MOLECULAR PHYLOGENETIC POSITION OF TURKISH *ABIES* (PINACEAE) BASED ON NONCODING *trn* REGIONS OF CHLOROPLAST GENOME

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Approval of the thesis

MOLECULAR PHYLOGENETIC POSITION OF TURKISH *ABIES* (PINACEAE) BASED ON NONCODING *trn* REGIONS OF CHLOROPLAST GENOME

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ABSTRACT

MOLECULAR PHYLOGENETIC POSITION OF TURKISH *ABIES* (PINACEAE) BASED ON NONCODING *trn* REGIONS OF CHLOROPLAST GENOME

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September 2011, 81 pages

Abies is the second largest genus of family Pinaceae (after *Pinus*), consisting of about 51 species, all native to the Northern Hemisphere.

There are six native taxa belonging to this genus growing in pure and mixed stands in Turkey. *Abies cilicica* subsp. *isaurica*, *Abies nordmanniana* subsp. *bornmülleriana*, *Abies nordmanniana* subsp. *equi-trojani, Abies x olcayana* are endemic and considered as lower risk (LR) species.

To determine the genetic relationships in Turkish firs, 18 populations of different subspecies of *Abies* were collected from different regions of Turkey and non-coding *trn* regions of chloroplast DNA were sequenced to assess the genetic structure of the studied species. *trn*L, *trn*F *and trnV* region were examined. All the *trn* regions of *Abies* species in the world (aproximately 300 species that found in the IPNI (The International Plant Names Index) were investigated in the database of NCBI. The available *trn* sequences of 23 *Abies* species worldwide included into the analyses. All analyses to estimate molecular diversity parameters were carried out with the MEGA software.

The constructed phylogenetic tree with the *trn* sequences revealed that Turkish firs formed a monophyletic group with almost no sequence divergence.

Since sequence data for all three sectors of *trn* were not available from the NCBI data base, the phylogentic analysis with the sequence data of *trn*L regions were compartively analyzed in all firs. The results showed that Turkish- European species formed a single clade, which clearly differentiated them from the others, such as Japanese species, *A. veitchii*. Similarly, according to the sequence data of *trn*F, Turkish fir species were grouped together and distinctly separated from Asian-American Fir species.

The results suggest that all Turkish firs may have evolved from single ancestral fir species, most likely from *Abies nordmanniana.*

Key words: *Abies* spp., Turkey, cpDNA, *trn* regions, molecular phylogenetic.

ÖZ

KLOROPLAST GENOMUNDAKİ KODLANMAYAN *trn* BÖLGELERİNİN KARŞILAŞTIRILMASI YAPILARAK TÜRKİYE GÖKNARININ FİLOGENETİK ANALİZİ

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Pinaceae (*Pinus*' dan sonra) ailesinin 51 türden oluşan ikinci en büyük cinsi *Abies* olup kuzey yarım kürede yayılış göstermektedir.

Bu cinsin 6 doğal taksonu ülkemizde saf ve karışık meşçereler halinde bulunmaktadır. *Abies cilicica* subsp. *isaurica, Abies nordmanniana* subsp. *bornmülleriana, Abies nordmanniana* subsp. *equi-trojani, Abies x olcayana* endemik olup az tehdit altında (LR: lower risk) tür kategorisinde değerlendirilmektedir.

Türkiye' deki göknar türlerinin genetik ilişkisini belirlemek için, göknar türlerini temsilen 18 farklı göknar populasyonu Türkiye'nin farklı bölgelerinden toplandı ve kloroplast DNA' sının kodlanmayan *trn* bölgesi çalışıldı. Türlerin genetik yapısının değerlendirilmesi için *trn*L, *trn*F *ve trnV* bölgeleri incelendi. Dünyadaki tüm göknar türlerinin (Uluslararası Bitki Adları İndeksi' nde yer alan yaklaşık 300 tür) *trn* bölgeleri NCBI veritabanında (Ulusal Biyoteknoloji Bilgi Merkezi, 2010) araştırıldı. Türkiye göknarlarının filogenetik konumunu netleştirmek için 23 tane göknar türünün *trn* dizileri analiz edildi. Bu çalışmadaki bütün moleküler analizler MEGA bilgisayar yazılımı ile yapılmıştır.

Kloroplast DNA’sının *trn* bölgelerinin verileriyle elde edilen filogenetik ağaca göre; Türkiye göknarlarının monofiletik bir grup oluşturduğu gözlemlenmiştir.

*trn*L bölgesi ile elde edilen filogenetik ağaca göre, en önemli bulgulardan biri Türkiye-Avrupa türlerininin aynı grupta yer almasıdır. Aynı zamanda, tek Japon türü olan *A. veitchii* onlardan farklı grupta yer almaktadır. *trn*F bölgesi verileriyle elde edilen filogenetik ağaca göre ise; Türkiye göknar türleri aynı grupta yer alarak Asya-Amerikan göknar türlerinden ayrıldığı görülmüştür.

Sonuçlar Türkiyedeki göknar türlerinin tek bir ata göknar türünden (muhtemelen *Abies nordmanniana*) evrimleşmiş olabileceğini düşündürmektedir.

Anahtar kelimeler: *Abies* türleri, Türkiye, cpDNA, *trn*, moleküler filogenetik

to my precious family...

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Special thanks to my mother Mukadder Adaçay, my sister Sevgi Adaçay, my son Orhan Arslan Değirmenci, my lovely cousin Kübra Özdemir, my teachers Prof.Dr. Orhan Arslan and Dr. Çiğdem Alev Özel, my friend Mevlüde Alev Ateş and Değirmenci Family for their love, support and patience over the years. Last but not the least, special thanks go to my beloved husband Nuri Değirmenci for his endless understanding, patience and taking care of me during this hectic period. 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

NCBI National Center for Biotechnology Information

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 on genus *Abies*

Abies is the second largest genus of family Pinaceae after *Pinus*, consisting of about 51 species, all native to the Northern Hemisphere. They are naturally found in temperate and boreal regions of the northern hemisphere, chiefly in mountainous regions in North America, Central America, Europe, North Africa, Asia (Himalaya, South China, and Taiwan), (LIU, 1975, www.conifers.org, 2010) (Figure 1.1). The genus was revised by LIU (1971) and RUSHFORTH (1989), with a full review of previous classification schemes provided by Farjon and Rushforth (1989). According to the taxonomic account by LIU (1971), the genus comprises 39 species, 23 varieties and 8 natural hybrids between sympatric species growing in different parts of the world. A more recent classification (FARJON and RUSHFORTH, 1989) proposed the existence of as many as 49 species, 23 varieties and one natural hybrid in *Abies*. Because of the great variability of morphological traits, the taxonomy of the genus has been in a state of confusion for many years.

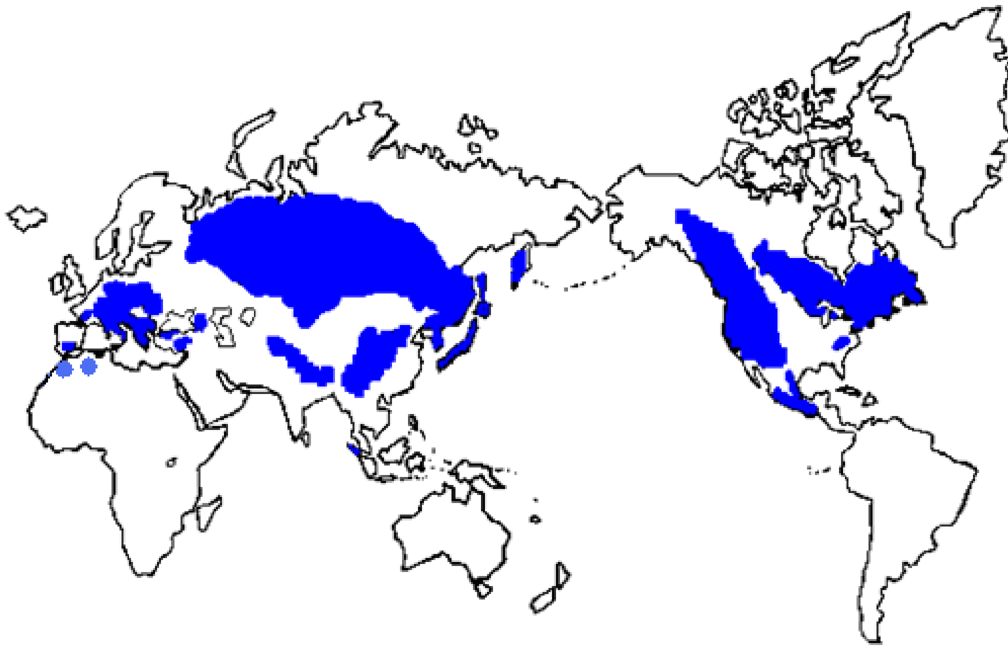

Figure 1.1 Map Showing the Natural Distribution of *Abies* genera (FARJON *et al.*, 1990)

Species of *Abies,* like most other genera of Pinaceae, are typically monoecious evergreen trees. They have a spire-like or conic crown, however, they become flattened or rounded in old trees.

All members of *Abies* species are generally 10–80 m tall and have 0.5–4 m trunk diameters when mature. Firs can be distinguished from other members of the pine family due to their needle-like leaves, attached to the twig by a base that resembles a small suction cup, and by erect, cylindrical cones 5–25 cm long (Figure 1.2).

Abies typically have a single straight trunk with regularly spaced branch whorls which are produced annually. Therefore, to determine the age of a tree it is possible by counting branch whorls. The branch pattern is also exceptionally regular, with a single terminal and two lateral shoots produced each year at the tip of most active branches. As a result, *Abies* displays a

form of geometric regularity. This form is less common in *Picea* and *Larix*, and rather unusual in *Pinus*.

For identification of the species, the followings are the main characteristics: size and arrangement of leaves, size and shape of the cones, and whether the bract scales of the cones are long and exserted, or short and hidden inside the cone (www.conifers.org, 2010).

Civilizations overused conifer forests since the ancient times in the Mediterranean Basin. Some species have continued to live in inaccessible areas, but the ranges of other species have been greatly reduced due to clearance for agriculture, livestock raising, illegal felling and, in some cases, fire. The firs are no exception and some now exist only as relict species (ESTEBAN *et.al.*, 2010).

The wood of most firs is often used as pulp or for the manufacture of plywood and rough timber, but it is not suitable for general timber use. It is generally suggested for construction purposes as indoor use only because its wood is not resistant to insect or rot after logging. Nordmann Fir *(Abies nordmanniana)*, Noble Fir *(Abies procera),* Fraser Fir *(Abies fraseri)* and Balsam Fir *(Abies balsamea)* have aromatic foliage that does not shed many needles on drying out; therefore it is generally considered to be the best for Christmas trees. Many are also very decorative garden trees, notably Korean Fir (*Abies koreana*) and Fraser Fir (*Abies fraseri*), which produce brightly coloured cones even when very young, still only 1–2 m tall. Moreover, the caterpillars of some *Lepidoptera* species feed on those species (www.conifers.org, 2010).

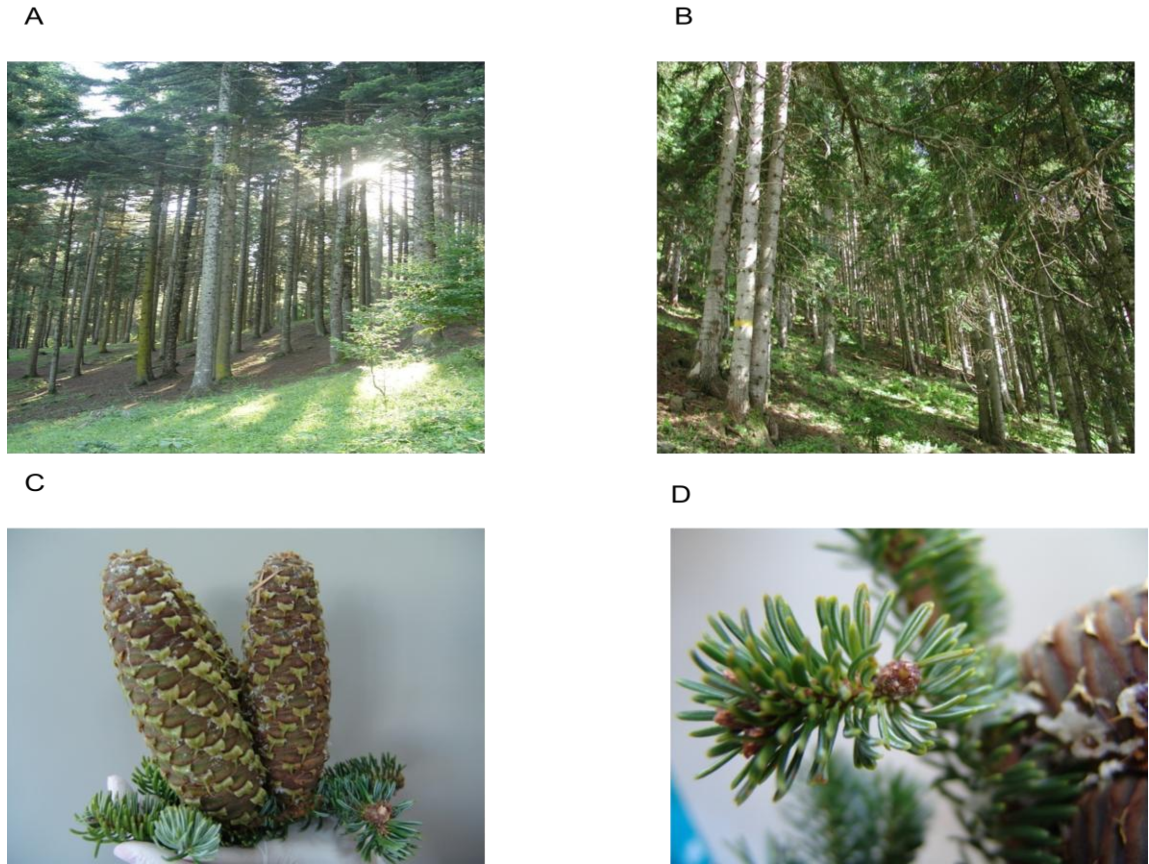

Figure 1.2 General appearance of *Abies* and some of its features A. General appearance *(A. nordmanniana* subsp. *bornmulleriana*, Bolu-Kökez*)*, B. Trunk of mature tree, C. One-year old female conelet, D. Male cone (*A. nordmanniana* subsp. nordmanniana*,* Şavşat-Artvin*)* (Photo: Forest Tree Seeds and Tree Breeding Research Directorate)

1.2. Literature review on *Abies spp.*

Fir (*Abies*) species are widely distributed forest tree species in Turkey where biodiversity and endemism ratio is very high. There are approximately 10 500 taxa in Turkey, 3 500 of which are endemic (KAYA and RAYNAL, 2001). There are six *Abies* taxa growing as pure and mixed stands in Turkey. These are *Abies cilicica* subs. *isaurica*, *Abies cilicica* subsp. *cilicica*, *Abies nordmanniana* subsp. *bornmuelleriana*, *Abies nordmanniana* subsp. *equi-trojani, Abies nordmanniana* subsp. *nordmanniana* and *Abies x olcayana.* Four of them, *Abies cilicica* subs. *isaurica*, *Abies nordmanniana* subsp.

bornmuelleriana, *Abies nordmanniana* subsp. *equi-trojani, Abies x olcayana* are endemic and considered as low risk (LR) species according to IUCN categories (EKIM *et al.*, 2000). Fir species cover 626 647 ha natural distribution area which consists of 3% of total forest existence of Turkey (386 203 ha high forest, 240 444 ha. degraded or unproductive stands) (ANONYMOUS, 2006). Allowable annual cut is approximately 532 913 cubic meters and 4712.3 hectares area is rejuvenated annually using *Abies* species (ANONYMOUS, 2006).

It is also used as Christmas tree. Therefore, fir species have ecological, silvicultural and economical importance in Turkey. Although they are important species, there is little genetic knowledge and related research. Taxonomic position of some species is not very clear; consequently, taxonomic classification of species is also under question.

