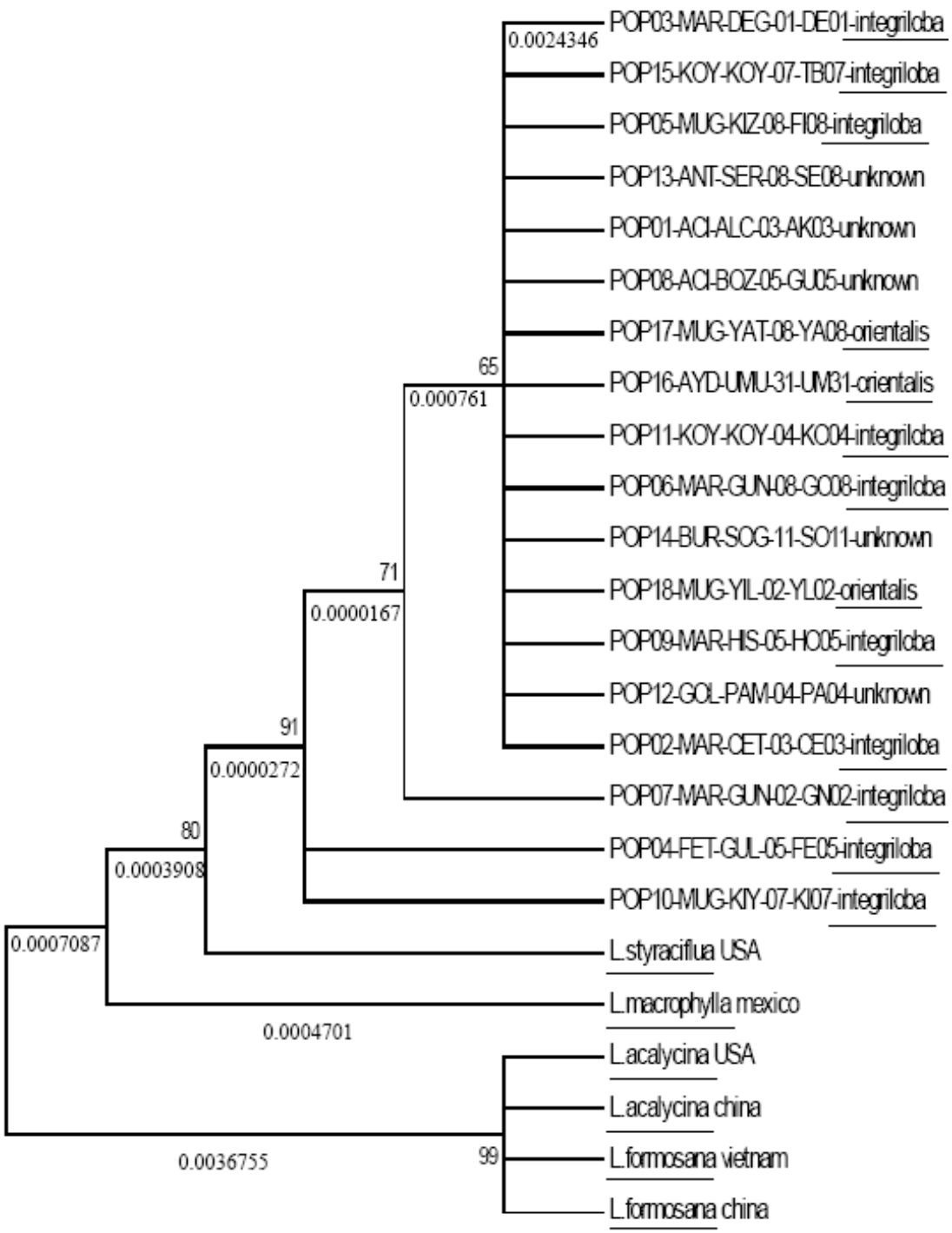

Figure 3. 2 Phylogenetic tree constructed by one representative from each population and all the species of *Liquidambar* (The values above and below branches are the bootstrap values and the branch lenghts, respectively. The branch length is not indicated if it is zero.)

