MOLECULAR PHYLOGENETICS OF TURKISH *ABIES* (PINACEAE)
SPECIES BASED ON *mat*K GENE REGIONS OF CHLOROPLAST
GENOME

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ABSTRACT

MOLECULAR PHYLOGENETICS OF TURKISH *ABIES* (PINACEAE) SPECIES BASED ON *mat*K GENE REGIONS OF CHLOROPLAST GENOME

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Pineacea is the largest family of conifers that includes 51 species of *Abies* which is the second largest genus after *Pinus*.

There are six native taxa in Turkey belonging to this genus. Four of these taxa (*Abies cilicica* subsp. *isaurica*, *Abies nordmanniana* subsp. *bornmülleriana*, *Abies nordmanniana* subsp. *equi-trojani, Abies x olcayana*) are endemic and considered as low risk (LR) species according to the IUCN criteria.

To determine the phylogenetic relationship in *Abies* spp. in Turkey, 18 populations of different taxa were collected from their natural distribution areas in Turkey. The *mat*K gene regions of chloroplast DNA (cpDNA) were studied comparatively to reveal the genetic relationship among Turkish fir species. The available sequences from the NCBI database for the *mat*K region of the other *Abies* species in the world were also investigated

comparatively with the sequences from Turkish firs. With the *mat*K sequence data, a phylogenetic tree was constructed for the fir species and the molecular diversity parameters such as conserved and variable sites, nucleotide diversity, and evolutionary divergence were estimated using the MEGA software.

The results indicated that there are no variable sites among Turkish firs with regard to *mat*K regions of cpDNA. It appears that the *mat*K region of cpDNA for Turkish firs is highly conserved.

Since sequence data for all *mat*K regions were not available from the NCBI data base, the phylogentic analysis with the sequence data of *mat*K were comparatively analyzed in all firs including Turkish firs. According to *mat*K1 region, the results showed that there were three major clades. One of the clades included all Turkish fir taxa and one species from European firs, *A.numidica*; however, *A.holopylla, A.firma, A.veitchii, A.sachalinensis, A.nephrolepis, A.lasiocarpa, A.koreana, A.homolephis, A.fraseri, A.fargesii, A.sibirica* and *A.fabri* formed in another clade. In addition to this, *A.mariesii, A.hidalgensis, A.bracteata, A.alba* formed in different major clade. According to *mat*K1 region the results showed that Turkish firs and European firs are closer to each other. Furthermore, based on *mat*K2 region, the results indicated that Turkish firs formed a monophyletic group.The other fir species, with respect to *mat*K2 regions, formed different clades from Turkish firs.

The results based on *mat*K region suggest that all Turkish firs may have evolved from single ancestral fir species and the *mat*K gene region appears to be highly conserved region in fir species.

Key words: *Abies*, Turkish firs, cpDNA, *matK region*, phylogenetic tree.

ÖZ

KLOROPLAST GENOMUNDAKİ *mat*K GEN BÖLGESİNİN KARŞILAŞTIRILMASI YAPILARAK TÜRKİYE GÖKNARLARININ FİLOGENETİK ANALİZİ

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Pineacea ailesi tüm dünyada çok sayıda türle temsil edilen kozalaklı ağaçlar grubunun en yüksek türle temsil edilen ailesidir. 51 türe sahip olan *Abies* cinsi ise *Pinus* cinsinden sonra bu ailenin ikinci büyük üyesidir.

Ülkemizde bu cinse ait altı adet doğal takson saf ve karışık meşçeler halinde yer almaktadır. Bu taksonlardan dört tanesi (*Abies cilicica* subsp. *isaurica*, *Abies nordmanniana* subsp. *bornmülleriana*, *Abies nordmanniana* subsp. *equi-trojani, Abies x olcayana*) endemik olup az tehdit altında (LR: low risk) tür kategorisinde yer almaktadır.

Türkiye'de bulunan *Abies* türlerinin filogenetik ilişkilerini yeniden belirlenmesi amacıyla ülkenin farklı bölgelerinden türleri temsilen 18 farklı populasyon örneklenmiştir. Türk göknar türlerine ait kloroplast DNA'sının *mat*K gen bölgesi kullanılarak türler arasındaki genetik ilişki değerlendirilmiştir. Bunlara ek olarak tüm dünyada çalışılmış ve NCBI veri bankasında yer alan tüm

Abies türlerine ait *mat*K gen bölgesi dizileri de analiz için kullanılmıştır. Kloraplast DNA 'sının *mat*K bölge dizileri Göknar türlerinin ilişkilerini yansıtan filogentik ağaçlar, korunmuş bölge, çeşitlilik gösteren bölge vb. parametreler MEGA bilgisayar yazılımını (versiyon 5) yardımı ile değerlendirilmiştir.

Sonuçlar göstermiştir ki Türkiye göknarları, *mat*K gen bölgesinin verilerine göre bir farklılık göstermemektedir ve bu bölgenin evrimsel olarak oldukca korunmuş bir bölge olduğuna işaret etmektedir.