Firs establishes pure or mixed forests in high mountainous areas in southern latitudes, even at sea level and lower altitudes in northern latitudes (KAYACIK, 1980; YALTIRIK and EFE, 2000). Firs are pyramidal in young ages, and a conical crowns in later ages. Buds are mostly resinous. Fir forests are quite resistant to wind and storms by means of their tap roots. They require moist soil and temperate climate. They are susceptible to spring frost since they grow on shade.

Firs show differences from each other according to their habitats, external morphology (forms of cones, leaves, buds, young shoots, hairy or glabrous young shoots and the body form etc.), internal morphology (cell number and maximum heights of tracheids) and pollen morphology. These characteristics vary in natural distribution areas (ATA, 1975). The two Turkish species, *A. cilicica* and *A. nordmanniana*, can only be differentiated by ripe cone's characteristics. For example, if bracts exserted from between the cone scales, it is classified as *A. nordmanniana* while if bracts are not so exserted, it could be categorized as *A. cilicica*. These characters are well correlated with geography that is *A. cilicica* occurs only in Southern Anatolia and *A. nordmanniana* only in north. Both species exhibit parallel variation in

indumentums and presence or absence of resinous buds. Thus, Uludağ (*A. nordmanniana* subsp. *bornmulleriana*) and Kazdağı Fir (subsp. *equi-trojani*) are accepted as a subspecies of Nordman Fir (DAVIS, 1965).

Uludağ Fir [*Abies nordmanniana* (Stev.) subsp. *bornmuelleriana* (Mattf.)] is one of the four endemic species in Turkey. It grows up to 30–40 m length. Distribution range of Uludağ Fir is in Black Sea Region, from west of Kızılırmak river to Uludağ mountain. Its elevation ranges from 1000 m to and 2000 m. Uludağ Fir forms pure and mixed stands with beech, and pine in Uludağ, Köroğlu, Bolu, Ilgaz and Küre Mountains (SAATÇİOĞLU, 1976). Bolu, Zonguldak and Kastamonu provinces are the optimum distribution area of this species (GENÇ, 2004). Its distribution area consists of two ecotypes: coastal type which is resistant to moisture (between Zonguldak-Ayancık) and inland type which is highly resistant to drought (between Kızılcahamam-Gerede) (SAATÇİOĞLU, 1976). Multiple factor such as climate change and elevation of the land does not prevent species distribution (SEVİM, 1962). Uludağ Fir has been intensively used in forest industry. The improved characteristics of heat-treated timber of Uludağ Fir offer several potential opportunities for timber industry (YALTIRIK and EFE, 2000).

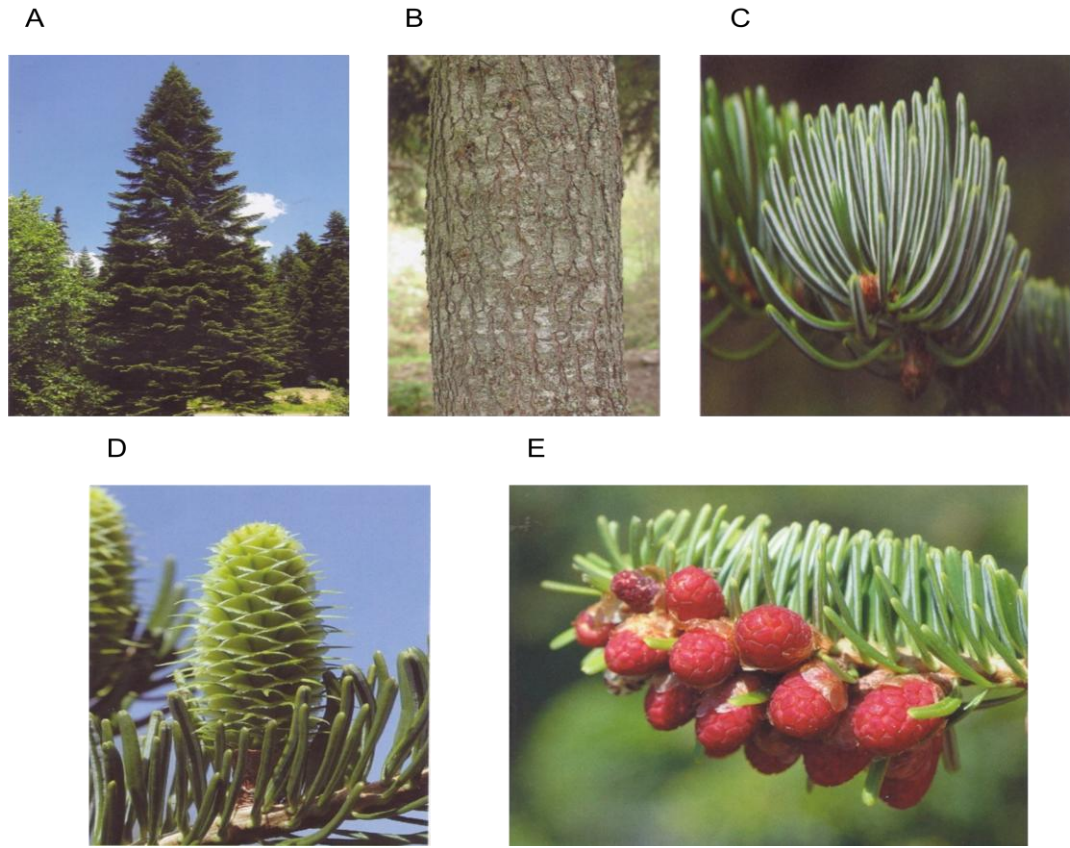

Figure 1.3 General appearance of *A.nordmanniana* subsp. *bornmuelleriana* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (MAMIKOĞLU, 2007).

Kazdağı Fir [*A. nordmanniana* subsp. *equi-trojani* (Asch. *Et* Sint.)] is distributed in a limited area, 3 600 ha (GÜLBABA *et al.*, 1998) with a number of small isolated populations ranging from 120 ha to 2 400 ha at elevations of 400 m – 1 650 m. This fir, a narrow endemic, is found only in the Kazdağı in western Turkey (SAATÇİOĞLU, 1969; VIDAKOVIC, 1991). Therefore, it has the most limited natural distribution among the Turkish Fir species. Kazdağı Fir forms mixed forests with *Quercus* species, Anatolian black pine (*Pinus nigra* Arnold subsp. *pallasiana* (Lamb) Holmboe) and Oriental beech (*Fagus orientalis* Lipsky) (ATA, 1975). In “National Plan for *in situ* Conservation of Plant Genetic Diversity In Turkey”, it is determined as a priority plant species to be conserved since Kazdağı Fir has great potential as a genetic resource for planting and breeding programs due to the fact that it has unique growth

form and ability to grow faster than other fir species in Turkey (KAYA *et al.*, 1997)

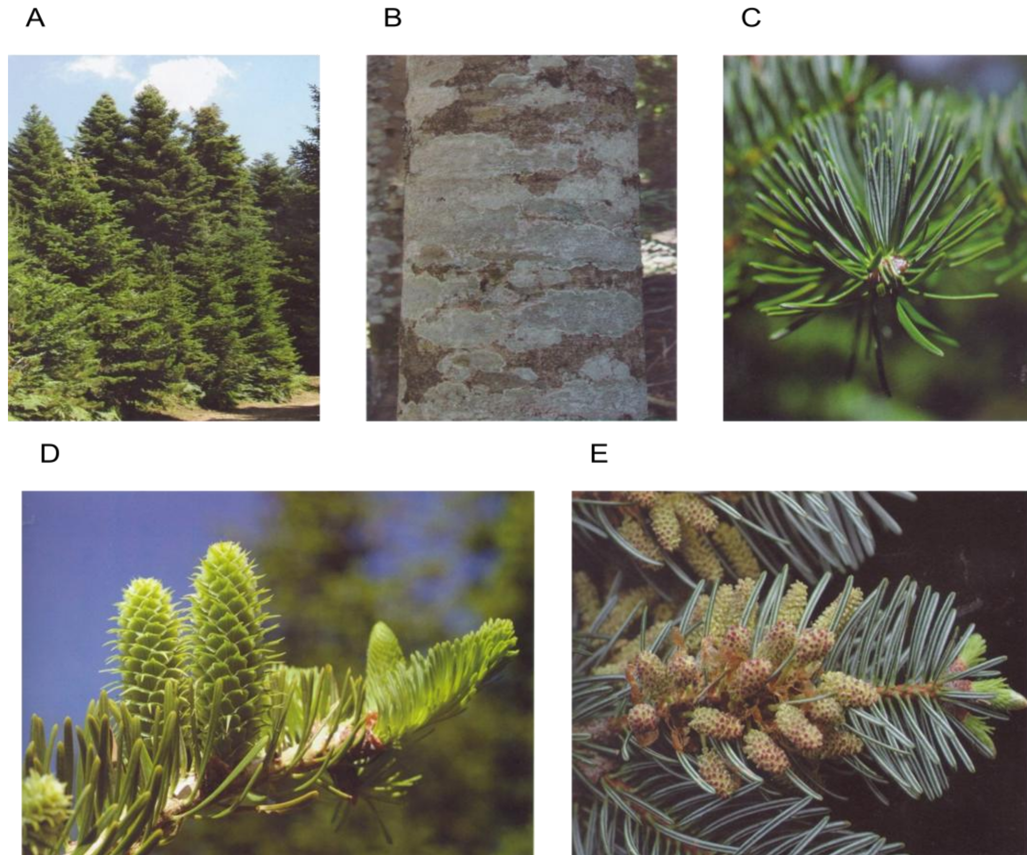

Figure 1.4 General appearance of *A.nordmanniana* subsp. *equi-trojani* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (MAMIKOĞLU, 2007).

The natural distribution area of Cilician Fir (*Abies cilicica* Carr.) is in Southern Anatolia (Middle and East Taurus Mountains, Amanos Mountains), Syria and Lebanon (SAATÇİOĞLU, 1976; KAYACIK, 1980; MAYER and AKSOY, 1988). Its altitudinal range is generally from 1 150m up to 2 000m in Taurus Mountains.There are two subspecies of *Abies cilicica* in Turkey; *Abies cilicica* subsp. *isaurica* Coode & Cullen and *A. cilicica* (Ant. & Kotschy) Carr. subsp. *cilicica*. The differences between those subspecies are the former has glabrous young shoots and has the resinous buds; and the latter has hairy

young shoots and has the no resinous buds. *A. cilicica* subsp. *isaurica* is an endemic species and grows in the area of West Taurus Mountain (Antalya-Konya) and subspecies *cilicica* grows in East Taurus Mountain (Mersin, Adana, Kahramanmaraş, Hatay) (BOZKUŞ, 1988). The buds of *A. cilicica* subsp. *cilicica* trees are not resinous except for its female cones. Cone is subsessile, cylindrical and somewhat tapered above, up to 15 cm or more (DAVIS, 1967). The resin has been traditionally used as an antiseptic, anti-inflammatory, antipyretic, antibacterial and antiviral medicine and as chewing gum against some stomach diseases (e.g., ulcer), lip-dryness, asthma and curing wounds in the form of ointment and plaster (BAYTOP, 1999).The fir forests in the Taurus mountain are both pure stands or mixed with *Cedrus libani* A.Rich., *Juniperus excelsa* M. Bieb., *J. foetidissima* Willd. and *Pinus nigra* Arnold subsp. *pallasiana* (DOĞAN *et al*., 2010).

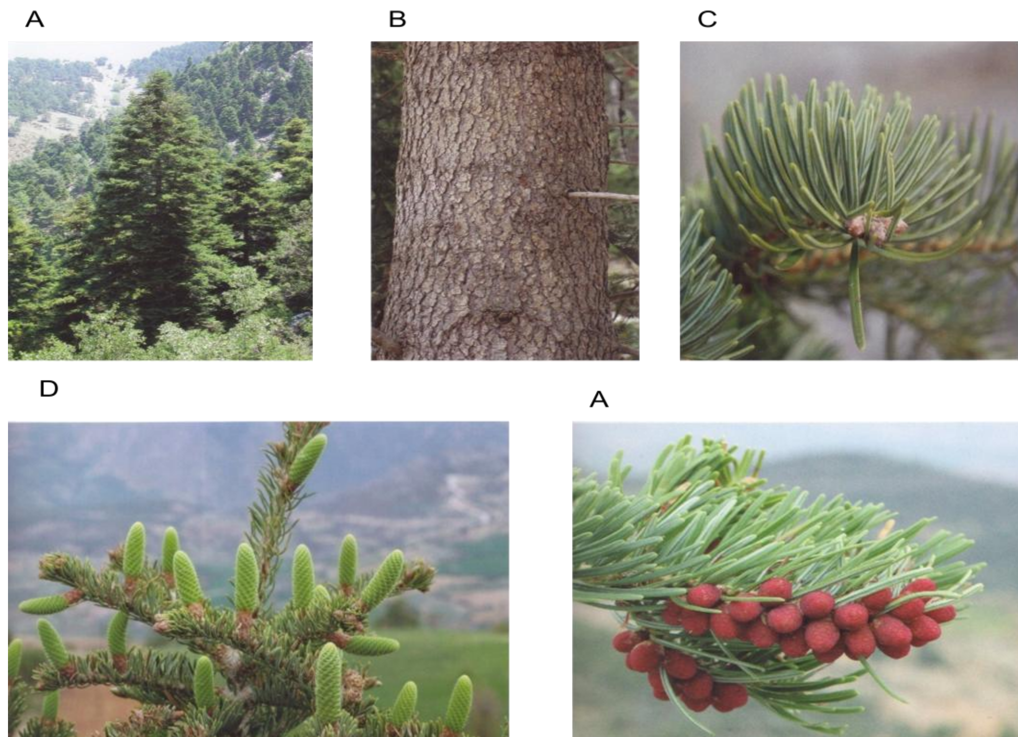

Figure 1.5 General appearance of *A. cilicica* supsp. *cilicica* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (MAMIKOĞLU, 2007).

Nordman Fir (*A. nordmanniana* subsp. *nordmanniana* (Stev.) Spach) is found the eastern Black Sea regions, including the Caucasus of Russia, Georgia and north-eastern parts of Turkey (LIU, 1971). They usually form mixed forests with Oriental beech (*Fagus orientalis* Lipsky), oriental spruce (*Picea orientalis*) and Scots pine (*Pinus sylvestris*) or pure forests (MATTFELD, 1928; KAYACIK, 1980). This situation reveals that Nordman Fir may have some degree of drought resistance (SARAÇOĞLU, 1988). Its normal altitudinal distribution ranges from 1000 m to 2000 m, meaning that it is adapted to mountainous environments. Species draws considerable commercial interest since it is widely used as a Christmas tree in North European countries (NIELSEN, 1993). Besides, the leaves of Nordmann Fir can provide inexpensive source as biosorbents for toxic metal removal from natural or wastewaters (SERENCAM *et al.,*2000).

Figure 1.6 General appearance of Fir *A.nordmanniana* supsp. *nordmanniana* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (MAMIKOĞLU, 2007).

In addition, ATA and MEREV (1987) reported a distinct endemic taxon that spread in 915 hectares in Çataldağ area, located between Susurluk (Balıkesir) and Mustafa Kemal Paşa (Bursa) provinces. Mixed and pure stands of Çataldağ Fir are seen between 800 m and 1300 m elevation. The area of mixed stands is larger than those of pure stands. External morphological features of Çataldağ Fir are very similar to *Abies nordmanniana* supsp.*bornmuelleriana* Mattfeld and *Abies nordmanniana* supsp.*equi-trojani,* but the features of pollen and wood anatomy are different. These differences show that this fir taxon, distributed on Çataldağ, is not *Abies equi-trojani,* but a new fir taxon in Turkey, and it is called as "*Abies x olcayana* Merev and Ata". They concluded that it is a natural hybrid developed during geological time, and grows faster than *Abies nordmanniana* Spach, *Abies bornmuelleriana* and *Abies cilicica* Carr.

The subspecies of *A. nordmanniana* show a replacement pattern of distribution. If *A. nordmanniana* subsp. *equi-trojani* is intermediate (and of hybrid origin) between *A. nordmanniana* subsp. *bornmuelleriana* and the *A. cephalonica* from Greece, then a whole replacement series from the Caucasus to Western Europe may be distinguished: *A. nordmanniana* (subsp. *nordmanniana,* -subsp. *bornmuelleriana,* -subsp. *equi-trojani*), *A. cephalonica*, *A. borisii-regis*, and *A. alba* (DAVIS, 1965).