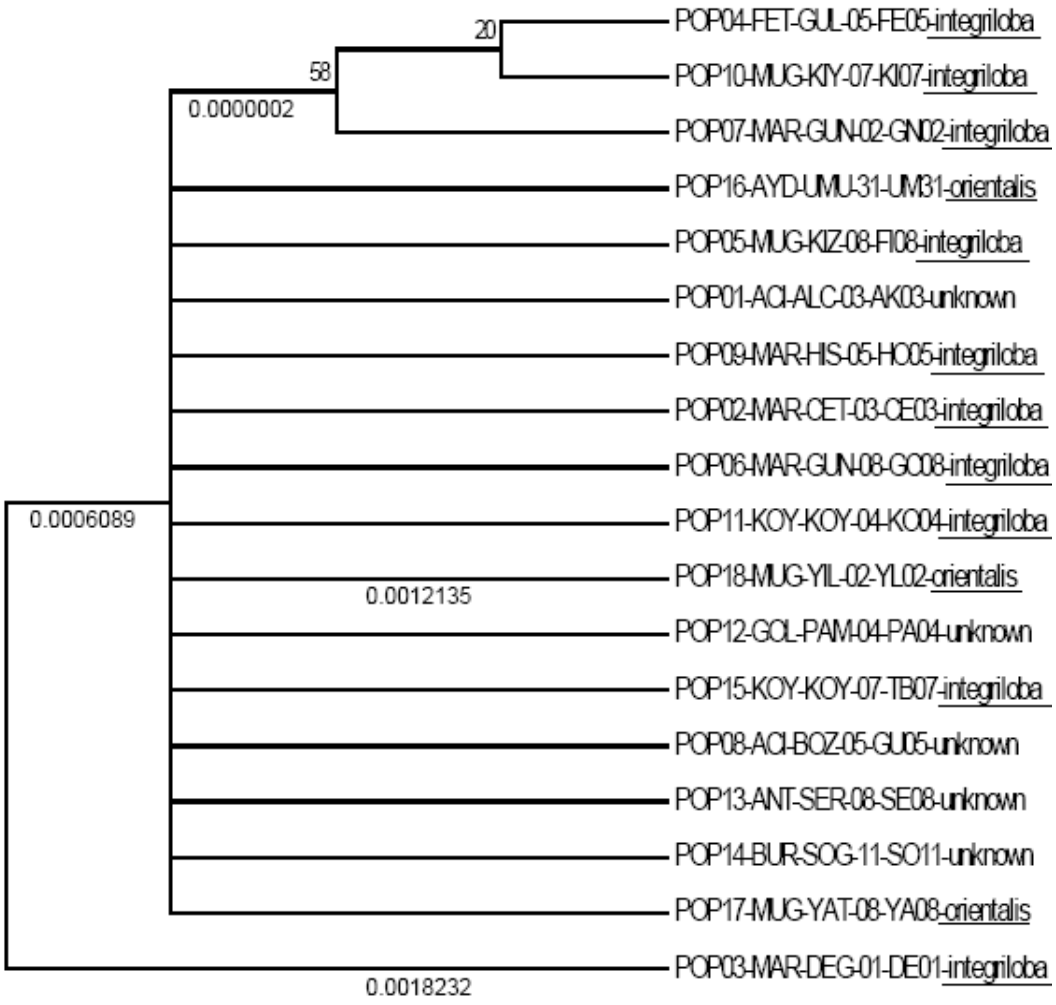

Figure 3. 3 Phylogenetic tree constructed with just populations of Turkish *L. orientalis* (The values above and below branches are the bootstrap values and the branch lenghts, respectively. The branch length is not indicated if it is zero.)

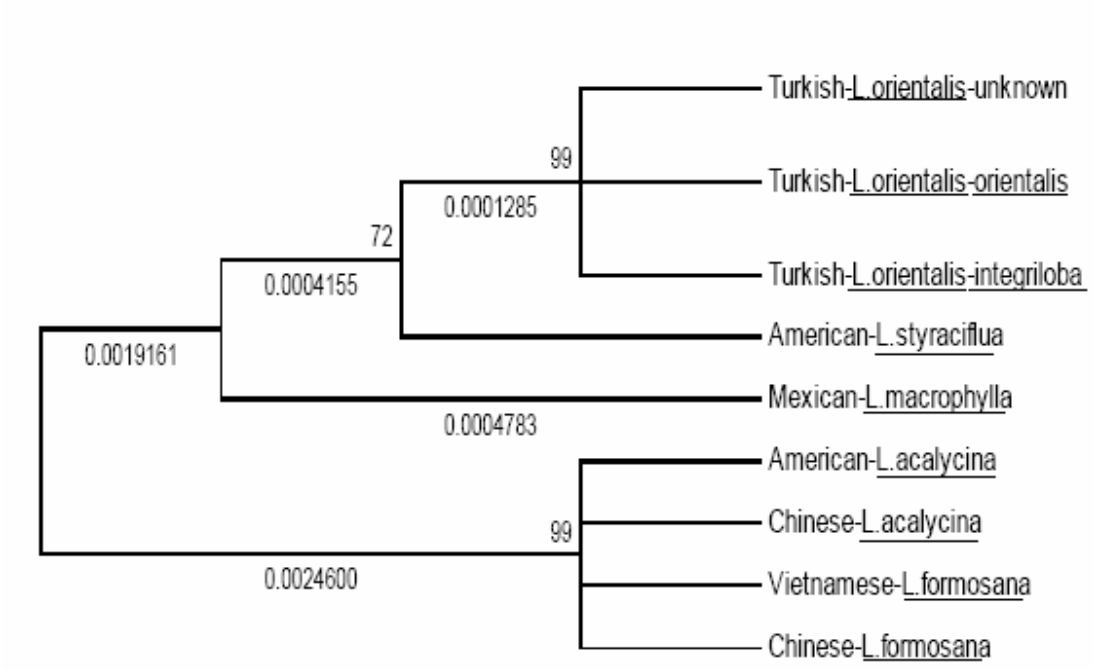

Figure 3. 4 Phlogenetic tree constructed with the use of species of genus *Liquidambar* and varieties of *L. orientalis*

3.3.5. Evolutionary relationship among sweet gum species: The Minimum Spanning Tree (MST)

Minimum spanning tree (Figure 3.5) was constructed by the Operational Taxonomic Units (OTUs) between 58 individuals, 52 being from Turkish populations of *L.orientalis* and the others from the database representing other species of *Liquidambar*.

There were four main groups in the MST; Group A, Group B, Group C and Group D. Group A represents samples from 14 populations of Turkish sweet gum. There were 3 individuals emerging from Group A with a connection length of 1, which were Population 9 (Marmaris-Hisarönü), Population 16 (Aydın-Umurlu) and Populaion 18 (Muğla-Yılanlı). Population 17 (Muğla-

Yatağan) was separated from the group with a connection length of 2. the second major group, Group B that was separated from the first major group with a connection length of 1, is consisted of 5 sweet gum populations from Turkey which were Population 4 (Fethiye-Günlükbaşı), Population 6 and 7 (Marmaris-Günnücek), Population 10 (Muğla-Kıyra) and Population 18 (Muğla-Yılanlı). There were two individuals separated from B; Population 7 (Marmaris-Günnücek) and Population 18 (Muğla-Yılanlı). The third major group C was branched from Group B by a connection length of 1 and this group was represented by Population 4 (Fethiye-Günlükbaşı) and Population 10 (Muğla-Kıyra).