NCBI veri bankasından dünyadaki göknar türlerinin tüm matK gen bölgesine ulaşılamamasına rağmen, elde edilinebilen kısmı Türkiye göknarlarının matK gen bölgesi ile karşılaştırmalı olarak analiz edilmiştir. *mat*K1 gen bölgesi verilerine göre sonuçlar göknar türlerini üç ana gruba ayırmaktadır. Ana grupların bir tanesi, Türkiye göknarlarının tüm türlerini ve bir adet Avrupa göknarlarından olan *A.numidica* türünü kapsamaktadır. İkinci ana grupta dünyadaki diğer göknar türlerinden *A.holopylla, A.firma, A.veitchii, A.sachalinensis, A.nephrolepis, A.lasiocarpa, A.koreana, A.homolephis, A.fraseri, A.fargesii, A.sibirica* and *A.fabri* yer almaktadır. Üçüncü ana grubu ise *A.mariesii, A.hidalgensis, A.bracteata, A.alba* oluşturmaktadır. *Mat*K1 gen bölgesi sonuçlarına gore Türkiye göknarlarının Avrupa göknarlarına yakın olduğu söylenebilir. Bunlara ek olarak, *mat*K2 gen bölgesi verilerine gore yapılan analizlerde ise filogenetik ağaçta Türkiye göknarları tek bir ana grup oluşturmakta ve *mat*K2 gen bölgesi verilerine ulaşılabilen *A.veitchii, A.firma* ve *A.holopylla* türleri ise diğer grupta yer almaktadırlar.

Sonuçlar Türkiye göknarlarının ortak atadan evrimleştiklerini ve *mat*K gen bölgesinin de bu türler için korunaklı bir bölge olduğunu düşündürmektedir.

Anahtar kelimeler: Abies türleri, Türkiye Göknar türleri, cpDNA, *mat*K, filogenetik ağaç

to my family...

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

β-ME   Beta Mercaptoethanol

cpDNA   Chloroplast DNA

CTAB   Cetyl Trimethyl Ammonium Bromide

DNA   Deoxyribonucleic Acid

dNTP   Deoxyribonucleotide Tri-Phosphate

EDTA   Ethylene Diamine Tetra Aceticacid

ETOH   Ethanol

ITS   Internal Transcribed Spacer Region

IUCN   The World Conservation Union

MEGA   Molecular Evolutionary Genetic Analysis

mtDNA Mitochondrial DNA

matK   Maturase K

NCBI   National Center for Biotechnology Information

NJ   Neighbour-joining

nrDNA   Nuclear ribosomal DNA

OTUs   Operational Taxonomical Units

PCR   Polymerase Chain Reaction

rbcL   RuBisCo Large subunit

SDS   Sodium Dodecyl Sulphate

TE   Tris EDTA Buffer

t-RNA   Transfer Ribonucleic Acid

trnK   Lysine tRNA,

CHAPTER 1

INTRODUCTION

1.1 General Review on Pinaceae Family and Genus *Abies*

Pinaceae is the largest family of conifers that includes millions of individuals all over the world. This family is composed of approximately 260 species which naturally occure in temperate forests of the Northern Hemisphere. The ancestors of Pinaceae family were evolved during the Cretaceous Era according to fossil records.

In the Mediterranean Basin, conifer forests were excessively utilized for centuries. Individuals of the family are economically valuable and many species are sources of timber, paper pulp, oils and resins. In addition, some species are used as ornamentals. For that reason, materials from *Abies* in the Pinecea family are widely used (Esteban *et al.*, 2010).

Abies is the second largest genus of family Pinaceae composing about 51 species, which are natural to the Northern Hemisphere. *Abies* species are naturally distributed in North America, Central America, Europe, and North Africa, Asia (South China, Himalaya and Taiwan). They mostly prefer the mountainous lands that are boreal and temperate regions of the northern hemisphere (Liu, 1971) (Figure 1.1).

Figure 1.1 Distribution map for *Abies* genera (Adapted from Farjon *et al*. 1990)