Three subspecies of *A. nordmanniana* are discriminated. If leaves are somewhat acute, buds are not resinous and young shoots are glabrous, then it is subsp. *equi-trojani* while If leaves are truncate or emarginated, and shoots are hairy; buds are usually not resinous, it is classified as subsp. *nordmanniana,* and shoots are glabrous; buds are usually resinous, it could be categorized as subsp. *bornmuelleriana* (DAVIS, 1965).

Two subspecies of *A. cilicica* were identified if buds are not resinous, and young shoots are hairy, it is classified as subsp. *cilicica* while buds are resinous, and young shoots are glabrous, it could be categorized as subsp. *isaurica* (DAVIS 1965) (Table 1.1).

Table 1.1 The features of *Abies* taxa

A.nordmanniana* subsp. *equi-trojani	Buds are not resinous Young shoots are glabrous	Leaves are somewhat acute
A.nordmanniana* subsp. *nordmanniana	Shoots are hairy buds are usually not resinous	Leaves truncate or emarginated
A.nordmanniana* subsp. *bornmuelleriana	Shoots are glabrous buds are usually resinous	
A.cilicica* subsp. *cilicica	Buds are not resinous Young shoots are hairy	Leaves truncate or emarginated
A.cilicica* subsp. *isaurica	Buds are resinous Young shoots are glabrous	

As a result, it is very difficult to distinguish *Abies* taxa just by their morphological characteristics (AYTUĞ, 1959a; ATA, 1975; BOZKUŞ and ÇOBAN, 2006). For this reason, microscopic wood properties were investigated in detail by several authors (AYTUĞ, 1959a; ATA, 1975; ATA and MEREV, 1987). According to AYTUĞ (1959a), four fir species are very different from each other especially in terms of cell number and maximum heights in vascular rays.

1.2. Genetic Variation and Molecular Markers

Studies on the genetic structure of Turkish fir species are very limited in Turkey. The first fir studies started with MİRABOĞLU (1955 and1957) and AYTUĞ (1958). After that many researchers worked on silvicultural, morphological traits; pollen, seed and cone characteristics; and chemical features of Turkish fir species (AYTUĞ, 1959a;b; IRMAK, 1961; KAYACIK, 1964; TANK, 1964; ELİÇİN, 1967; BEŞKÖK, 1968; BOZKURT, 1971; ANONYMOUS, 1973; ATA, 1975; KANTARCI, 1978; ÇEHRELİ, 1979; ASLAN, 1982,1986; ASAN, 1984; BOZKUŞ, 1988; SARAÇOĞLU, 1988; ERKULOĞLU, 1993; TOSUN *et al.*, 1999; KESKİN and ŞAHİN, 2000;

VELİOĞLU and ARSLAN, 2001; KARAŞAHİN *et al.*, 2001; KARAŞAHİN *et al.*, 2002; KARAŞAHİN *et al.*, 2003; SERİN *et al.*, 2005).

Isozyme variation of six Nordman Fir, Uludağ Fir and Kazdağı Fir populations were studied by Şimşek (1992). Six enzyme systems were used in this study. He reported that Kazdağı Fir is not genetically variable and there is no relationship with Uludağ Fir. Moreover, *Abies alba* was genetically different from Nordman Fir, Uludağ Fir and Kazdağı Fir populations. The genetic structure of these three species was found to be different from each other. He reported that there was no difference between studied Eastern Black Sea populations. Finally, Kazdağı Fir is not identified as a kind of hybrid.

FADY and CONKLE (1993) investigated the genetic diversity of *A. cephalonica, A. borisii regis, A. bornmuelleriana* and *A.alba* by assaying a total of 22 loci using horizontal starch gel electrophoresis. They determined that *A.alba* appeared genetically closer to *A. bornmuelleriana.*

Isozyme variation of four *Abies equi-trojani* populations was investigated by GÜLBABA *et al.* (1998). Results revealed that studied populations have remarkable genetic diversity and expected heterozygosity ranges between 0.084 and 0.152. In another isozyme study; high genetic diversity and low genetic distance between populations were reported in four populations of the Taurus Fir in Bolkar Mountains (ÖZER, 2000). The study with the same populations used by GÜLBABA *et al* (1998) dealt with adaptive seedling traits and reported that within population variation is high. Furthermore, Çan population was genetically different from other populations (ÇİÇEK *et al.*, 2005).

Nineteen natural Mediterranean Fir populations, belonging to eight species *(A. alba, A. cephalonica, A. bornmuelleriana, A. nordmanniana, A. equi-trojani, A. pinsapo, A. numidica, A. cilicica)* and one natural hybrid (A. x *borisii-regis),* were investigated using starch and polyacrylamide gel electrophoresis by SCALTSOYIANNES *et al.* (1999). They observed

considerable variation in the heterozygosity among studied populations and ranged from 0.010 *(A. pinsapo)* to 0.328 *(A. cephalonica).* The interpopulation genetic diversity was about 26% of the total genetic diversity. Dendrogram revealed new phylogenetic relationships. According to their results, high genetic similarity was observed between the Calabrian Fir population and the one from north-west Greece as well as between *A. equi-trojani* grown in Turkey and the southern Greek populations. Species specific alleles were found in *A. cilicica.*

ISODA *et al.* (2000) determined DNA sequences of the chloroplast spacer region between the *trn*L and *trn*F genes in 18 species of *Abies* MILL. and another five species of Pinaceae. They reported that 18 *Abies* species were divided into nine groups by the tandem repeat type, which was defined by both the number of repeats and the unit type. According to Liu and Farjon (1975, 1989) two European species, *A. alba* and *A. nordmanniana*, are classified into sect. *Abies.* They concluded that these two species possessed same TRT (Tandem repeat type), differentiating them from the other 16 species.

SUYAMA *et al*. (2000) investigated the phylogenetic positions of Japanese *Abies* species (*A. firma, A. homolepis, A. veitchii, A. sachalinensis,* and *A. mariesii*) by chloroplast DNA. First, they analyzed *rbc*L sequences of 24 *Abies* species worldwide to clarify the phylogenetic position of the Japanese *Abies* within this genus. They reported that while 4 of the Japanese species formed a monophyletic group with almost no sequence divergence, *A. mariesii* was placed into a clearly different group with North American species. Second, to clarify the relationships among the species in the monophyletic group, they analyzed sequences of *rbc*L, *mat*K, and six spacer regions (total ca. 5 kb) for 12 species of the group, with *A. mariesii* as an out-group. They concluded that *A. veitchii* and *A. sachalinensis* gave identical sequences for all the sequenced regions; however, a unique sequence and some species-specific sequences were detected in *A. mariesii, A. firma* and *A. homolepis*. Their results clearly indicate that Japanese *Abies* species differ greatly from the other *Abies* species (including *A. nordmanniana).*

KORMUTAK *et al.* (2004) studied chloroplast DNA diversity in 15 Asian (*A. firma, A. holophylla, A. homolopis, A. mariesii, A. kawakamii, A. chensiensis, A.fargesii, A. squamata, A. devayi, A. devayi* var*. georgei, A. recurvata, A. koreana, A. nephrolepis, A. sachalinensis,* and *A. veitchii)*, 6 North American (*A. procera, A. magnifica, A. concolar, A. grandis, A. amabilis* and *A. lasiocarpa)* and 7 Mediterranean firs (*A. alba, A. cephalonica, A. nordmanniana;* from Turkey*, A. nebrodensis, A. cilicica, A. pinsapo* and *A. numidica*). Phylogenetic relationships between *Abies* species were determined with the survey of *cp-rbcl*, *cp-trn*, *cp-psb* primers. Difference between Mediterranean, Asia and North America firs is shown at molecular level. According to their results, the Mediterranean species, *A. alba* and *A. nordmanniana* were found to be the most differentiated from other species in their tandem repeat sequence.

ZIEGENHAGEN *et al*. (2005) investigated the relationships between Mediterranean *Abies* species using primers *nad5-4* intron region of mitochondrial DNA. They showed that Western Mediterranean species (*A. pinsapo* and *A. numidica*) were differentiated types of Eastern Mediterranean Firs. Moreover, *A. bornmulleriana* and *A. equitrojani* showed same haplotype with *A. nordmanniana* and *A. cephalonica.*

Fifteen populations of *Abies nordmanniana*, originating from all main parts of its distributional area in the Caucasian region, were genotyped for three chloroplast microsatellites as well as one mitochondrial marker by HANSEN *et al.* (2005). They concluded that the chloroplast microsatellites were highly variable, resulting in a total of 111 haplotypes in 361 analyzed individuals, while the mitochondrial marker showed no variation. Furthermore, analysis of molecular variance attributed 2.1% of the variation was among populations, and no correlation between geographic distribution and genetic distances among populations could be observed.

Eleven polymorphic nuclear microsatellite markers for *Abies alba* Mill. were developed from an enriched genomic library by CREMER *et al*. (2006). They indicated that these loci can be used in studies of genetic diversity for parentage analysis and for estimation of gene flow in Silver Fir populations. Moreover, successful amplifications were obtained for eight other Mediterranean *Abies* species (including *Abies bornmuelleriana* and *Abies equitrojani)*, suggesting that these loci may be useful for similar applications in other fir species.

KAYA *et al.* (2008) studied RAPD (randomly amplified polymorphic DNA) and *cp*SSR (simple sequence repeats) markers for in situ gene conservation programs for fir species. Moreover, they investigated genetic similarities between *Abies nordmanniana* Spach species complex (*A. nordmanniana, A. bornmuelleriana* Matff., *A. equi-trojani* (Asch. & Sint. ex Boiss) Mattf.) and *A. equi-trojani*. They concluded that depending on genetic similarity and distance values, studied Turkish Fir species, are genetically well differentiated. Fragmented distribution of 3 fir species in northern Turkey belonging to the Nordmanniana fir complex, also supports the possible existence of an ancestral species.

LIEPELT *et al*. (2010) studied the variation and geographic distribution of paternally inherited chloroplast DNA markers in nine Mediterranean *Abies* taxa. They used *A. bornmuelleriana* (Kastamonu), *A. equi-trojani* (Kazdağı Mountain) from Turkey. They applied markers with high and low mutation rates in order to differentiate among a scenario of secondary genetic contact and a scenario of complete isolation after speciation.They extrapolated that the central and widespread *A. alba* retained all ancient lineages, represented by markers with low mutation rates, whereas other species exhibited fewer, down to a single lineage. In contrast, modern lineages, depicted by markers with high mutation rates, were largely separated among species. According to their results, the western Mediterranean *A. pinsapo* and *A. numidica* were clearly separated from each other and from the remaining *Abies* species. This indicates the absence of secondary contact. They apply the same scenario to the eastern Mediterranean *Abies* species. Their results indicate

that an exception is the parapatric complex of *A. alba, A. cephalonica,* and their supposed hybrid *A. borisii regis*, which exhibited evidence of secondary contact. For the group *A. nordmanniana, A. bornmuelleriana,* and *A. equi-trojani,* which has traditionally been hard to classify, they can not conclude safely on either of the scenarios, although they think that it seems scenario 1 is more probable. Some species are reduced to very small relictual populations (*e.g. A. pinsapo, A. numidica, A. equi-trojani*), which might be critical for their long-term survival. Their results indicate there is still a chance to maintain genetic diversity, because most species still exhibit high levels of diversity.

Efforts to focus attention on the conservation of species or populations depend in part on our ability to recognize genetic and evolutionary distinctiveness. *In situ* and *ex situ* gene conservation efforts will be facilitated by techniques that estimate diversity and genetic discontinuity for closely related groups. Molecular genetic markers may better reveal genetic discontinuities and distinctiveness among or between taxa with slight morphological or reproductive differentiation.

Several studies have been done on ITS, *trn, mat*K, *SSRs, rpoC, and rbcL* to understand of the genetic characteristics and phylogenetic positions of *Abies* species until now. However, *trn* sequence divergence and its phylogenetic application in Turkish fir species have not been previously investigated.

1.4. Phylogenetic Classification and Chloroplast DNA

Molecular systematics encompasses a series of approaches in which phylogenetic relationship are inferred using information from macromolecules of the organisms under study. The development of DNA barcodes is gaining more and more in species identification (HEBERT *et al.*, 2003) and commonly starts with sequence analysis of candidate DNA region. Specifically, the types of molecular data acquired include that from DNA sequences, DNA restriction sites, allozymes, microsatellites, RAPDs, and

AFLPs. A revolution in inferring the phylogenetic relationships of life is occurring with the use of molecular data. The next step is review of the types of data, methods of acquisition, and methods of analysis of molecular systematics. DNA sequences data basically refers to the sequence of nucleotides in a particular region of the DNA of a given taxon. Comparisons of homologous regions of DNA among the taxa under study yield the characters and character state that are used to infer relationships in phylogenetic analyses.

The advantages of phylogenetic classification are immediately obvious because:

(1) it is not based on intuition about which category of information best reflected natural relationships;

(2) it ends competition between systems based on differing emphases;

(3) the analysis could be repeated by other researchers using either the same or different data (other genes or categories of information); and

(4) it could be updated as new data emerged, particularly from studies of how chromosomes are organized and how morphology and other traits are determined by the genes that code for them (GRAHAM and WILCOX, 2000).

DNA data became more widely available as the basis for establishing a classification. Thus, the use of data allows a more explicit methodology for turning the results of a phylogenetic analysis into a formal classification. This methodology, called cladistics, allowed a large number of botanists to share ideas of how the various taxonomic categories could be better defined. Although there remain a number of dissenting opinions about some minor matters of classification, it is now impossible for scientists to propose alternative ideas based solely on opinion.

For plants, the tree basic types of DNA sequence data system from the tree major sources of DNA: nuclear (nDNA), chloroplast (cpDNA), and mitochondrial (mtDNA) (SIMPSON, 2006). Because of having a complex and

highly repetitive characteristic, the nuclear genome is used in systematic botany less frequently. The mitochondrial genome is used at the species level since it has rapid changes in its structure, size, configuration, and gene order. cpDNA has three main features. First of all, it is a relatively abundant component of plant total DNA, so facilitating extraction and analysis. Secondly, it contains primarily single copy genes. Finally, cpDNA has a conservative rate of 2 nucleotide substitution; and extensive background for molecular information on the chloroplast genome is available. Therefore, molecular data generated from the cpDNA genes are the most useful data that conducted in phylogenetic reconstructions in plant systematics (LIANG, 1997).

1.5. Transfer Ribonucleic Acid region of the chloroplast DNA

Chloroplast DNA sequences are the primary source of data for inferring plant phylogenies (BALDWIN, 1992; BALDWIN *et al.*, 1995 ÁLVAREZ and WENDEL, 2003). Noncoding regions should be under less functional constraint than coding regions and should provide greater levels of variation for phylogenetic analyses. Therefore, they were explored for lower level taxonomic studies (GIELLY AND TABERLET, 1994). One of these coding regions; the t-RNA (*trn*L-*trn*F and *trn*V) region is the most widely discussed cpDNA fragment due to their extensive use in addressing phylogenetic relationships at the levels below family (TABERLET *et al.,*1991; KELCHNER, 2000).

The *trn*L-F region is composed of *trn*L (UAA) gene and an intergenic spacer which is *trn*L-F. The *trn*L gene, which consist of two highly conserved exons, split by a group I intron, an intergenic spacer that characterized by a highly conserved core structure encoding the active site. In plants, the *trnL* intron generally displays sequence conservation in the regions flanking both *trnL* exons. However, the central part is highly variable. The *trn*V gene consists of an exon that is split by a group III intron. The *trn*L-*trn*F and *trn*V regions

exhibit a quite high substitution rate in many plant taxa (BAYER and STARR, 1998; BAKKER *et al.,*2000; MANSION and STRUWE, 2004).

The region between the *trn*L and *trn*F and the region *trn*V are particularly suitable for evolutionary studies due to; 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, and iii. And the absence of gene rearrangements among many species (WOLFE *et al*., 1987).

In this study, the evolutionary relationships among fir species 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 (Figure1.7). The first region is between *trn*L5' and *trn*L3' amplified by *trn*c and *trn*d primers. The second one is between *trn*L3' and *trn*F that is amplified by *trn*e and *trn*f primers. The last region that lies between *trn*V5' and *trn*V3' is amplified by *trn*VF and *trn*VR primer set.