The closest neighbor of Turkish sweet gum, *L. styraciflua* is branched from the third main group, Group C, by a connection length of 2 and the second closest neighbor *L.macrophylla* from Mexico is separated from *L. styraciflua. L.formosana* from Vietnam, *L.acalycina* from China, *L.formosana* from China, *L.acalycina* from USA are in another major group branched from *L. styraciflua* separated by a connection length of 4.

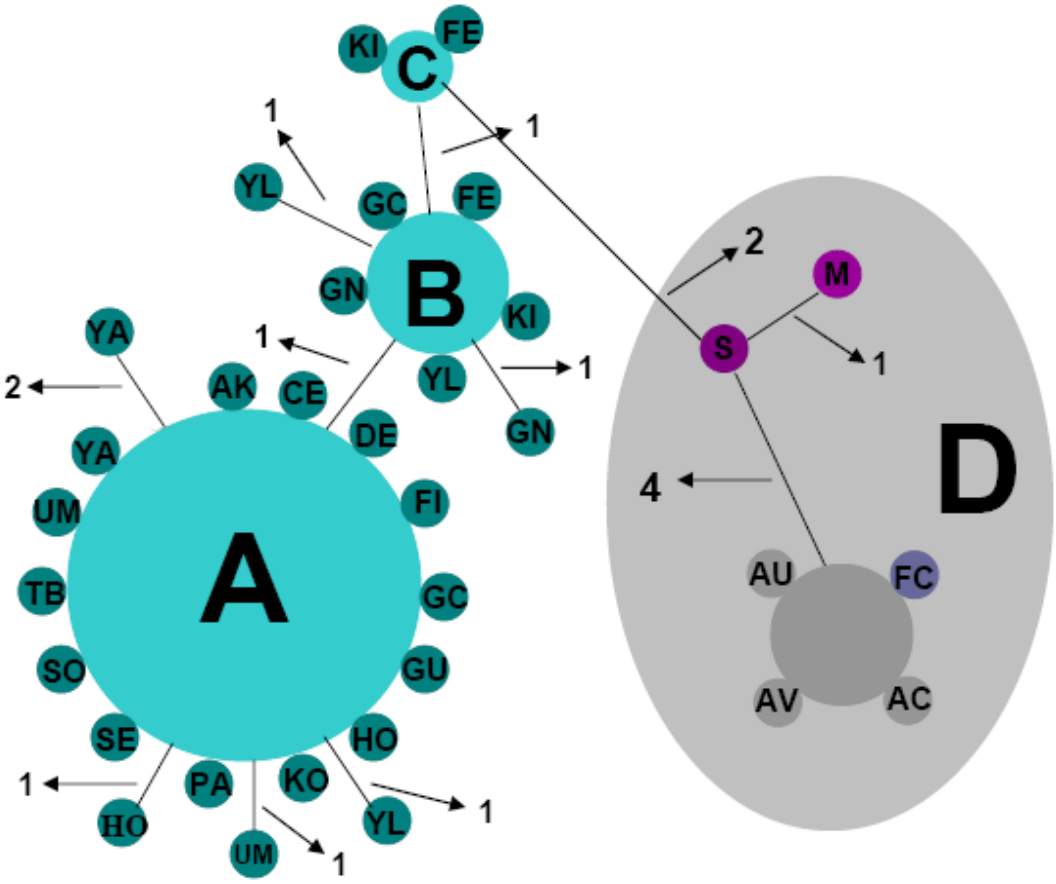

Figure 3. 5 Minimum spanning tree constructed by operational taxonomic units by Arlequin 2.000. The distances are indicated by arrows. The abbreviations are as follows;

AK: Acıpayam-Alcı, **CE**: Marmaris-Çetibeli, **DE**: Marmaris-Değirmenyanı, **FE**: Fethiye-Günlükbaşı, **FI**: Muğla-Kızılkaya, **GC**: Marmaris-Günnücek (Population6), **GN**: Marmaris-Günnücek (Population7), **GU**: Acıpayam-Bozdağ, **HO**: Marmaris-Hisarönü, **KI**: Muğla-Kıyra, **KO**: Köyceğiz-Köyceğiz (Population11), **PA**: Gölhisar-Pamucak, **SE**: Antalya-Serik, **SO**: Burdur Söğütdağ, **TB**: Köyceğiz-Köyceğiz (Population15), **UM**: Aydın-Umurlu, **YA**: Muğla-Yatağan, **YL**: Muğla-Yılanlı, **S**: American *L. styraciflua*, **M**: American *L. macrophylla*, **AU**: American *L. acalycina*, **AV**: Vietnamese *L. acalycina*, **AC**: Chinese *L. acalycina*, **FC**: Chinese *L. formosana*

CHAPTER 4

DISCUSSION

4.1. Molecular diversity in Turkish sweet gum populations

The highest number of polymorphic sites which is an indication of variation is found within Population 17 located in Muğla-Yatağan. Population 13 (Antalya-Serik) has the second highest number of polymorphic sites followed by Population 1 (Acıpayam-Alcı), Population 2 (Marmaris-Çetibeli) and Population 7 (Marmaris-Günnücek). The high number of polymorphic sites indicates that these populations have more variation within and relatively higher mutation rates. This could lead the populations to separate more and become new taxonomic entities and this situation increases the importance of the populations in a conservation point of view.

From the diversity indices it can be seen that the least variable and rate of mutations were observed in the Populations 4, 5, 6, 8, 9, 10, 11, 12, 14, 15 and 16.

4.2. Genetic distances and sequence diversity of Turkish sweet gum

When the genetic distances were computed for all the populations, population number 7, located in Marmaris-Günnücek seems to be the population harbouring the highest variation, followed by Population 1 (Acıpayam-Alcı) and 7 (Marmaris-Günnücek). In some of the populations, (4, 5, 6, 8, 9, 10, 11, 12, 14, 15, 16), individuals showed no variation, which could be attributed to the small size of the habitat. As the size of the population decreases, the variation in the intra-level decreases significantly (Deka *et al*., 2005).

Considering the molecular diversity pattern among populations of Turkish sweet gum, new *in-situ* conservation areas could be set aside. From these data, Population 7 which is located in Marmaris-Günnücek could be considered as one of the priority sites with the highest diversity values according to *trn* sequence data. This population is located in Datça-Bozburun Nature Conservation Area, but it could be more appropriate to consider this area as a genetic reserve area for the future establishments of Turkish sweet gum. Population 1 located in Acıpayam-Alcı and Population 17 located in Muğla-Yatağan are the second divergent populations that are not under any conservation measures and they could be good candidates to be included in the future conservation programs.

Based on genetic distances, the variety *orientalis* was the most diverse, and the variety *orientalis* and unknown were the most distant groups from each other. The average genetic diversity was computed within varieties, which is found to be 85.71 % of the total diversity and 14.29 % of the total diversity was attributed to the intervariety diversity. This means that the *trn* sequences within the same variety, among populations, were more divergent when compared to the divergence of the sequences between the varieties.

The average genetic distances were computed for populations that were grouped with respect to the geographic locations; the most divergent geographic groups were Group 1 (Denizli) and Group 4 (Muğla-Yatağan-Yılanlı). These two geographic groups also showed the highest variation when compared to each other. This means that those populations are the most distant groups from each other with the highest variation within the groups. When the average diversities were computed, it can be seen that 81.82% of the total diversity is within the geographic groups, and 18.18% of the total diversity is between the geographic groups.

The genetic distances in the species level showed that the closest neighbor species of Turkish sweet gum is *L. styraciflua*. The second closest neighbor is Mexican *L. macrophylla*., followed by other species, American *L. acalycina,* Vietnamese *L. formosana*, Chinese *L. acalycina* and *L. formosana*.

These results and previous studies (Ickert-Bond and Wen, 2005) show that *L. styraciflua* and *L. orientalis* are quite similar although their early divergence occured in Oligocene/Miocene. This similarity despite of the intercontinental disjunct could be due to morphologicai statis via convergence or low rates of morphological evolution (Ickert-Bond and Wen, 2005). The proposed migration route in the middle Miocene is another explanation for the species to be intercontinentailly disjunct, but morphologically and phylogeneticlly similar (Wen 1998, 1999, 2001). Most intercontinental disjunct taxa do not have sister species relationships in flowering plants unlike *L. orientalis* and *L. styraciflua* (Wen, 2001). However, in ferns, the disjuncts seem to have a closer relationship, and a number of fern disjuncts are conspecific (Yatabe *et al*. 1999). These results also indicate that there is no or low hybridisation between the populations of *L. orientalis* and the other species of *Liquidambar* located in Asia. It is also shown that Asian species which are *L. acalycina* and *L. formosana* are more close to American *L. styraciflua* in comparison with *L. orientalis*.

4.3. Partition of total molecular variation in Turkish sweet gum populations

According to the AMOVA analysis for all the individuals grouped according to their varieties, the percentage of variation among varieties was found to be 2.38, whereas when the AMOVA results for populations grouped according to their geographic locations is considered, the value is found to be 1.97. This difference is meaningful when the ease of physical and therefore genetical mixing is considered between geographically near populations. When the AMOVA results according to the varieties are considered, it is shown that the percentage of variation among populations within varieties is higher than the percentage of variation among varieties. However, the great percentage of total variation was found within populations (91.97%).

For the sequences of the nuclear DNA internal transcriped spacer (ITS), it was also shown that most of the variation is found within populations when compared to among populations for Hamamelidaceae (Shi *et al*., 1998). In the majority of the studies on *trn* region of the chloroplast DNA, it was also shown that the high percent of the variation is attributed to within population level for *trn* region of *Pinus* (Wang *et al*., 1999), of *Lepidium* (Mummenhoff *et al*., 2001) and for *Morus* (Weiguo *et al*., 2005).

4.4. Phylogenetic trees constructed by MEGA 3.1

From the constructed phylogenetic tree (Figure 3.2), it was shown that Turkish sweet gum populations formed three branches with bootstrap values of 65, 71 and 88 meaning that those topologies are just phylogenetically informative. In the main branch, having a bootstrap value of 65, 15 populations of Turkish sweet gum were grouped together. This proposes that there were no or very little variation between those Turkish sweet gum samples when *trn* region is used as a molecular evolutionary tool.

Populations 4 (Fethiye- Günlükbaşı) and 10 (Muğla-Kıyra) forms another branch with population number 7 (Marmaris-Günnücek) with a relatively high bootstrap value, 91. This shows that those populations have significantly separated from the other populations of Turkish sweet gum phylogenetically. Population 3 located in Marmaris-Değirmenyanı has the greatest branch length and although Population 3 is defined as *integriloba*, it could be another or a new variety.

The other informative branches include *L. styraciflua* from USA and *L. macrophylla* from Mexico being the closest neighbors of *L. orientalis*. The second major branch with a bootstrap value of 99, meaning that this topology is correct, includes all the remaining out-groups that are to be; *L. formosana* from Vietnam, *L. acalycina* from China and *L. formosana* from China. These results are also consistent with the results of genetic distances.

Our results are also consistent with the results of Hoey and Parks (1991) who studied the isozyme divergence between Eastern Asian, North American and Turkish species of *Liquidambar* by estimating the divergence times among species of *Liquidambar* using Nei's (1987) and Thorpe's (1982) methods. They showed that *Liquidambar styraciflua* diverged ca. 7mya (Nei's) or 13mya (Thorpe's) from the Turkish *L. orientalis*, and 10mya (Nei's) or 17mya (Thorpe's) from the Eastern Asian *L. formosana;* meaning that *L. formosana* is a further neighbor when compared to *L.orientalis*.