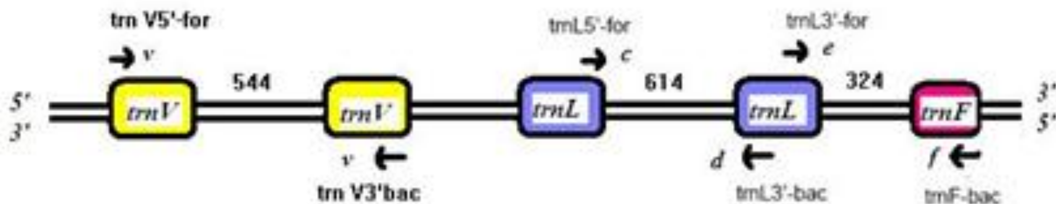

Figure 1.7 *trn* region of the cpDNA (TABERLET *et al.,* 1991; WAKASUGI *et al.*, 1994)

1.6. Significance of The Study

Taxonomic situation of *Abies* species distributed in Turkey is not clear yet. It is not apparent that *A. equi-trojani* and *A. bornmuelleriana* are separate

species or subspecies of *A. nordmanniana.* On the other hand, *A. equi-trojani* is considered by some authors as a hybrid between *A. nordmanniana* and *A. cephalonica* (LUI, 1971). Also, it is not clear that *A. cilica subsp. isaurica* is subsp. of *A. cilicica.* With such taxonomic confusion and wide morphological variation, there is hope that molecular data would give some idea for the relationships with in *Abies* genus. With the advance of molecular markers, it is now possible to use DNA markers to solve the problems of conservation genetics and plant taxonomy.

The most appropriate indicator for the ability of forests to adapt in new climatic conditions is the existence of high levels of biological diversity and especially genetic diversity. In the last decade, there are some studies dealing with the species' genetic diversity by means of isozymes variation (ŞİMŞEK, 1992; ÇİÇEK *et a*l., 2005); allozyme variation (SCALTSOYIANNES *et al.*, 1999); chloroplast variation (ISODA *et al.*, 2000; SUYAMA *et al.*, 2000; KORMUTAK *et al.,* 2004 and LIEPELT *et al.*, 2010); mitochondrial DNA variation (ZIEGENHAGEN *et al.*, 2005) and RAPD and *cp*SSR variation (KAYA *et al.,* 2008). In spite of limited studies on genetic diversity of Turkish Firs, there is no molecular systematics study with the species. In this way, there is a need to assess systematic states of Turkish Firs.

This study deals with the phylogeny of the *Abies* taxa distributed in Turkey. Moreover, it brings out phylogenetic relationships between Turkish firs and from other European, American and Asian fir species.

CHAPTER 2

OBJECTIVES OF THE STUDY

The general objective of the study is to determine the phylogenetic relationships of Turkish Fir Species, and their phylogenetic divergence from other fir species with use of molecular diversity in tRNA region of cpDNA.

The specific objectives of the study are:

- To obtain molecular diversity data from tRNA region of cpDNA that may help to solve taxonomic problems of Turkish Firs,
- To provide information on pattern of tRNA diversity with respect to Turkish firs as well as overall fir species,
- To determine the phylogenetic relationships among Turkish firs and their evolutionary divergence from other European, American and Asian fir species.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant material

In this study, there are three populations of *Abies nordmanniana* subsp. *nordmanniana*, five populations of *Abies nordmanniana* subsp. *bornmuelleriana* (endemic), three populations of *Abies nordmanniana* subsp. *equi-trojani* (endemic), three populations of *Abies cilicica* subsp. *isaurica* (endemic) and three populations of *Abies cilicica* subsp. *cilicica* and one population of *AbiesXolcayana.* Open pollinated seeds were obtained from 18 populations representing six *Abies* taxa distributed in Turkey. Cones were collected by Forest Tree Seeds and Tree Breeding Research Directorate, Ministry of Environment and Forestry in 2007-2009 years (Figure 3.1). Cones from 20-25 mother trees were sampled for each population (Figure 3.2). Detailed information on studied population, with respect to location, type of population, altitude and latitude of populations, are given in Table 3.1. Seeds were extracted from cones in open air and kept in cold storage (4°C) until use.

Figure 3.1 Fir cone (*A. nordmanniana* subsp. *bornmuelleriana*, Bolu-Kökez) (Photo: Forest Tree Seeds and Tree Breeding Research Directorate)

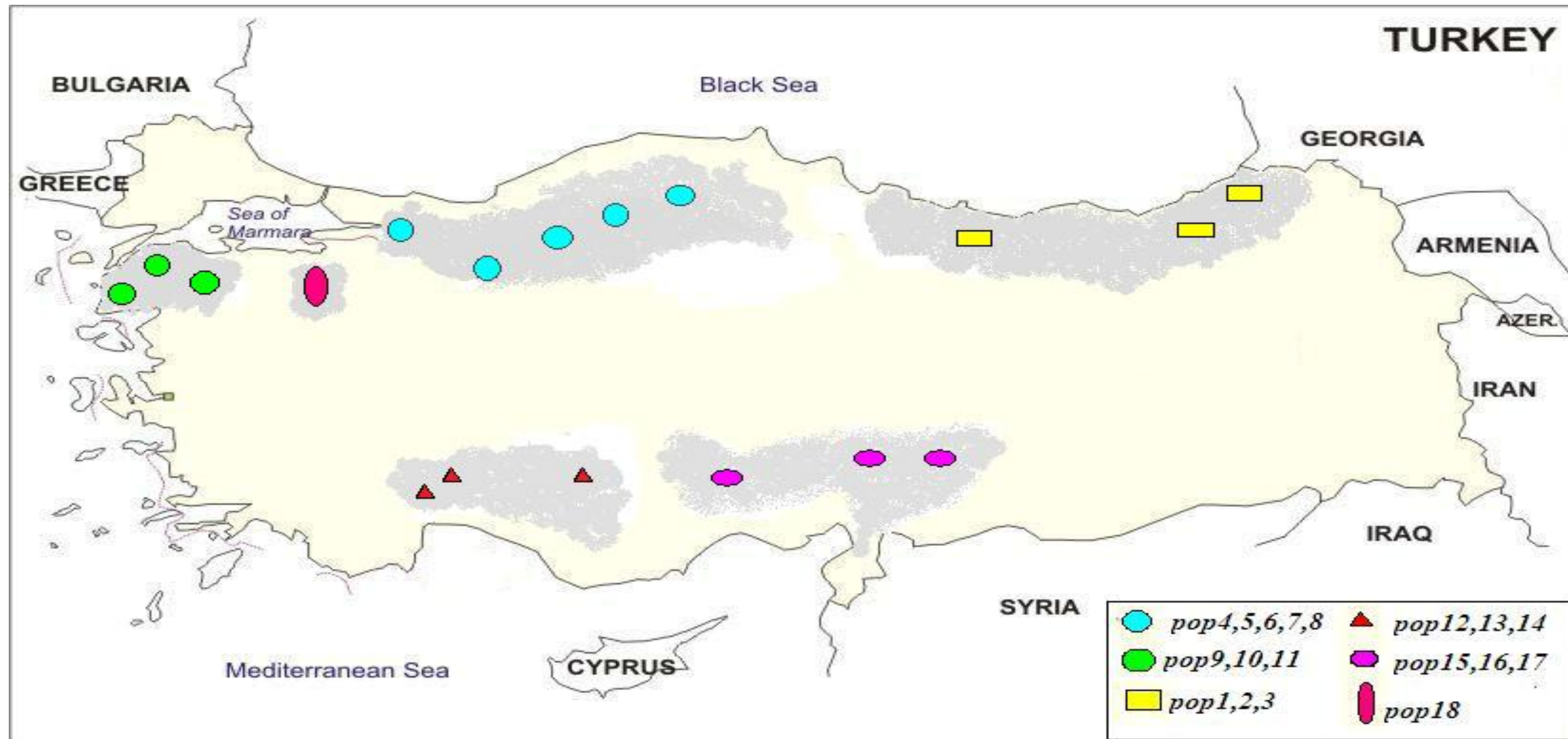

Figure 3.2 Map showing distribution and sampling sites for the 18 Turkish fir populations. Populations 1-3 are representing ANN. Population 4-8 are representing ANB. Populations 9-11 are representing ANE. Populations 12-14 are representing ACI. Populations 15-17 are representing ACC. Population 18 are representing AXO. See Table3.1 for the codes.

Tablo 3.1 Description of studied Turkish Firs seed sources/populations

The Codes	Taxon	Population	Type	Latitude (N)	Longitude (E)	Altitude (m)	Geographic exposure
ANN-1	*A. nordmanniana* subsp. *nordmanniana*	Artvin-Ortaköy	Seed Stand	41°16'37"	41°57'47"	1600	N
ANN-2	*A. nordmanniana* subsp. *nordmanniana*	Mesudiye-Arpaalan	Seed Stand	40°20'40"	37°51'40"	1850	N-NW
ANN-3	*A. nordmanniana* subsp. *nordmanniana*	Artvin-Yayla	Seed Stand	41°13'25"	42°27'20"	1800	N
ANB-1	*A. nordmanniana* subsp.*bornmuelleriana*	Uludağ Milli Parkı	Seed Stand	40°07'04"	29°07'35"	1600	N
ANB-2	*A. nordmanniana* subsp. *bornmülleriana*	Akyazı-Dokurcun	Seed Stand	40°37'30"	30°51'00"	1300	SW-SE
ANB-3	*A. nordmanniana* subsp.*bornmuelleriana*	Bolu-Kökez	Seed Stand	40°39'05"	31°36'56"	1300	N-W
ANB-4	*A. nordmanniana* subsp.*bornmuelleriana*	Karabük-Sarıçiçek	Seed Stand	41°20'30"	32°36'24"	1500	N-NE
ANB-5	*A. nordmanniana* subsp.*bornmuelleriana*	Bursa-Baraklı	Natural Stand	39°52'03"	28°21'10"	700	NW
ANE-1	*A. nordmanniana* subsp. *equi-trojani*	Edremit-Gürgendağı	Seed Stand	39°45'48"	26°57'50"	1300	S
ANE-2	*A. nordmanniana* subsp.*equi-trojani*	Kalkım-Eybekli	Gene Conservation Forest	39°42'35"	27°07'42"	950	W
ANE-3	*A. nordmanniana* subsp.*equi-trojani*	Çan-Çan	Gene Conservation Forest	39°56'00"	27°05'33"	750	E
ACI-1	*A. cilicica* subsp. *isaurica*	Akseki-Akseki(Kuyucak)	Seed Stand	37°06'51"	31°46'52"	1350	NE
ACI-2	*A. cilicica* subsp. *isaurica*	Bucak-Uğurlu	Seed Stand	37°19'52"	30°37'41"	1200	N-W
ACI-3	*A. cilicica* subsp. *isaurica*	Bucak-Y.Bademli	Natural Stand	37°19'52"	30°37'41"	1200	N-W
ACC-1	*A. cilicica* subsp. *cilicica*	Saimbeyli-Tufanbeyli	Natural Stand	37°19'52"	30°37'41"	1200	N-W
ACC-2	*A. cilicica* subsp. *cilicica*	Göksun-Göksun	Natural Stand	37°06'51"	31°46'52"	1350	NE
ACC-3	*A. cilicica* subsp. *cilicica*	Tarsus-Cehennemdere	Natural Stand	37°06'51"	31°46'52"	1350	NE
AXO-1	*Abies x olcayana*	MKP-Paşa	Gene Conservation Forest	39°52'03"	28°21'10"	700	NW

3.2. DNA Extraction

Sample size for each population (number of trees that embryo tissues obtained) was approximately 20-25 trees which were set for DNA marker studies. From this larger sampling scheme, five parent trees with highest DNA yield for each population of species were chosen randomly for this study.

Figure 3.3 Turkish *Abies* Seeds (Photo: Forest Tree Seeds and Tree Breeding Research Directorate)

CTAB (Cetyl Trimethyl Ammonium Bromide) DNA isolation method (DOYLE and DOYLE, 1990) was used for DNA isolations from embryo tissues. Steps *of* DNA isolation are shown in Figure 3.4. Seeds were soaked in distilled

water at 4°C for 48 hours (Figure 3.4.A) before excising and removing seed coat and endosperm (Figure 3.4.B). Then, the megagametophyte of each seed was carefully dissected away and preserved separately at -20°C for potential subsequent analysis (Figure 3.4.C, D). After that, embryos were homogenized in 200 µl CTAB (0.1 M Tris HCl pH: 8.0, 0.1 M EDTA, 0.25 M NaCl) in 1.5 ml eppendorf tubes (Figure 3.4.E). After homogenization, they were subjected to 600 µl of preheated CTAB and vortexed. Homogenized tissues were incubated for about 1 hour at 65°C in water bath (Figure 3.4.F). As the duration of incubation gets longer, isolated DNA becomes purer since it helps solubilization of lipids and protein dissociation from DNA. The tubes were centrifuged at 14 000 rpm for 20 minutes and then (Figure 3.4.G), supernatant was transfered to clean microfuge tubes (Figure 3.4.H) and mixed with 500 µl chloroform–octanol (24:1) solution to denature proteins and inactivate DNAse (Figure 3.4.I, J). By the centrifugation at 14 000 rpm for 15 min, resulting solution consists of two phases; one aqueous phase which contains the DNA and a lower chloroform phase that contains some damaged proteins, lipids, and many secondary compounds. The aqueous phase, which is clear and colorless, is taken to a new centrifuge tube and 600 µl cold isopropanol solution was added to precipitate the DNA.

The tubes were placed at -80°C for at least 60 min. After then, centrifugation at 14 000 rpm for 20 min was performed, the supernatant was discarded and the pellet was washed twice with 70% ice-cold ethanol to remove chemical residues. The pellets were left to dry at room temperature and re-suspended in 50 µl TE buffer to solve DNA.The DNA samples were stored at -20°C. DNA presence was detected by 2% agarose gel electrophoresis (Figure 3.5).