The close similarity of the *trn* regions of these two species, Asian *L. orientalis* and North American *L. styraciflua*, further corroborate the sister-group relationship that was already supported in previous phylogenetic analyses based on allozymes (Hoey and Parks, 1991, 1994) and DNA sequence data (Li *et al*., 1997a,b; Li and Donoghue, 1999; Shi *et al*., 1998; Shi *et al*., 2001, Ickert-Bond, Wen, 2005).

The phylogenetic tree constructed by Ickert-Bond and Wen (2005) is presented in Figure 4.1. As can be seen from this figure, the bootstrap value for the topology between *L. styraciflua* and *L. orientalis* is between 100–91% meaning that this topology is a true evolutionary branching. *L. formosana* and *L. acalycina* seem to appear in another branch showing the far-neighbourhood relationship.

In the phylogenetic tree represented in Figure 3.3, it can be seen that the four specialized populations 4 (Fethiye- Günlükbaşı), 7 (Marmaris-Günnücek), Population 3 (Marmaris-Değirmenyanı) and 10 (Muğla-Kıyra) are varieties of var. *integriloba*. These results show that the most genetically divergent samples are from var. *integriloba*. This means that, this variety could be considered as a different and true taxonomic entity. Considering the *in-situ* conservation programs, the results of this study could be taken into account to protect these specialized populations located in Fethiye-Günlükbaşı, Marmaris-Günnücek, Marmaris-Değirmenyanı and Muğla-Kıyra.

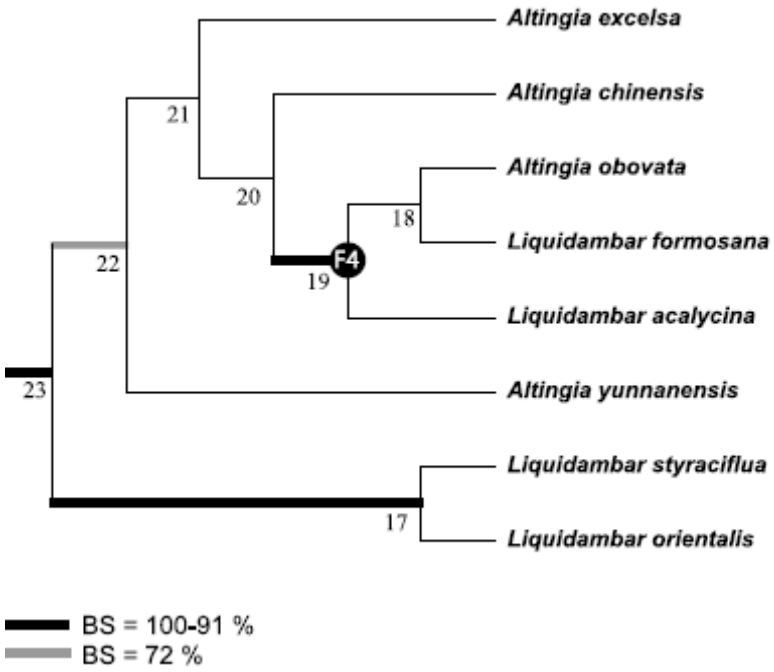

Figure 4. 1 Phylogenetic tree constructed by Ickert-Bond and Wen, 2005

4.5. Evolutionary relationship among sweet gum species: The Minimum Spanning Tree (MST)

The minimum spanning tree constructed by Arlequin 2.000 showed consistent results with the phylogenetic tree constructed by MEGA 3.1. In both of the trees, *L. styraciflua* from USA is the closest neighbor with *L. macrophylla* from Mexico, emerging from it. The second major group in the minimum spanning tree is consisted of all the other species from the *Liquidambar*.

It has been proposed that, the separation of Europe and North America before early Oligocene period led to the divergence and formation of *L. orientalis* and *L. styraciflua* (Wen, 2001). But there appears to be a land bridge in the early Oligocene between North America and Europe since the faunas of the two regions are very similar which explains the occurence of sister-species in the two continents (Wen, 2001). The close relationship between *L. orientalis* and *L. styraciflua,* also represented in our study, supports the presence of the land bridge which led to the genetic hybridization of the two species before the continents separated completely. As the two species were genetically mixed they did not turn out to be very distinct species both morphologically and geneticaly.

In addition to the specialized populations stated in the previous section (Population 3, 4, 7 and 10), Population 18 located in Muğla-Yılanlı is also a divergent population when compared to the other populations of Turkey. According to these results, Population 18 could also be included in to the probable conservation programs as this population is separated from the other populations of Turkish sweet gum.

CHAPTER 5

CONCLUSION

The goal of this study was to detect the phylogenetic relationships among *L. orientalis* varieties from Turkey, by comparing wide range sampled Turkish sweet gum populations according to their variety groups and geographic locations as well as among other neighbor-species within the genus *Liquidambar*.

Based on the results of molecular diversity analysis; the most divergent population was found to be sampled population number 7 of Turkish sweet gum that is located in Marmaris-Günnücek. The other populations with high diversity values were Population 1 (Acıpayam-Alcı), Population 3 (Marmaris-Değirmenyanı) and Population 17 (Muğla-Yatağan). Among the varieties, the most divergent variety was var. *orientalis* followed by the unknown variety group and then var. *integriloba.* However, the most specialized or variant populations or both were from the variety *integriloba*. These populations could be members of true taxonomic entity that is var. *integriloba*. However, the existing taxonomic variety groups should be revised based on systematic data obtained from this study.

The most differentiated populations, that show differences in *trn* sequences when compared to other populations are Population 3 (Marmaris-Değirmenyanı), Population 4 (Fethiye- Günlükbaşı), Population 7 (Marmaris-

Günnücek), Population 10 (Muğla-Kıyra) and Population 18 (Muğla-Yılanlı). All of these populations could be considered as potential candidates for *in-situ* gene conservation programs due to their high variation and differentiation properties.