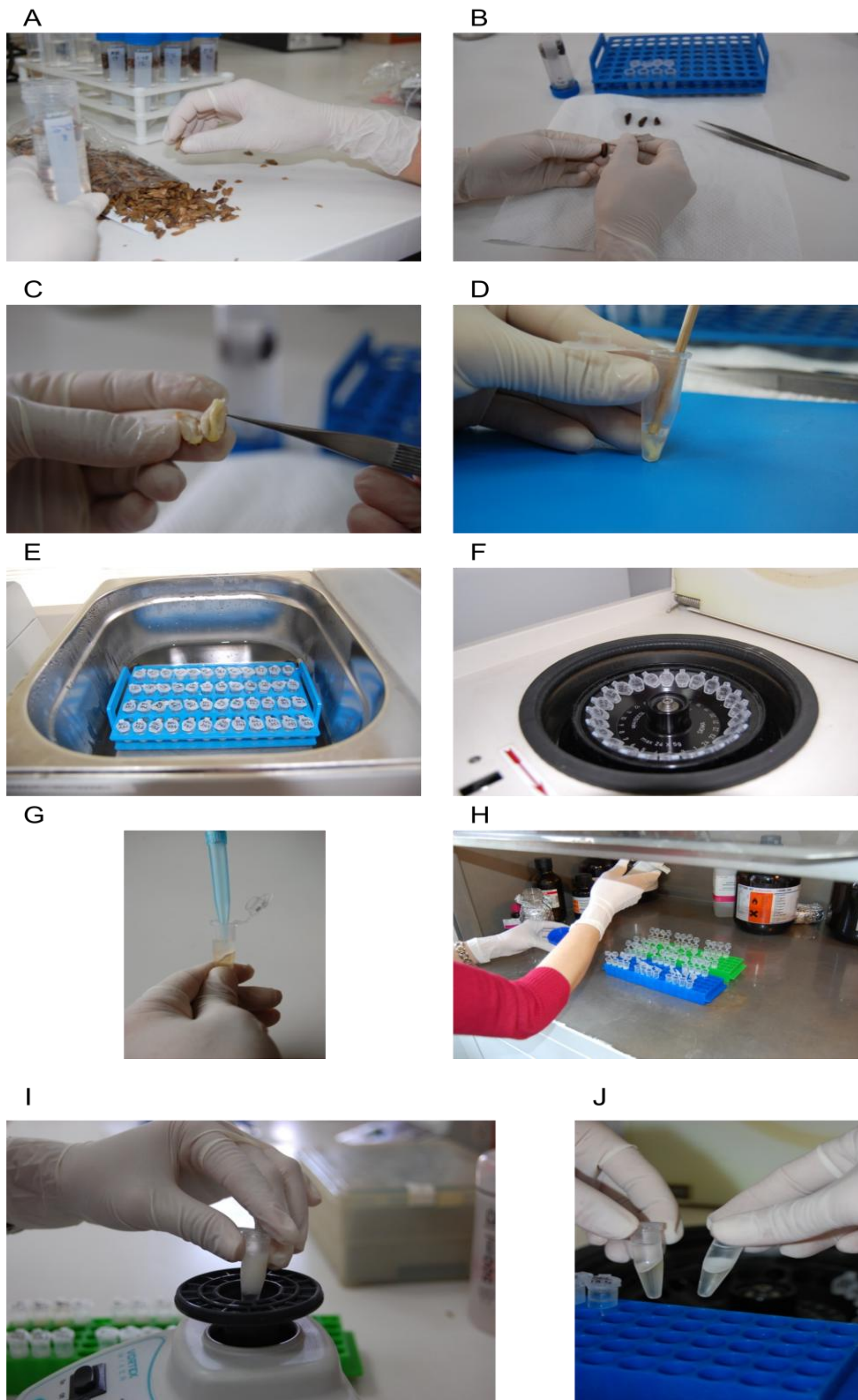

Figure 3.4The Steps of DNA isolation (Photo: Forest Tree Seeds and Tree Breeding Research Directorate)

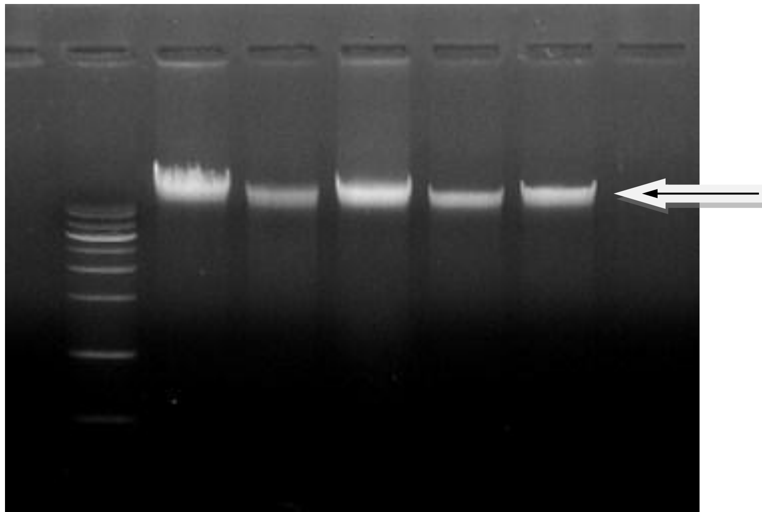

Figure 3.5 The photo showing 2% agarose gel electrophoresis of genomic DNA (indicated with arrow) with a 100 bp ladder

3.3. DNA quantification

DNA quantification of five individuals from each population was performed with GeneQuant pro RNA/DNA Calculator Spectrophotometer. Firstly, 2 ml of distilled water was put in the cuvette, and its outer surface was dried with tissue paper. After drying, cuvette was placed into the instrument and it was calibrated as blank. 10 microliters of DNA sample was diluted to 2 ml with distilled water and then the DNA measurements were performed (Table 3.2). After quantification, the samples with the highest DNA concentraion were selected.

Tablo 3.2 Mean DNA concentrations obtained for six Turkish fir taxa (total of 18 Turkish *Abies* populations)

				DNA Concentration ng/µL	
ANN		Sample Size	Mean±SD*	Minimum	Maximum
ANN – 1		11	1618±154	634	2480
ANN – 2		25	1269±43	1412	2525
ANN – 3		14	1098±54	326	1882
(n=3)	Species Mean	50	1328±216	1098	1618
ANB					
ANB – 1		20	1167±128	362	3511
ANB – 2		21	953±58	199	1756
ANB – 3		20	783±31	217	1683
ANB – 4		21	1815±112	470	3855
ANB – 5		25	871±60	217	1919
(n=5)	Species Mean	107	1118±371	871	1815
ANE					
ANE – 1		20	1675±80	271	3041
ANE – 2		25	1225±30	489	2407
ANE – 3		21	1249±124	253	4272
(n=3)	Species Mean	66	1383±207	1225	1675
ACI					
ACI – 1		25	2665±205	290	4652
ACI – 2		25	1811±116	597	3801
ACI – 3		25	3125±86	1213	5032
(n=3)	Species Mean	75	2534±544	1811	3125
ACC					
ACC- 1		25	2625±82	561	4054
ACC – 2		25	1849±119	181	4941
ACC – 3		25	2390±50	760	4181
(n=3)	Species Mean	75	2288±325	1849	2625
AXO					
AXO – 1					
(n=3)	Species Mean	25	1426±34	652	3095

*SD=Standard deviation

3.4. Primers used to amplify the t-RNA region of the chloroplast DNA

Three sets of primers (*trn*c and *trn*d, *trn*e and *trn*f, *trn*VF and *trn*VR) were used for PCR amplification of tRNA region of cpDNA. The primer sequences are as follows:

For *trn*L5'-*trn*L3' region [tRNA- Leucyl (*trn*L) gene], the primer pair was:
*trn*c (Forward): 5' CGA AAT CGG TAG ACG CTA CG 3'
*trn*d (Reverse): 5' GGG GAT AGA GGA CTT GA AC 3'

For *trn*L3'-*trn*F region [tRNA- Phenylalanyl (*trn*F) gene], the primer pair was:
*trn*e (Forward): 5' GGT TCA AGT CCC TCT ATC CC 3'
*trn*f (Reverse): 5' ATT TGA ACT GGT GAC ACG AG 3'

For *trn*V5'-*trn*V3' region [tRNA- Valin (*trn*V) gene], the primer pair was:
*trn*V5' (Forward): 5' GTA GAG CAC CTC GTT TAC AC 3'
*trn*V3' (Reverse): 5' CTC GAA CCG TAG ACC TTC TC 3'

3.5. Optimization of PCR (Polymerase Chain Reaction) conditions for Turkish Fir

For the optimization of PCR conditions, different concentrations of $MgCl_2$, dNTP (deoxyribonucleotide triphosphate) mixture, primers, and template DNA were tested for 25 µl of reaction volume. The combinations of these parameters in different concentrations are provided in Table 3.3.

Tablo 3.3 Tested reaction mixtures for optimization of PCR conditions

The reaction mixture number	dH2O (µl)	10X Buffer	MgCl2 (25mM)	dNTP (mM)	Primer Pairs (100 µM)	*Taq* DNA polymearse (5u/ µl)	DNA (3ng/ µl)	PCR Amplification
1	**16.75µl**	**2.5µl**	**2.5µl**	**0.5µl**	**1.0µl + 1.0µl**	**0.25µl**	**1µl**	**Excellent**
2	16.75µl	2.5µl	3.0µl	1.0µl	0.25µl + 0.25µl	0.25µl	1µl	Good
3	17.25µl	2.5µl	3.0µl	0.5µl	0.25µl + 0.25µl	0.25µl	1µl	Good
4	16.25µl	2.5µl	3.0µl	1.0µl	0.50µl +0.50µl	0.25µl	1µl	Good
5	16.75µl	2.5µl	3.0µl	0.5µl	0.50µl +0.50µl	0.25µl	1µl	Good
6	**17.25µl**	**2.5µl**	**2.5µl**	**1.0µl**	**0.25µl +0.25µl**	**0.25µl**	**1µl**	**Excellent**
7	17.75µl	2.5µl	2.5µl	0.5µl	0.25µl +0.25µl	0.25µl	1µl	Good
8	16.75µl	2.5µl	2.5µl	1.0µl	0.50µl +0.50µl	0.25µl	1µl	Good
9	**16.25µl**	**2.5µl**	**2.5µl**	**0.5µl**	**1.0µl + 1.0µl**	**0.25µl**	**1µl**	**Excellent**
10	15.25µl	2.5µl	2.5µl	0.5µl	1.5µl + 1.5µl	0.25µl	1µl	Fair
11	14.75µl	2.5µl	2.5µl	0.5µl	1.75µl + 1.75µl	0.25µl	1µl	Poor
12	14.25µl	2.5µl	2.5µl	0.5µl	2.0µl + 2.0µl	0.25µl	1µl	Poor

Since 50µL mixture was needed for the sequence analysis, reaction mixture was modified to 50µL for *trn*V5'-*trn*V3' regions. Among the total PCR reaction mixture, 5 µL of 10X Buffer ($MgCl_2$ free), 5µL of 25mM $MgCl_2$, 1.0µL of 10mM dNTPs (Fermentas, Ontorio, Canada), 2µL of ~3ng/µL of DNA, 2.0µL of 100µM each of the primers and 0.5µL of 5u/el Taq polymerase (Fermentas, Ontorio, Canada); and 33.5µL of dH_20 (Sterile water) were used for the 50µL of PCR mixture for sequence analysis of *trn*V5'-*trn*V3' regions.

The reaction mixtures of 2 to 8 were tested for *trn*c and *trn*d primer pairs and the sixth reaction mixture was found to be the best and was modified to 50μL for the sequence analysis of these primers. For these primers, PCR conditions were optimized as; 2 μL template DNA, 5 μL of 10X Buffer ($MgCl_2$ free), 0.5 μL (1 unit) of *Taq* DNA polymerase (Fermentas, Ontorio, Canada), 1.0 mM of dNTP mix (Fermentas, Ontorio, Canada), 5mM $MgCl_2$, 1.0μL of 100μM each of the primers and 35.5μL of dH_20 for 50 μL reaction mixture.

The reaction mixtures of 9 to 12 were tested for *trn*e and *trn*f and 9th condition was found to be the best and was modified to 50μL reaction mixture, containing 5 mM $MgCl_2$, 1.0μL of 100μM each of the primers, 0.5 μL (1 unit) *taq* DNA polymerase (Fermentas, Ontorio, Canada), 0.1 mM of dNTPs mix (Fermentas, Ontorio, Canada)(Table 3.4). About 5μL of the PCR mixture were run in 2% agorose gel to analyze band quality. Gels were run in 1XTAE (0.4M Tris Acetate) buffer at 90 volts for 2 hours. After electrophoresis, DNA bands were stained with 5μg/ml ethidium bromide and visualized under UV light. The gels were also photographed and digitialized by using a gel imaging system (Vilbor Lourmat, France). After the good band quality, remainig 50μL mixture was stocked for sequencing analysis later. The reaction mixtures were prepared in thin-walled 0.2 mL eppendorf tubes. The thermal cycler (Eppendorf-Mastercycler, Eppendorf, Canada) programs were optimized as indicated in Table 3.5 for each of the three sectors of trn region of cpDNA.

Tablo 3.4 Optimized PCR conditions for *trn* region of chloroplast genome of Turkish Firs

PCR Mixture contents	*trn*L		*trn*F		*trn*V	
	Volume used in PCR (μL)	**Final Concentration**	**Volume used in PCR (μL)**	**Final Concentration**	**Volume used in PCR (μL)**	**Final Concentration**
PCR Grade Water	34.5	NA	31.5	NA	33.5	NA
10X PCR Buffer	5	1X	5	1X	5	1X
MgCl2 (25mM stock)	5	2.5 mM	5	2.5 mM	5	2.5 mM
dNTP (10mM of each dNTP)	2	0.4 mM	2	0.4 mM	1	0.2 mM
Forward primer (100μM)	0. 5	1 μM	2.0	4 μM	2.0	4 μM
Reverse primer (100μM)	0.5	1 μM	2.0	4 μM	2.0	4 μM
Taq DNA polymerase (5u/μL)	0.5	0.5u	0.5	0.5u	0.5	0.5u
DNA	2	6 ng/μL	2	6 ng/μL	2	6 ng/μL
Total Volume	50		50		50	

Tablo 3.5 Optimized thermal cycler program used for amplification of *trn* region of chloroplast genome of Turkish Firs

trn regions	Temperature (°C)	Duration	Number of Cycle	Purpose
*trn*L and *trn*V	94	5 minutes	1	Initial denaturation
	94	30 seconds	35	Internal denaturation
	55	30 seconds		Annealing
	72	50 seconds		Extension
	72	5 minutes	1	Final Extension
*trn*F	94	5 minutes	1	Initial denaturation
	94	30 seconds	35	Internal denaturation
	60	30 seconds		Annealing
	72	50 seconds		Extension
	72	5 minutes	1	Final Extension

3.6. Sequencing t-RNA region of the chloroplast DNA

After amplification of t-RNA region of the chloroplast DNA, PCR products were stored at -20°C until sequence analysis. Both forward (*trn*c, *trn*e and *trn*VF) and reverse (*trn*d, *trn*f and *trn*VR) primers were used to sequence the three regions. A PCR purification process was done before the sequence analysis. Both purification and sequencing of samples were performed by Refgen Biotechnology, METU Teknokent, Ankara. In sequence analysis, ABI 310 Genetic Analyser User's Manual was followed. The sequencing was performed using the Big Dye Cycle Sequencing Kit (applied biosystems) with ABI 310 Genetic Analyser (PE applied Biosystem) automatic sequencer. For purification of PCR product Nucleospin Extract Kit (Clontech Laboratories, Inc.) was used.

In sequence Analysis, the purification processes were as follows:

- 2 volumes of buffer NT (contains chaotropic salt) with 1 volume of sample was mixed.
- A NucleoSpin® Extract II column was placed into a 2 ml collecting tube and the sample was loaded
- It was centrifugated at 11,000g for 1min.
- Flow-through was discarded and NucleoSpin® Extract II column was placed into the collecting tube.
- 600 μL ethanolic NT3 buffer was added and centrifugated at 11,000g for 1min.
- Flow-through was discarded and the NucleoSpin® Extract II was placed column back into the collecting tube.
- Centrifugation was done for 2min at 11,000g to remove buffer NT3 quantitatively.
- The NucleoSpin® Extract II column was placed into a clean 1.5 ml microcentrifuge tube.
- 15-50 *μl* elution buffer NE (5mM Tris-Cl pH: 8.5) was added and It was incubated at room temperature for 1 min to increase he yield of eluted DNA.
- Centrifugation was done for 1 min at 11,000g.

Tablo 3.6 Reaction conditions for sequencing

Reagent	Concentration	Volume
Ready Reaction Premix	2.5X	4µL
BigDye Sequencing Buffer	5X	2µL
Primer	-	3.2 pmol
Template	-	5-20ng
Water	-	to 20µL
Final Volume	1X	20µL

Tablo 3.7 Thermal cycler program for sequencing

<table>
<tr><th>Temperature (°C)</th><th>Duration</th><th>Number of cycles</th><th>Purpose</th></tr>
<tr><td>96</td><td>1</td><td>1</td><td>Initial Denaturation</td></tr>
<tr><td>96</td><td>10</td><td rowspan="3">25</td><td>Denaturation</td></tr>
<tr><td>50</td><td>5</td><td>Annealing</td></tr>
<tr><td>60</td><td>4</td><td>Extension</td></tr>
<tr><td>4</td><td>∞</td><td>1</td><td>Hold</td></tr>
</table>

After thermal cycling for sequencing, precipitation procedure was performed as follows:

1. Addition of 2 µl of 125 mM EDTA.
2. Addition of 2 µl of 3 M sodium acetate.
3. Addition of 50 µl of 100% ethanol.
4. Inverting 4 times.
5. Incubating for 15 min at room temperature.
6. Centrifugation at 2000-3000g for 30min.
7. Inverting the plate and spinned up to 185g.
8. Addition of 60 µl 70% ethanol.
9. Centrifugation at 4C for 15 min at 1650g.
10. Inverting the plate and spinned up to 185g for 1 min.

To prepare extension product purification, the following procedure was:

1. Preparation of 2.2% SDS in deionized water.
2. Addition of appropriate amount of SDS solution to sample to reach the volume of 0.2% SDS concentration.
3. Heating the tubes at 98C for 5min and cooling at 25C for 10 min.

For spin column purification, the following procedure was used:

1. Addition of 0.8 ml of deionized water.
2. Hydrating the gel at room temperature for at least 2 hours
3. Inserting the column to wash tube.
4. Spinning the column in a microcentrifuge at 730g for 2 minutes.
5. Removal of the column from wash tube and inserting into a sample collection tube. (Refgen Biotechnology, METU Teknokent, Ankara)

The t-RNA region was amplified as tree parts with the help of six primers. Before the analysis, these parts were aligned visually. For viewing the chromotogram data, Finch TV Version 1.4.0 developed by the Geopiza Research Team was used (PATTERSON *et al.*, 2004-2006). After the collection of the data, the sequences from forward primer and the sequences from the reverse primer were aligned and the accuracy of the bases was checked manually due to the Multiple Alignment (algorithm) that is the arrangement of several protein or nucleic acid sequences with postulated gaps so that similar residues (in one-letter code) are juxtaposed. If there is any inconsistency between the two series, the sample has been neglected from the analysis.

3.7. Collection and analysis of data

Phylogenetic and molecular evolutionary analysis was conducted using MEGA (Molecular Evolutionary Genetics Analysis) Version 5 (KUMAR *et al.*, 2008). The sequences were pre-processed in the form of the Fasta by aligning “- “to gaps and “N” to the unknown bases between the three regions

*trn*L, *trn*F and *trn*V. It was found that the analyzed sequences were shorter than unprocessed sequence data since the beginning and the ends of the data were trimmed. The unreliability of these parts is the reason for shortening. By using automatic sequencing systems, the quality of sequencing decreases dramatically at the begining and at the end of the sequencing.

All the *trn* Leu (trnL), Phe (trnF) and Val (trnV) regions of *Abies* species in the world (aproximately 300 species that found in IPNI (The International Plant Names Index), 2010) were investigate in the database NCBI (National Center for Biotechnology Information, 2010). The *trn*L, *trn*F and *trn*V sequences of 23 *Abies* species worldwide were available for the analyses to clarify the phylogenetic position of the Turkish *Abies* within the genus. The analysis was done including these species of *Abies* that were available in the database as well.

The names and the gene bank accession numbers of these sequences are below (Table 3.8, Table 3.9).

Tablo 3.8 Sequence of leucyl-tRNA gene in the species

Species Name	Genbank accession number	Reference
Abies alba	DQ126327.1	Ziegenhagen,B., Fady,B., Kuhlenkamp,V. and Liepelt,S.2005. Differentiating groups of *Abies* species with a simple molecular barcode. Silvae Genetica 54, 3.
Abies cephalonica	DQ126328.1	
Abies bornmuelleriana	DQ126329.1	
Abies nordmanniana	DQ126330.1	
Abies numidica	DQ126331.1	
Abies pinsapo	DQ126332.1	
Abies cilicica	DQ126333.1	
Abies nebrodensis	DQ126334.1	
Abies veitchii	EF395416.1	Havill,N.P.,Campbell,C.S., Vining,T.F., LePage,B., Bayer,R.J. and Donoghue,M.J.2007 Phylogeny and Biogeography of Tsuga (Pinaceae) Inferred from Nuclear Ribosomal Its and cpDNA Sequence Data. Systematic Botany 33(3):478-789.

Tablo 3.9 Sequence of phenylalanyl-tRNA gene in the species

Species Name	Genbank accession number	Reference
Abies fabri	AB029735.1	Suyama,Y., Yoshimaru,H. and Tsumura,Y. 2000 Molecular phylogenetic position of Japanese Abies (Pinaceae) based on chloroplast DNA sequences. Mol. Phylogenet. Evol. 16 (2), 271-277.
Abies fargesii	AB029736.1	
Abies firma	AB029737.1	
Abies fraseri	AB029738.1	
Abies holophylla	AB029739.1	
Abies homolepis	AB029740.1	
Abies koreana	AB029741.1	
Abies lasiocarpa	AB029742.1	
Abies mariesii	AB029743.1	
Abies nephrolepis	AB029744.1	
Abies sachalinensis	AB029745.1	
Abies sibirica	AB029746.1	
Abies veitchii	AB029747.1	
Abies kawakamii	DQ116573.1	Shih,F.L., Hwang,S.Y., Cheng,Y.P., Lee,P.F. and Lin,T.P. 2007 Uniform genetic diversity, low differentiation, and neutral evolution characterize contemporary refuge populations of Taiwan fir (*Abies kawakamii*, Pinaceae). Am. J. Bot. 94 (2), 194-202.
Abies spectabilis	DQ116576.1	

3.8. Construction of Phylogenetic Trees for Turkish Fir Taxa

Phylogenetic trees show the evolutionary interrelationships among various species or other entities based upon similarities and differences in their physical or genetic characteristics. These species are believed to have a common ancestor. There are two types of phylogenetic tree. A directed tree

with a unique node is rooted phylogenetic tree. The most common method for rooting trees is use of an uncontroversial outgroup that close enough to allow inference from sequence. It is also possible to draw a tree without a root. This is called an unrooted tree that illustrates the relatedness of the leaf nodes without making assumptions about ancestry at all. Unrooted trees can always be generated from rooted ones due to omitting the root.

The phylogenetic tree was also constructed by using neighbor joining (NJ) method together with bootstrap test analysis (SAITOU and NEI, 1987). It is usually used for trees based on DNA or protein sequence data. To construct the topology of a tree is the algorithm of the method. Furthermore, the NJ method provides both the topology and the branch lengths of the final tree. SAITOU AND NEI (1987) found that different than the standard algorithm for minimum-evolution trees, the NJ method minimizes the sum of branch lengths at each stage of clustering of OTUs (Operational Taxonomic Unit) starting with a starlike tree. Thus, the final tree produced may not be the minimum-evolution tree among all possible trees. Their data indicated that the NJ method is quite efficient compared with other tree-making methods that produce a single parsimonious tree. Morever, a root is estimated as part of the procedure.

The bootstrap test was applied in this study. It is a popular way of evaluating the reliability of an inferred phylogenetic tree. To re-sample the alignment columns with replacement is the first step. In this alignment, a given column in the original alignment may occur two or more times, while some columns may not be represented in the new alignment at all. The re-sampled alignment show an opinion of how a different set of sequences from the same genes and the same species may have evolved on the same tree.

If a new tree reconstruction on the re-sampled alignment results in a tree similar to the original one, this increases the confidence in the original tree. If, on the other hand, the new tree looks very different, it means that the inferred tree is unreliable. By re-sampling a number of times it is possibly to put reliability weights on each internal branch of the inferred tree. If the data was

bootstrapped a 100 times, a bootstrap score of 100 means that the corresponding branch occurs in all 100 trees made from re-sampled alignments. Thus, a high bootstrap score is a sign of greater reliability. 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 informative (NEI and KUMAR, 2000). The phylogenetic tree was constructed by MEGA 5.

CHAPTER 4

RESULTS

4.1. Amplification of the t-RNA Region of the Chloroplast DNA

Good quality single bands were observed for *trn*L, *trn*F and *trn*V primer sets. Due to the good quality of the bands, amplified fragments by all three primers were selected for sequencing (Figure 4.1).

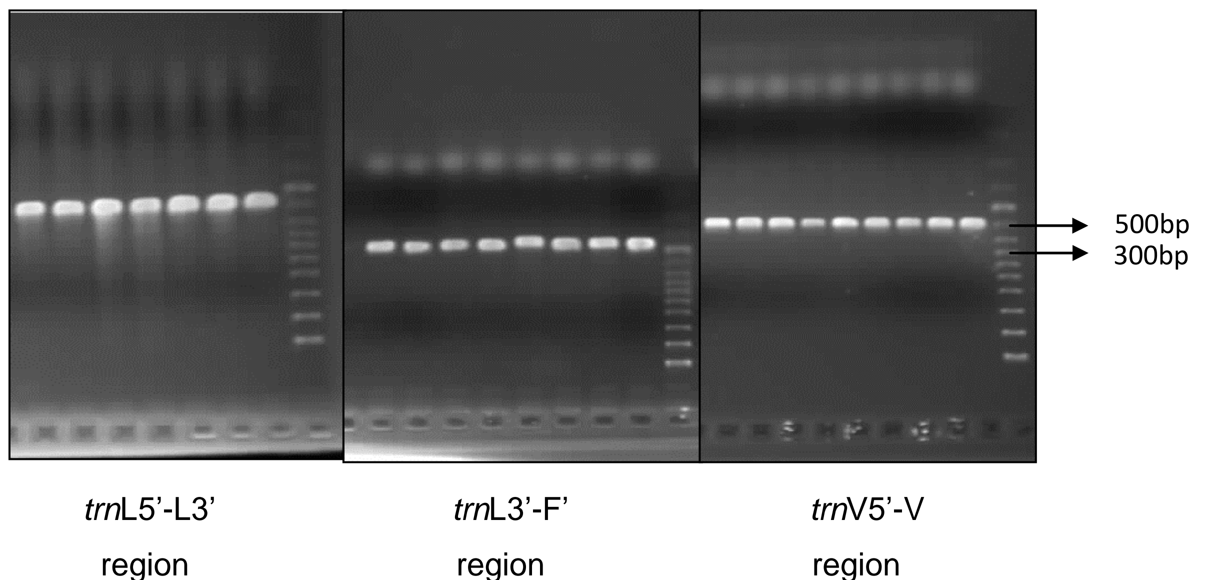

Figure 4.1 Photograph showing the amplified DNA of three regions of tRNA

4.2. Sequencing t-RNA region of the chloroplast DNA of Turkish Firs

Sequencing reactions were performed as described in Chapter 3 and the purified sequence products were run on 2% polyacrylamide gel (Visible Genetics Automated Sequencing System). The sequence data were very

good in quality, so there were no confusion on determination of the bases according to the chromatogram data.

4.3. Phylogenetic Analysis in the t-RNA Region

4.3.1. Data evaluation and results for only Turkish firs with regard to three regions and combination of three regions.

In the sequence analysis total length of whole region was found to be 1378 bp with 36.1% GC content. Since all individuals within Turkish Fir populations gave identical sequences, there is no variable sites, parsimony – informative sites and singleton sites. The first region (also called *trn*L region) between *trn*L5'-L3'is about 491 base pairs (bp) while the second "*trn*F" region between *trn*L3'-F' and the third "*trn*V" region between *trn*V5'-V3' were about 391 bp and 496 bp long, respectively. Among 491 bp, of "cd" region, GC content was 37.3% and transition / transversion bias (R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)].) was 0.476. The second region (*trn*F region) had 34.8%GC content and transition / transversion bias(R) was 0.455. The third region (v) had 38.5% GC content and transition / transversion bias(R) was 0.475 (Table 4.1).

Tablo 4.1 Estimated molecular diversity parameters for *trn*L5'-L3', *trn*L3'-F and *trn*VF5'-VR3'region for Turkish Fir taxa.

Molecular diversity parameters	Total	*trn*L5'-L3' region	*trn*L3'-F region	*trn*VF5'-VR3' region
Total Length (bp)	1378	491	391	496
GC content (%)	36.1	37.3	34.8	38.5
Conserved sites	1378	491	391	496
Variable sites	0	0	0	0
Singleton sites	0	0	0	0
Parsimony informative sites	0	0	0	0
Transitional pairs	33.32	33.34	33.34	33.29
Transversional pairs	66.66	66.68	66.68	66.68
Transition / Transversion Bias(R)	0.463	0.476	0.455	0.475

4.3.2. Data evaluation and results for Turkish firs, European firs and Asian firs with regard to *trn*L5'-L3' region

*trn*L sequences were available for only 9 species from NCBI database (6 species from Europe, 2 species from Asia and 1 species from Africa).When Turkish Fir species compared to European species (*A. alba, A. cephalonica, A. bornmuelleriana, A. nordmanniana, A. pinsapo, A. nebrodensis)*, among 491 bp, there were no variable, parsimony – informative and singleton sites. The transition / transversion bias(R) 0.476 and GC content 37.3.The *trn*L5'-L3' region had 37.3% GC content, 2 variable sites, 489 conserved sites, 0 parsimony – informative sites and 2 singleton sites with Asian fir (*A. veitchii*) Moreover, transition / transversion bias(R) was 1.004 (Table 4.2.A).

4.3.3. Data evaluation and results for Turkish firs, North American firs and Asian firs with regard to *trn*L3'-F region

The *trn*F sequences were available for only 15 species from NCBI database (13 species from Asia and 2 species from North America). Among 508 bp, there were 5 variable sites (V), 414 conserved sites, 4 parsimony – informative sites and 1 singleton sites. The transition / transversion bias(R) and GC content were 0.284 and 34.5% with North American firs (*A. lasiocarpa* and *A. fraseri)*. There were 12 variable sites (V), 395 conserved sites, 5 parsimony – informative sites, 7 singleton sites and transition / transversion bias(R) was 0.179 when compared Turkish firs with Asian firs (*A. fabri, A. fargesii, A. holophylla, A. nephrolepis, A. spectabilis, A. homolepis, A. mariesii, A. veitchii, A. firma, A. sachalinensis, A. koreana, A. sibirica,* and *A. kawakamii)* GC content was 34.7% (Table 4.2.B).

Tablo 4.2 Molecular diversity parameters. A) *trn*L5'-L3'region of *Abies* species. B) *trn*L3'-F *region* of *Abies* species (data included from this study and Genebank database).

A)

Sequence characteristics of ***trn*L5'-L3'** region	Turkish Firs	Turkish Firs+ European Firs	Turkish Firs+ Asian firs	Turkish firs and all other firs combined
Total Length (bp)	491	491	491	491
GC content (%)	37.3	37.3	37.3	37.3
Conserved sites	491	491	489	489
Variable sites	0	0	2	2
Singleton sites	0	0	2	2
Parsimony informative sites	0	0	0	0
Transitional pairs	33.34	33.34	49.26	33.34
Transversional pairs	66.68	66.68	50.74	66.68
Transition / Transversion Bias(R)	0.476	0.476	1.004	0.476

B)

Sequence characteristics of *trn*L3'-Fregion	Turkish Firs	Turkish Firs+ American Firs	Turkish Firs+ Asian firs	Turkish firs and all other firs combined
Total Length (bp)	391	508	508	487
GC content (%)	34.8	34.5	34.7	34.6
Conserved sites	391	414	395	407
Variable sites	0	5	12	14
Singleton sites	0	1	7	8
Parsimony informative sites	0	4	5	6
Transitional pairs	33.34	22.96	14.76	20.3
Transversional pairs	66.68	77.04	85.24	79.7
Transition / Transversion Bias(R)	0.455	0.284	0.179	0.221

4.4. Phylogenetic Trees

4.4.1. Phylogenetic Tree Based on three region

When the distances among all individuals within populations were computed by MEGA 5 software, there was no divergency within each population of a Turkish fir taxon and for that reason, phylogenetic tree construction was performed by choosing just one representative individual from each population of each taxon of Turkish fir species. Phylogenetic trees were constructed by including *P.abies* as an outgroup along Turkish Fir Taxa (Genebank accession number AF327585.1). All individuals within Turkish Fir populations gave identical sequences for all three regions.

According to phylogenetic tree (Figure 4.2) which was constructed by using Neigborjoining method together with bootstrap test analysis, it was seen that 18 Turkish fir populations were formed a monophyletic group with almost no sequence divergence.