When the geographic distributions are considered, the most variant populations are in geographic group 1 (located in Denizli) and geographic group 4 (located in Muğla-Yatağan-Yılanlı). The most distant groups are again Denizli and Muğla-Yatağan-Yılanlı. Thus, conservation programs could also take these diverse populations regardless of variety and geographic location into the consideration when new *in-situ* or *ex-situ* conservation programs for Turkish sweet gum is concerned.

The results of phylogenetic and minimum spanning trees were consisted with each other that Turkish sweet gum and *L. styraciflua* were the closest neighbors.

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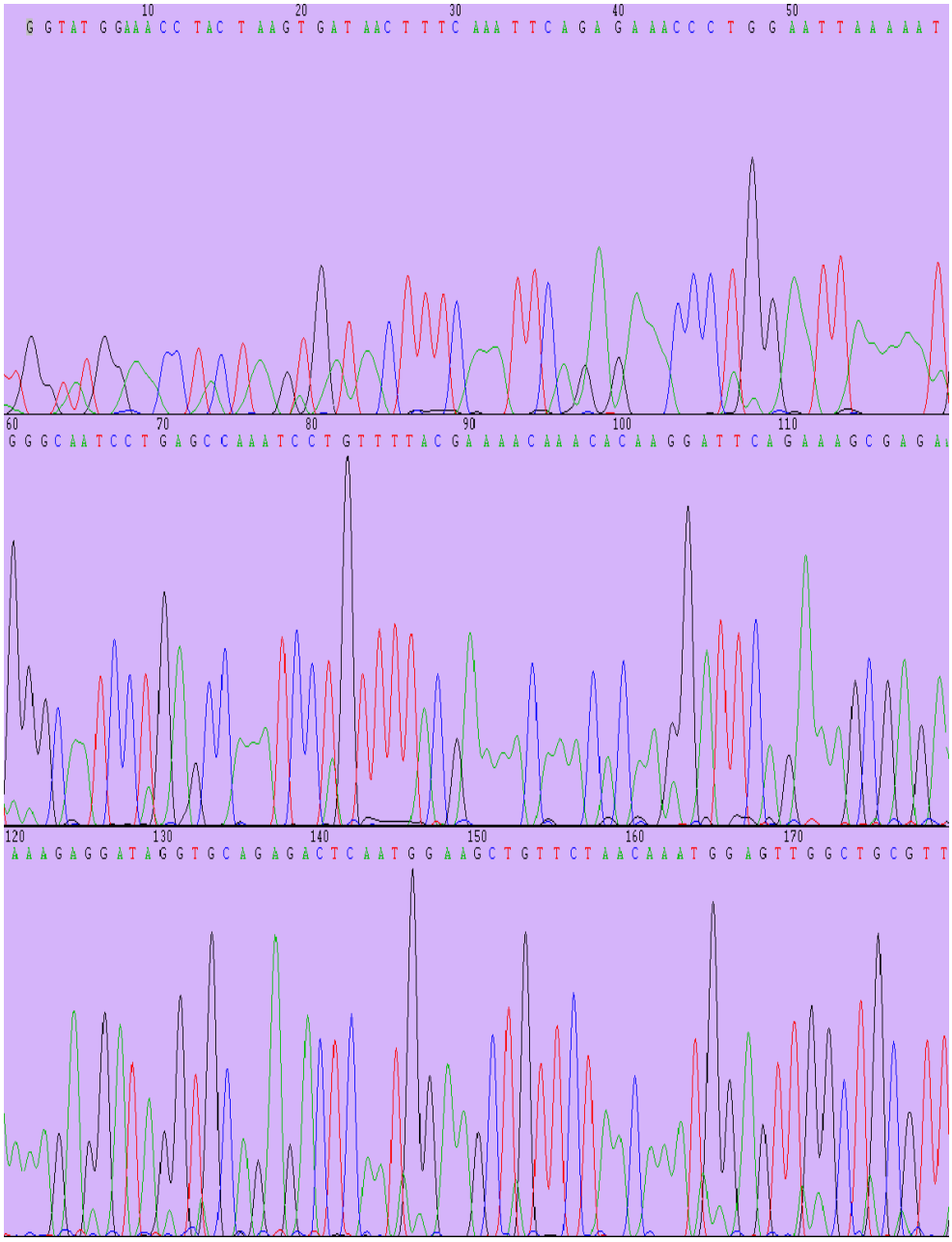
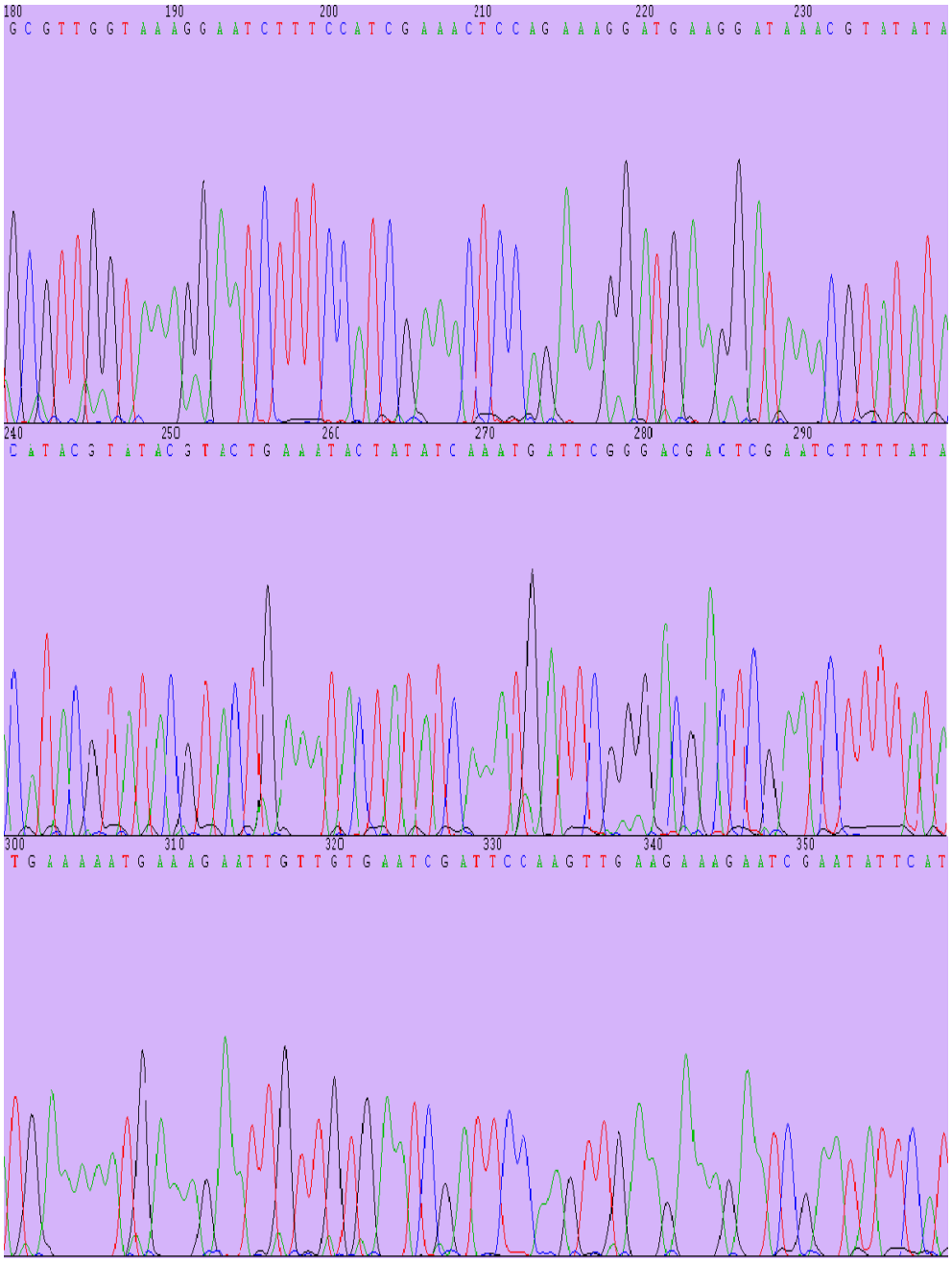
APPENDIX A