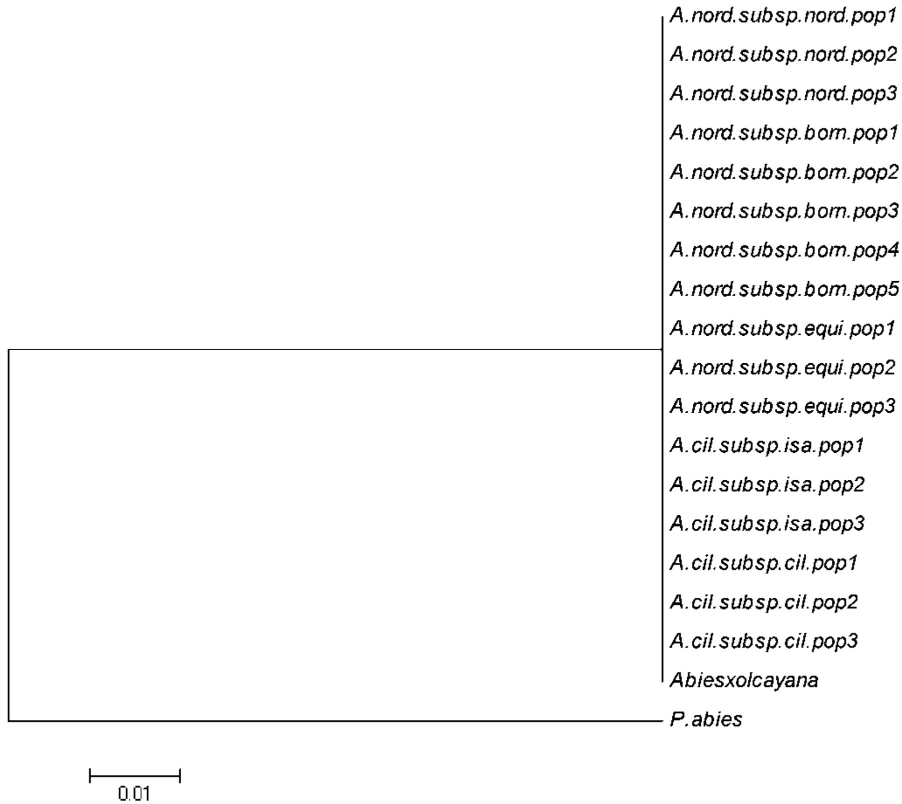

Figure 4.2 Phylogenetic tree was constructed by using Neighborjoining method together with bootstrap test analysis for *Abies* spp. in Turkey with the sequences of *trn*L, F, V regions of cpDNA .

4.4.2. Phylogenetic Tree for the fir species (including Turkish firs) Based on *trn*L5'-L3' region

In this study, a total of 491 nucleotides in the *trn*L5'-L3' region were sequenced for six Turkish *Abies* taxa (*A. nordmanniana* subsp. *nordmanniana, A.nordmanniana* subsp*. bornmuelleriana, A. nordmanniana* subsp*. equi-trojani, A.cilicica* subsp*. isaurica, A. cilicica* subsp. *cilicica* and *Abies x olcayana*) were sequenced. In addition, the *trn*L sequences of *A. alba, A. cephalonica, A. numidica, A. pinsapo, A. nebrodensis, A. veitchii, A. bornmuelleriana, A. nordmanniana,* and *A. cilicica* from NCBI database under

Accession No. DQ126327.1. to DQ126334.1. and EF395416.1. were also used for comparison (Table 4.3).

According to constructed phylogenetic tree*, Abies* species were divided into two clades: one Turkish- European species, and one *Abies veitchii* alone. The Turkish- European Clade consisted of Turkish *A. nordmanniana* subsp. *nordmanniana, A.nordmanniana* subsp*. bornmuelleriana, A. nordmanniana* subsp*. equi-trojani, A.cilicica* subsp*. isaurica, A. cilicica* subsp. *cilicica* and *Abies x olcayana,* with no variation in *trn*L sequences and *Abies numidica, Abies pinsapo, Abies nebrodensis, Abies alba, Abies cephalonica* (Figure 4.3). The Clade 2 consisted of only one Japanese species, *Abies veitchii*; however, the bootstrap reliability was low.

Tablo 4.3 Materials Used in the Phylogenetic Analysis of *Abies* (*trn*L5'-L3' region)

Materials Used in the Phylogenetic Analysis of *Abies* (*trn*L5'-L3' region)				
Species	**Section by Liu (1971)**	**Section by Farjon(1990)**	**Natural Distribution**	**Endemic(E)/Non-Endemic(NE)**
A. nordmanniana subsp. *nordmanniana*	*Abies*	*Abies*	Turkey	NE
A.nordmanniana subsp. *bornmuelleriana*				E (Bursa, Bolu, Zonguldak and Kastamonu)
A. nordmanniana subsp. *equi-trojani*				E (Çanakkale, Balıkesir)
A.cilicica subsp. *isaurica*				E(Between Antalya and Konya)
A. cilicica subsp. *cilicica*				NE
Abies x olcayana				E (Çataldağ)
Abies alba	*Abies*	*Abies*	Europe	NE
Abies cephalonica	*Abies*	*Abies*	Greece	E (Peloponnesos Mountain Kefallonia Island)
Abies bornmuelleriana	*Abies*	*Abies*	Turkey	E (Bursa, Bolu, Zonguldak and Kastamonu)
Abies nordmanniana	*Abies*	*Abies*	Turkey, Georgia, Russia, Armenia	NE
Abies numidica	*Piceaster*	*Piceaster*	Algeria	E (Djebel Babor)
Abies pinsapo	*Piceaster*	*Piceaster*	Spain	E (Andalusian mountains)
Abies cilicica	*Abies*	*Abies*	Lebanon, Syria, Turkey.	NE
Abies nebrodensis	*Abies*	*Abies*	Sicily	E (Nebrodi and Madonie mount ains)
Abies veitchii	*Elate*	*Balsamae*	Japan	E (Honshū and Shikoku Islands)

Figure 4.3 Phylogenetic tree was constructed by using Neighborjoining method together with bootstrap test analysis for *Abies* spp. with *trn*L primers.

4.4.3. Phylogenetic Tree for the fir species (including Turkish firs) Based on *trn*L5'-F region

A total of 487 nucleotides in the *trn*L3'-F region were used for 21 *Abies* species including the sequences from six Turkish *Abies* species .In addition, the sequences of *A. fabri, A. fargesii, A. holophylla, A. nephrolepis, A. spectabilis, A. homolepis*, *A. mariesii, A. veitchii, A. firma, A. sachalinensis, A. koreana, A. sibirica, A.kawakamii, A. lasiocarpa, and A. fraseri* from NCBI database under Accession Number AB029735.1 to AB029747.1, DQ116573.1 and DQ116576.1 were also obtained for comparison (Table 4.4). Bootstrap values were also given on the phylogenetic trees above the branches in figure 4.4.

In the strict consensus tree, shown with the 10,000 bootstrap replicates, *Abies* species were divided into four major clades: (1) Turkish species, (2) a combined group of Asian and North American species, (3) a Asian group and (4) *A.mariesii* alone. Clade 1 consisted of *A. nordmanniana* subsp. *nordmanniana, A.nordmanniana* subsp*. bornmuelleriana, A. nordmanniana* subsp*. equi-trojani, A.cilicica* subsp*. isaurica, A. cilicica* subsp. *cilicica* and *Abies x olcayana* with no variation in *trn*F sequences (bootstrap value, 87%). Clade 2 contained of four divergent species (*A.sibirica, A. firma, A. fabri, A. fargesii) and tree subclade,* one containing *A. fraseri and A. lasiocarpa; one containing A. holophylla and the other A. kawakamii.* Clade 3 contained of *A. homolepis, A. veitchii, A. koreana, A. nephrolepis, A. spectabilis and A. sachalinensis.* Clade 4 consisted of only *A. mariesii.*

Tablo 4.4 Materials Used in the Phylogenetic Analysis of *Abies* (*trn*L3'-F region)

Materials Used in the Phylogenetic Analysis of *Abies* (*trn*L3'-F region)				
Species	**Section by Liu (1971)**	**Section by Farjon(1990)**	**Natural Distribution**	**Endemic(E)/Non-Endemic(NE)**
A. nordmanniana subsp. *Nordmanniana*	*Abies*	*Abies*	Turkey	NE
A.nordmanniana subsp. *bornmuelleriana*				E (Bursa, Bolu, Zonguldak and Kastamonu)
A. nordmanniana subsp*. equi-trojani*				E (Çanakkale,Balıkesir)
A.cilicica subsp. *isaurica*				E (Between Antalya and Konya)
A. cilicica subsp. *Cilicica*				NE
Abies x olcayana				E (Çataldağ)
Abies fabri	*Elateopsis*	*Pseudopicea*	China	E (Sichuan)
Abies fargesii	*Elateopsis*	*Pseudopicea*	China	E (Qinling mountain)
Abies holophylla	*Homolepides*	*Momi*	China and Korea	E (Heilongjiang, Jilin, and Liaoning)
Abies nephrolepis	*Elate*	*Balsamae*	China, Korea and Russia	E (northeastern China North Korea, South Korea, and southeastern Russia)
Abies spectabilis	*Elateopsis*	*Pseudopicea*	China, Afghanistan, India, and Nepal	NE
Abies homolepis	*Homolepides*	*Momi*	Japan	E (Honshū and Shikoku, Mountains)

Table 4.1 (continued)

Abies mariesii	*Homolepides*	*Amabilis*	Japan	E (Honshū Mountain)
Abies veitchii	*Elate*	*Balsamae*	Japan	E (Honshū and Shikoku, Mountains)
Abies firma	*Momi*	*Momi*	Japan	E (Central and Southern Japan)
Abies sachalinensis	*Elate*	*Balsamae*	Japan and Russia	NE
Abies koreana	*Elate*	*Balsamae*	Korea	E (Mountains of South Korea, Jeju-do island)
Abies sibirica	*Pichta*	*Balsamae*	Siberia	NE
Abies kawakamii	*Balsamae*	*Balsamae*	Taiwan	NE
Abies lasiocarpa	*Balsameae*	*Balsamae*	North America	E (Western North America)
Abies fraseri	*Balsameae*	*Balsamae*	The United States and Canada.	E (Mountains of the eastern United States)

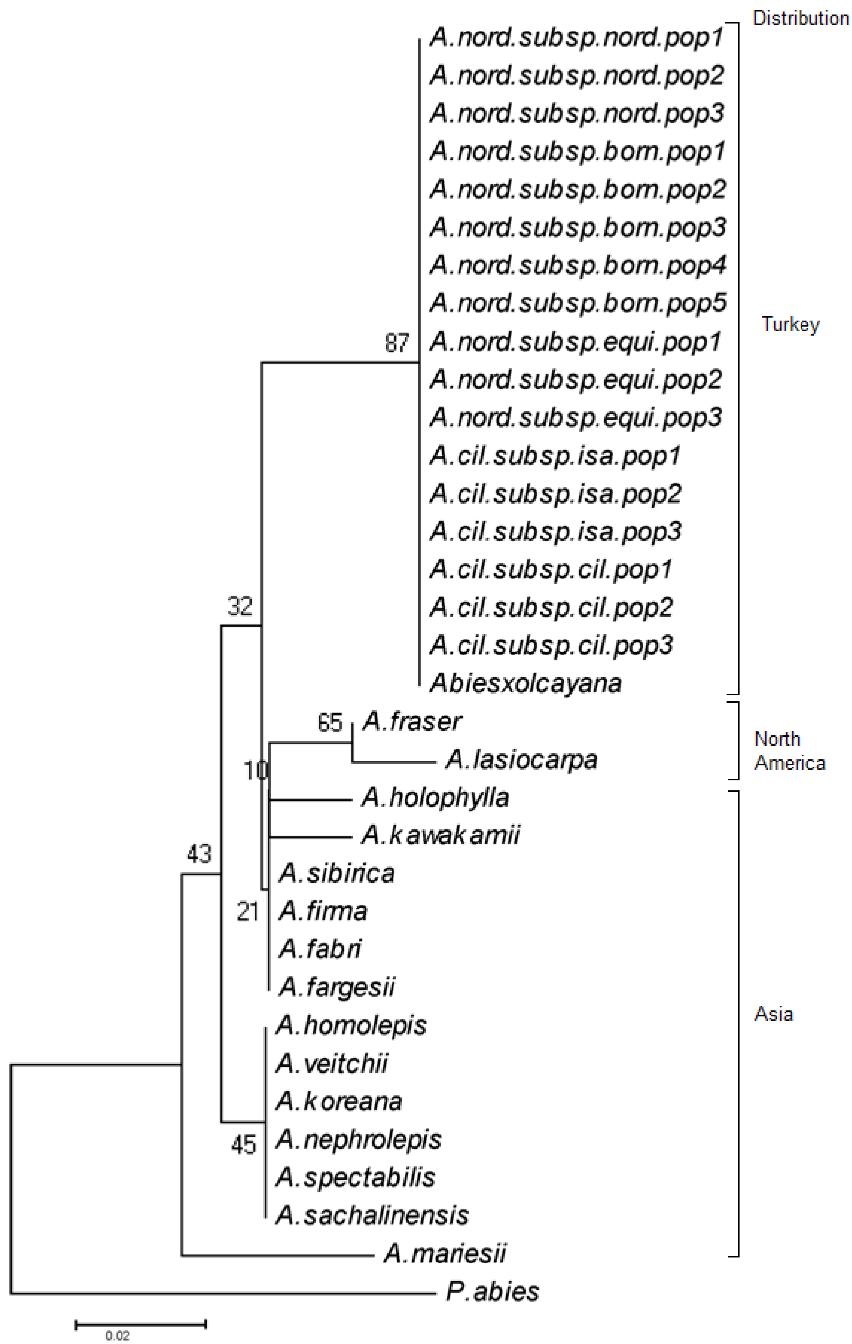

Figure 4.4 Phylogenetic tree was constructed by using Neighborjoining method together with 10 000 bootstrap test analysis for *Abies* spp. with *trn*L3'-F region.

CHAPTER 5

DISCUSSION

5.1. DNA sequence characteristics in the three spacer region of *trn*

The DNA sequence of the cpDNA spacer region between the *trn*L, *trn*F and *trn*V genes was determined for 18 populations representing 6 *Abies* taxa**.** In this study, cpDNA *trn* region was determined as about 1378 bp because of indels (insertion and deletion of bases). The size of the *trn*L intron varies from about 350 bp (genus *Avena*) to 600 bp (genus *Euphorbia*) (GIELLY and TABERLET, 1994). The length of *trn*L region was 491 bp in length in this study. However, the previous studies reported by Ziegenhagen *et al.* (unpublished) claimed that this region as 514 bp. In the study performed by Stech *et al.* (2003), they reported that the length of this region ranged from 448 bp to 520 bp in seed plants. The length of the *trn*L intron ranged from 477 to 537 bp and that of the intergenic spacer between *trn*L and *trn*F ranged from 307 to 378bp (BAYER et al., 1998).The length of *trn*F region in Turkish firs was found out as 391 bp. Suyama *et al.* (2000) reported that the length of this region was 444 bp in length. Moreover, the DNA sequence of the cpDNA spacer region between the *trn*L and *trn*F genes was determined for 18 populations representing 6 *Abies* taxa. This region varied in length from 403 bp to 455 bp among species (ISODA *et al.*, 2000). It was 464bp in length (FERRI *et al.*, 2008) and in the study performed by LOPEZ *et al.* (2001, 2002, 2006) it was about 400 bp in length as a result of various studies. The length of *trn*V region of cpDNA in Turkish Firs was resolved as 496 bp. According to the several studies, the length of this region is approximately

540bp (WAKASUGI *et al.*,1994; WANG *et al.*, 1999; ANN *et al.,* 2006; ECKERT *et al.*, 2006; LOPEZ *et al.,* 2001). WANG *et al.*(1999) studied the sequence divergence of chloroplast *trnV* intron among 32 *Pinus* species and representatives of six other genera in Pinaceae. They concluded that the length variation in this region is very minor. For example, the aligned sequence length for this region is 555 bp. and subgenus *Pinus* and subgenus *Strobus* differed only by 1 bp in length. These results and previous studies show that it may be possible that trn region is different in length among species since *trn* region is shown high variation.

5.2. The Constructed Phylogenetic Trees by MEGA 5

5.2.1. Phylogeny of the Genus Abies

Taxonomists have proposed a variety of different classifications for *Abies*, with Liu (1971) and Farjon (1989 and 1990) being the most recent. Their classifications are, however, different in many points, which may be indicative of the difficulty of classifying *Abies*.

The DNA sequence of the cpDNA spacer region between the *trn*L- *trn*F and *trn*V genes was determined for 6 *Abies* species (*A. nordmanniana* subsp. *nordmanniana, A.nordmanniana* subsp*. bornmuelleriana, A. nordmanniana* subsp*. equi-trojani, A.cilicica* subsp*. isaurica, A. cilicica* subsp. *cilicica* and *Abies x olcayana)*. According to the phylogenetic tree constructed with these sequences by Neigbor-joining method together with bootstrap test analysis in Figure 4.2 (for *trn*L, F, V primers), it was clear that 18 of the Turkish fir populations representing six taxa were formed a monophyletic group with no sequence divergence. Therefore, these regions have been well protected among Turkish Fir species. The origins of these species may be the same, most likely from *Abies nordmanniana.* Our results are also consistent with the results of BRUNNER (2001) who studie *trn* region divergence of *Picea abies.* They demonstrated they did not find any variation in 10 sequences of *Picea abies* originating from the entire species range throughout Europe. However,

SUYAMA *et al*. (2000) reported that *trn* region of Japanese *Abies* species ishighly informative for phylogenetic studies. Therefore, divergence of *trn* region may vary according to species.