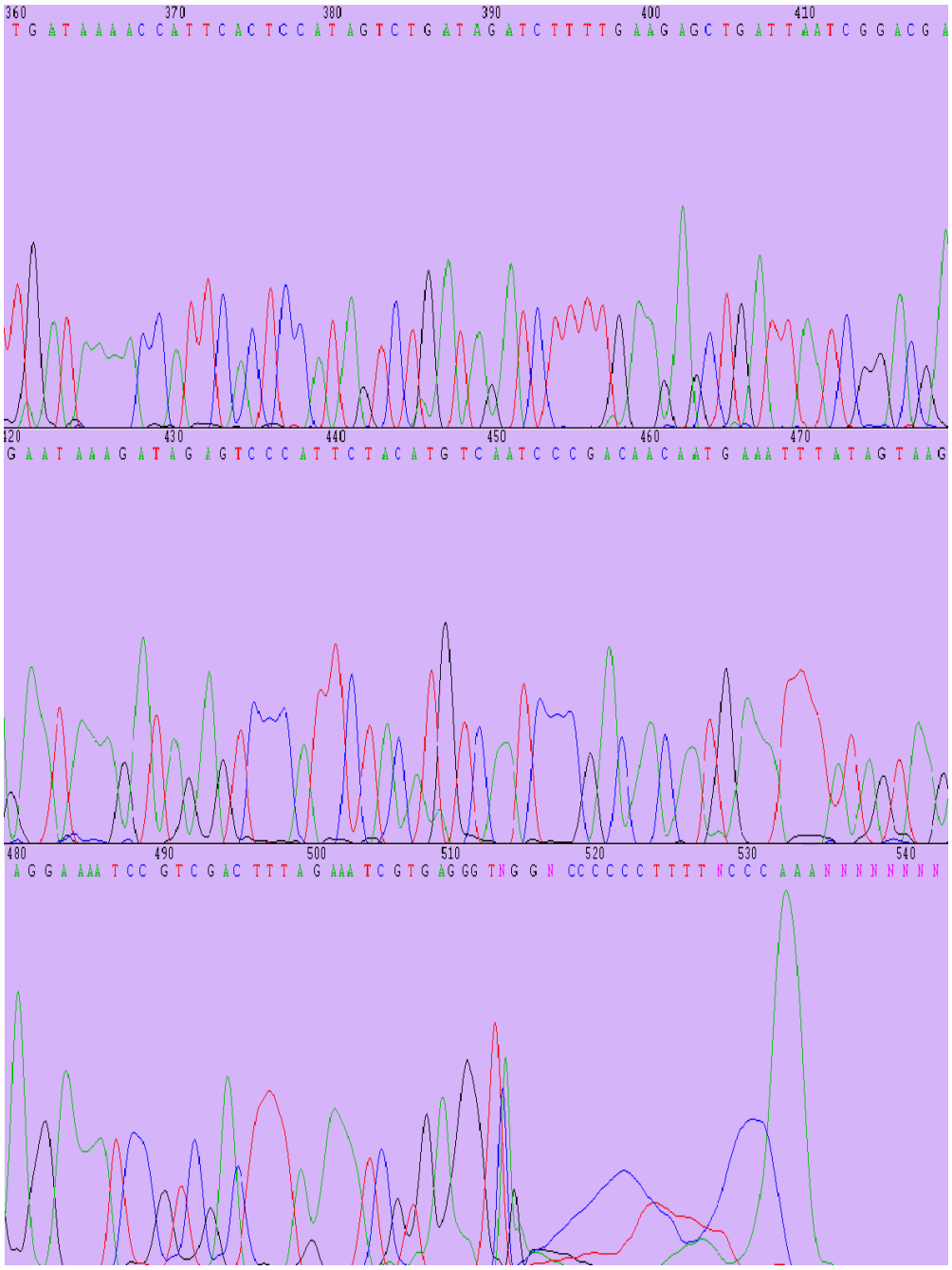
AN EXAMPLE OF CHROMOTOGRAM DATA



An example of chromotogram data continued



An example of chromotogram data continued



APPENDIX B

AN EXAMPLE OF MEGA SEQUENCE DATA

#POP01-ACI-ALC-03-AK03-integriloba

GGTATGGAAACCTACTAAGTGATAACTTTCAAATTCAGAGAAACCCTGG
AATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTACGAAAACAAACA
CAAGGATTCAGAAAGCGAGAAAAGAGGATAGGTGCAGAGACTCAATGG
AAGCTGTTCTAACAAATGGAGTTGGCTGCGTTGCGTTGGTAAAGGAATC
TTTCCATCGAAACTCCAGAAAGGATGAAGGATAAACGTATATACATACGT
ATACGTACTGAAATACTATATCAAATGATTCGGGACGACTCGAATCTTTT
ATATGAAAAATGAAAGAATTGTTGTGAATCGATTCCAAGTTGAAGAAAGA
ATCGAATATTCATTGATAAAACCATTCACTCCATAGTCTGATAGATCTTTT
GAAGAGCTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACAT
GTCAATCCCGACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGAC
TTTAGAAATCGTGAGGGT.....

APPENDIX C

AN EXAMPLE OF ARLEQUIN SEQUENCE DATA

(Profile)

Title="*trn*L-F intron"

NbSamples=26
GenotypicData=0
DataType=DNA
LocusSeparator=NONE
MissingData='N'

(Data)

((Samples))

SampleName="POP01-ACI-ALC-Integriloba"
SampleSize=1
SampleData= {
AK03 1 ----
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AATTAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTACGAAAACAAACA
CAAGGATTCAGAAAGCGAGAAAAGAGGATAGGTGCAGAGACTCAATGG
AAGCTGTTCTAACAAATGGAGTTGGCTGCGTTGCGTTGGTAAAGGAATC

TTTCCATCGAAACTCCAGAAAGGATGAAGGATAAACGTATATACATACGT
ATACGTACTGAAATACTATATCAAATGATTCGGGACGACTCGAATCTTTT
ATATGAAAAATGAAAGAATTGTTGTGAATCGATTCCAAGTTGAAGAAAGA
ATCGAATATTCATTGATAAAACCATTCACTCCATAGTCTGATAGATCTTTT
GAAGAGCTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACAT
GTCAATCCCGACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGAC
TTTAGAAATCGTGAGGGT.....

SampleName="POP02-MAR-CET-Integriloba"
SampleSize=1
SampleData= {
CE03 1 ----
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GTCAATCCCGACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGAC
TTTAGAAATCGTGAGGGT.....
}

SampleName="POP03-MAR-DEG-Integriloba"
SampleSize=1
SampleData= {
DE01 1 ----
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CAAGGATTCAGAAAGCGAGAAAAGAGGATAGGTGCAGAGACTCAATGG

AAGCTGTTCTAACAAATGGAGTTGGCTGCGTTGCGTTGGTAAAGGAATC
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TACGTACTGAAATACTATATCAAATGATTCGGGACGACTCGAATCTTTTA
TATGAAAAATGAAAGAATTGTTGTGAATCGATTCCAAGTTGAAGAAAGAA
TCGAATATTCATTGATAAAACCATTCACTCCATAGTCTGATAGATCTTTTG
AAGAGCTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTCTACATG
TCAATCCCGACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTT
TAGAAATCGTGAGGGT.....
}
----and the other populations are included as sampled above----

#Definition of the group structure:
((Structure))

StructureName="Turkey vs other"
NbGroups=7
IndividualLevel=0

#L.orientalis_Turkey_Integriloba
Group={
"POP01-ACI-ALC-Integriloba"
"POP02-MAR-CET-Integriloba"
"POP03-MAR-DEG-Integriloba"
"POP04-FET-GUL-Integriloba"
"POP05-MUG-KIZ-Integriloba"
"POP06-MAR-GUN-Integriloba"
"POP07-MAR-GUN-Integriloba"
"POP09-MAR-HIS-Integriloba"
"POP10-MUG-KIY-Integriloba"
"POP11-KOY-KOY-Integriloba"
"POP15-KOY-KOY-Integriloba"
}

#L.orientalis_Turkey_Orientalis
Group={
"POP16-AYD-UMU-Orientalis"
"POP17-MUG-YAT-Orientalis"
"POP18-MUG-YIL-Orientalis"
}
#L.orientalis_Turkey_Unknown
Group={
"POP08-ACI-BOZ-Unknown"
"POP12-GOL-PAM-Unknown"
"POP13-ANT-SER-Unknown"
"POP14-BUR-SOG-Unknown"
}
#*L. acalycina*
Group={
"*L. acalycina*_USA"
"*L. acalycina*_china"
}
#L.formosana
Group={
"L.formosana_vietnam"
"L.formosana_china"
}
#*L. macrophylla*
Group={
"*L. macrophylla*_mexico"
}
#L.orientalis
Group={
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