5.2.2. Phylogeny of Turkish *Abies* and Closely Related Species

All the *trnL*, trnF and trnV regions of cp DNA in *Abies* species of the world [aproximately 300 species that found in IPNI (The International Plant Names Index), 2010] were investigated in the NCBI database (National Center for Biotechnology Information, 2010). The *trn*L, *trn*F and *trn*V sequences of 23 *Abies* species worldwide were analyzed to clarify the phylogenetic position of the Turkish *Abies* species within this genus. The analysis was done including these *Abies* species that were available in the database.

5.2.2.1. Phylogeny of Turkish *Abies* and Closely Related Species Based on *trn*L5'-L3' region

The conventional systems proposed by LIU and FARJON (1971, 1989) are shown in Table 4.3 and 4.4. According to phylogenetic tree of *Abies* spp. with *trn*L primers the two conventional systems agree with each other in some selections. One of the most important findings is the placement of Turkish-European species (*A. nordmanniana* subsp. *nordmanniana, A.nordmanniana* subsp*. bornmuelleriana, A. nordmanniana* subsp*. equi-trojani, A.cilicica* subsp*. isaurica, A. cilicica* subsp. *cilicica* and *Abies x olcayana,* and *A. numidica, A. pinsapo, A. nebrodensis, A. alba, A. cephalonica)* in a clade, which clearly differentiated them from the Asian species though there was only one Japanese species available*, A. veitchii* (Figure4.3). However, the bootstrap reliability was low. The more sequences from the Asian species available in the analysis, it may improve the phylogentic relationship among European and Asian fir species.

Therefore, in the phylogenetic tree based on *trn*L sequences alone, Turkish-European *Abies* species formed a monophyletic group with strong support, containing no sequence divergence in the region. Both LIU (1971) and FARJON and RUSHFORTH (1989) classified the Turkish- European species into two closely related sections, *Abies* and *Piceaster*. Turkish Fir species, *A. nebrodensis, A. alba,* and *A. cephalonica* are classified as section *Abies* by both authors. Moreover, they have classified *A. numidica and A. pinsapo* into one section, *Piceaster.* FARJON (1989) has classified only one Japanese species *A. veitchii into* section *Balsamae.* However, it was classified into *Elate* by LIU. Our data on phylogentic analysis have strogngly supported the classification made by LIU and FARJON (1971, 1989) that Turkish firs and European firs are in the *Abies* section.

There are some studies that consistent with our results. FADY and CONKLE (1993) have reported that *A.alba* appeared to be genetically closer to *A.nordmanniana* subsp. *bornmuelleriana.* According to Scaltsoyiannes' results (1999), high genetic similarity was observed between the Calabrian Fir population and the one from north-west Greece as well as between *A. equi-trojani* grown in Asia Minor and the southern Greek populations based on allozyme divergence. In addition to SUYAMA *et al*. (2000) determined that *A. numidica, A. pinsapo, A. nebrodensis, A. alba,* and *A. nordmanniana* consisted one clade with no variation in *rbc*L sequences. Besides, ZIEGENHAGEN *et al.* (2005) showed that Western Mediterranean species (*A. pinsapo* and *A. numidica*) were differentiated types of Eastern Mediterranean Firs. Moreover, *A. bornmulleriana* and *A. equitrojani* showed same haplotype with *A. nordmanniana* and *A. cephalonica* using primers *nad5-4* intron region of mitochondrial DNA.

On the other hand, some do not supported by our phylogenetic results. For instance, it is reported by PARDUCCI and SZMIDT (1999) that the chloroplast genome of European Abies species is highly variable in their PCR-RFLP analysis. Besides, ISODA *et al.* (2000) and KORMUTAK *et al.* (2004)**,** concluded that, *A. alba* and *A. nordmanniana* were found to be the

most differentiated from other species in their tandem repeat sequence. Hansen *et al.* (2005) studied chloroplast microsatellites as well as one mitochondrial marker in fifteen populations of *Abies nordmanniana*. They concluded that the chloroplast microsatellites were highly variable, while the mitochondrial marker showed no variation. Likewise, KAYA *et al.* (2008) concluded that depending on genetic similarity and distance values, studied Turkish Fir species (*A. nordmanniana, A. bornmuelleriana*, and *A. equi-trojani*) are genetically well differentiated. Fragmented distribution of 3 fir species in northern Turkey belonging to the Nordmanniana fir complex, also supports the possible existence of an ancestral species. Liepelt *et al*. (2010) deduced that *A. pinsapo* and *A. numidica* were clearly separated from each other and from the remaining *Abies* species.

As a result, we can say that divergence of *trn* region may vary according to species. BRUNNER (2001) reported that phylogenetic analysis of *trn*L sequences demonstrated that this intron is not highly informative for distinguishing among families and genera. However, SUYAMA *et al*. (2000) reported that *trn* region of Japanese *Abies* species is highly informative for phylogenetic studies.

5.2.2.2. Phylogeny of Turkish *Abies* and Closely Related Species *trn*L3'-F region

From the constructed phylogenetic tree using the sequences available from all fir species (Figure 4.4), it was shown that Fir species formed four major clade. 18 populations of Turkish Fir species were grouped together, having a high bootstrap value of 87% which suggests that these species have significantly separated from the other *Abies* taxa. This result proposes that there were no or very little variation between those Turkish Fir species when *trn* region is used as a molecular evolutionary tool. Morever, 15 individuals of Asian and American Fir species were grouped together in another branch. This result also revealed that there were considerable genetic variation between Turkish Fir species and Asian-American Fir species.

The Turkish clade consisted of *A. nordmanniana* subsp. *nordmanniana, A.nordmanniana* subsp*. bornmuelleriana, A. nordmanniana* subsp*. equi-trojani, A.cilicica* subsp*. isaurica, A. cilicica* subsp. *cilicica* and *Abies x olcayana* with no variation in *trn*F sequences. Both LIU (1971) and FARJON and RUSHFORTH (1989) classified the Turkish species into section, *Abies*. Therefore, our phylogenetic analysis supported by Lıu and Farjon's classification.

One of the most important findings of this study is the placement of *A. mariesii* in only one clade, which clearly differentiated it from all other Asian, North American and Turkish fir species. Our result is also consistent with the results of Tsumura and SUYAMA (1998) who studied RFLP variation between Japanese fir species (*A. homolepis, A. veitchii*, *A. sachalinensis* and *A. firma)*. They showed that that *A. mariesii* is quite different from the other species.

According to our results, the two American species*, A. fraseri* and *A. lasiocarpa* grouped together. Our sequence data support Farjon and Rushforth's (1989) classification for the section *Balsamea* with other Asian species (*A.sibirica, A. firma, A. fabri, A. fargesii, A. holophylla* and *A. kawakamii)*. Besides, SUYAMA *et al*. (2000) supported our results. They reported *A. firma* and *A. sibirica* appeared as a sister group based on *rbc*L sequences. Similarly, ISODA *et al.* (2000) determined that TRTs (tandem repeat type) of *A. firma* and *A. Sibirica* are same. Therefore, the *trn*F sequences data presented here confirm this relationship and provide additional strong support. However, *A. kawakamii*, *A. firma*, and *A. sibirica* formed a divergent group in both LIU (1971) and FARJON and RUSHFORTH (1989) clasification.

Another clade contained of *A. homolepis, A. veitchii, A. koreana, A. nephrolepis, A. spectabilis and A. sachalinensis.* SUYAMA *et al*. (2000) also reported that *A. sachalinensis* and *A. veitchii* are positioned very closely within the genus. However, *A. holophylla, A. homolepis* formed a divergent

group in the Asian part of the tree. Both LIU (1971) and FARJON and RUSHFORTH (1989) have classified these two species into one section.

CHAPTER 6

CONCLUSION

The three sectors of *trn* region of was found to be 1378 bp in length in Turkish firs. The comparative analyses with three different *trn* regions (*trn*L, *trn*F and *trn*V), as well as whole *trn* region showed that Turkish Fir taxa have identical sequences, with no variable sites. Although the current study is the first study using *trn* region of cpDNA for Turkish Firs, it appears that this region is not very suitable for the studies dealing with the differentiation of Turkish *Abies* species.

The constructed phylogenetic tree indicated that Turkish Fir species were grouped together based on all the three sectors of *trn* region. According to phylogenetic tree of *Abies* spp. with *trn*L primers, Turkish-European *Abies* species are found to be closely related species and separated from the Japanese species of *A. veitchii.* These findings suggest that the existence of single chloroplast lineage with respect to *trn* region of cpDNA in Turkish and European fir species. Moreover, Turkish Firs were gruped in the same cluster and closer to Asian species with respect to *trn*F primers. The results suggest that all Turkish firs may have evolved from single ancestral fir species, most likely from *Abies nordmanniana* since they have similir sequences.

Although, additional DNA data would be needed to clarify the taxonomic relationship of *Abies* species, the DNA results are compatible with LIU and FARJON's classification of Turkish-European *Abies* into two closely related sections, *Abies* and *Piceaster*. At the very least, our results clearly show that

Turkish Fir species differs greatly from the other Asian-North American species.

As a conclusion it can be safe to say that constructed phylogenetic trees of the study supported the result of LIU (1971) and FARJON (1989, 1990) studies that shows compatibility between *trn*L region and the conventional classification systems. However, some of our results taken from *trn*F region did not support results of LIU (1971) and FARJON (1989, 1990). Therefore, to clarify the systematics of *Abies* genus, other suitable regions of cpDNA, mtDNA, nDNA and more sequence data from other fir species of the world would be needed in the future.

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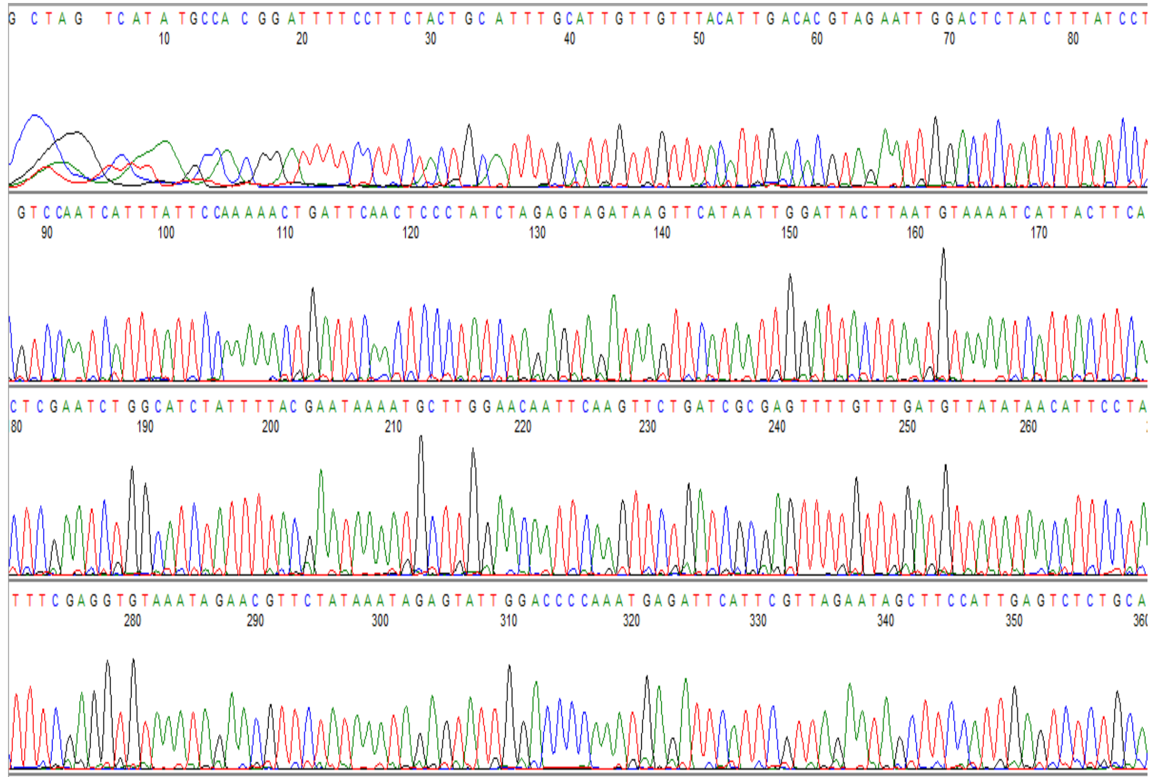
APPENDIX A

An Example of Chromotogram Data



APPENDIX B

An Example of *trn*LSequence

A. nordmanniana subsp. equi-trojani for *trn*L5'-L3'
GGTTGTTCGATTGAGCCTTGGTATGGACCCTACCAGTGATAGCTTCCAA
TCCAGGGAACCCTGGGATATTTTGAATGGGTAATCCTGAGCCAAATCCG
GTTCATAGAGAAAAGGGTTTCTCTCCTTCTCCTAAGGAAAGGGATAGGT
GCAGAGACTCAATGGAAGCTATTCTAACGAATGAATCTCATTTGGGGTC
CAATACTCTATTTATAGAACGTTCTATTTACACCTCGAAAGTAGGAATGT
TATATAACATCAAACAAAACTCGCGATCAGAACTTGAATTGTTCCAAGCA
TTTTATTCGTAAAATAGATGCCAGATTCGATTGAAGTAATGATTTTACATT
AAGTAATCCAATTATGAACTTATCTACTCTAGATAGGGAGTTGAATCAGT
TTTTGGAATAAATGATTGGACGAGGATAAAGATAGAGTCCAATTCTACGT
GTCAATGTAAACAACAATGCAAATTGCAGTAGAAGGAAAATCCGTTGGC
TTTATAGACCGTGAGGGTATCCCCCCCCTCATTCTCCACATT

A. nordmanniana subsp. equi-trojani for *trn*L3'-F'
GCAGGTTTCTTCCCGACGACTGATCTATTTTATCCATTCCGTTAGTTCAA
TCCATTCTCACTTCTCTTTGACCTCACTATTGAATTTATTCATGTTAATTT
ATTCATGAAGAGAAGAAATGGGAACATTAATCTTTCCATCTTATGACAAG
TTGAGTTGATCAGTGGATCAATTCATTTTGTCATATATGATCCACATAGA
TGTGATCATTTGGAAATTATTCGATCGCAGTCGATTTTTTATCGTATTAGT
TATTTCCAGATCGAAAAGAATAAAGATCATTCTAAAAACTGGGAAAAATC
CATTTCTTCCTTATTTTTAGTTGACACGAGTTAAAACCATGATCCACAGG
AAAGAGCCGGGATAGCTCAGTTGGTAGAGCAAAGGACTGAAAATCCTC
GTGTACCCAGTTCAAATAAG

A. nordmanniana subsp. equi-trojani *trn*VF5'-VR3'
GCGGGCATGGTTTTCGAGAGAGTTTATCGATTCGTCCGATCCACGAAAT
AGATTCTATGTGAAATAGTCTTACTCTATCAATTTGTTTCTCTGGGGAAC

AATAGCATGACAAAGATGAAGTTCGATTCGATTCGAATTACGAATCTAAT
TGATATGGTCAATCCCAGCTCCGTTCAATGCCAGGCATAATGAGTATAA
TACGGGGACCTCAAAATAGATTCTTTTCGCTCTATGAACTTTTAGGTGTA
TGAAGTGTCATATTTTACTTTTGGAGCGATAGAGGAGACTCTATTTGAGT
CAATCTATGCCCGAGCAAGGCAGACCTACGTCAAGGAAACCTTTTGAAT
AACTTTGGGATTGCTTCCGAAGGGTAATAATTTGGGGCACACGGAGCCA
TATTAGTATCTTCCTGGAAAGAGGAGAATGGCAAACTAACCGATCTTTCC
ATCAGTTAATGAAAGAGCCCAATGCGATAAAATGCATGTTGGGTTCTTG
GAACAGTTCAAATTATTTTGATAATAAGAATTTTGATCTGTTCTACCGGT