Abies species are significant for human utilization. Although *Abies* wood is not appropriate for general timber use, most firs' wood is used as pulp or for the production of plywood and rough timber. Since its wood is indurable to insect or rot after logging, firs are generally used for indoor construction purposes. Nordmann Fir *(Abies nordmanniana)*, Noble Fir *(Abies procera)*, Fraser Fir *(Abies fraseri)* and Balsam Fir *(Abies balsamea)* are the best choices for christmas tree farming, because of their aromatic foliage and non-spilled needles on drying out. Moreover, many fir species are used in gardens as decorative trees such as, Korean Fir (*Abies koreana*) and Fraser Fir (*Abies fraseri*), due to their brightly colored cones (www.conifers.org, 2010)(Figure 1.2).
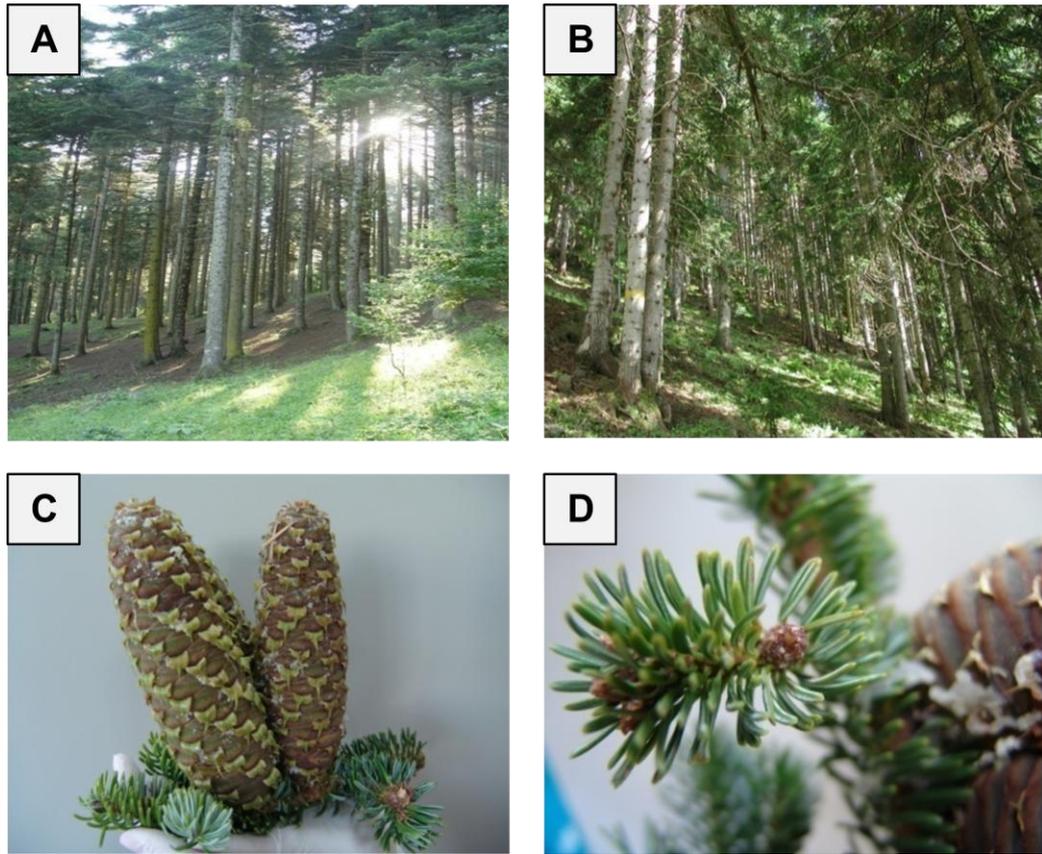
Figure 1.2 General appearance of Fir and some of its features A. General appearance *(A. bornmulleriana*, **Bolu-Kökez***)*, **B. Trunk of mature tree, C. One-year old female conelet, D. Male cone (***A. nordmanniana,* **Şavşat-Artvin) (Forest Tree Seeds and Tree Breeding Research Directorate)**

For quite a long time, due to the great variability of morphological traits, there was a conflict about the taxonomy of the genus *Abies*. Farjon and Rushforth (1989), has made revisions by using the previous classification schemes that supplied by Liu (1971). Liu (1971) indicated that there are 39 species, 23 varieties and 8 natural hybrids between sympatric species growing in all over the world. On the other hand, more recent classification by Farjon and Rushforth (1989) revealed that *Abies* has as many as 49 species, 23 varieties and 1 natural hybrid.

1.2. Biology of Turkish Firs

1.2.1. Natural Distribution of Turkish Firs

Turkey is located in European-Siberian, Mediterriannean and Iran-Turanian Plant Geographic Regions. Therefore, the ratio of the endemism in Turkey is very high. There are almost 10500 taxa and 3500 of them are endemic (Kaya and Raynal, 2001). Fir species are widely dispersed forest tree species in Turkey. Four *Abies* taxa are naturally found as endemic and evaluated as low risk (LR) species with respect to IUCN categories (Ekim *et al.*, 2000).

Both in high lands in southern latitudes, and lower latitudes even if sea levels in northern latitudes, firs prefer pure or mixed stands (Kayacik 1980; Yaltirik and Efe 2000). Firs comprise 3% of the total Turkish forests, covering 626647 ha natural dispersion area. Yearly, about 532913 m^3 wood have been harvested from 4712, 3 ha area by approved cuttings (Anonymous 2006).

There are four endemic fir species naturally occurring in Turkey. One of them is Uludağ Fir (*Abies nordmanniana* (Stev.) subsp. *bornmulleriana* (Mattf.)(Figure 1.3). The natural range of Uludağ Fir includes both coastal types with durable to moisture (in Zonguldak-Ayancık) and in land type with highly durable to drought (in Kızılcahamam-Gerede) (Saatçioğlu, 1976). Its natural distribution area includes Black Sea region from 1000 - 2000 m high and east-south of Marmara region (from Kızılırmak to Uludağ Mountain). This fir occurs as pure and mixed forests with beech, and pine in Uludağ, Köroğlu, Bolu, Ilgaz and Küre Mountains (Saatçioğlu 1976).
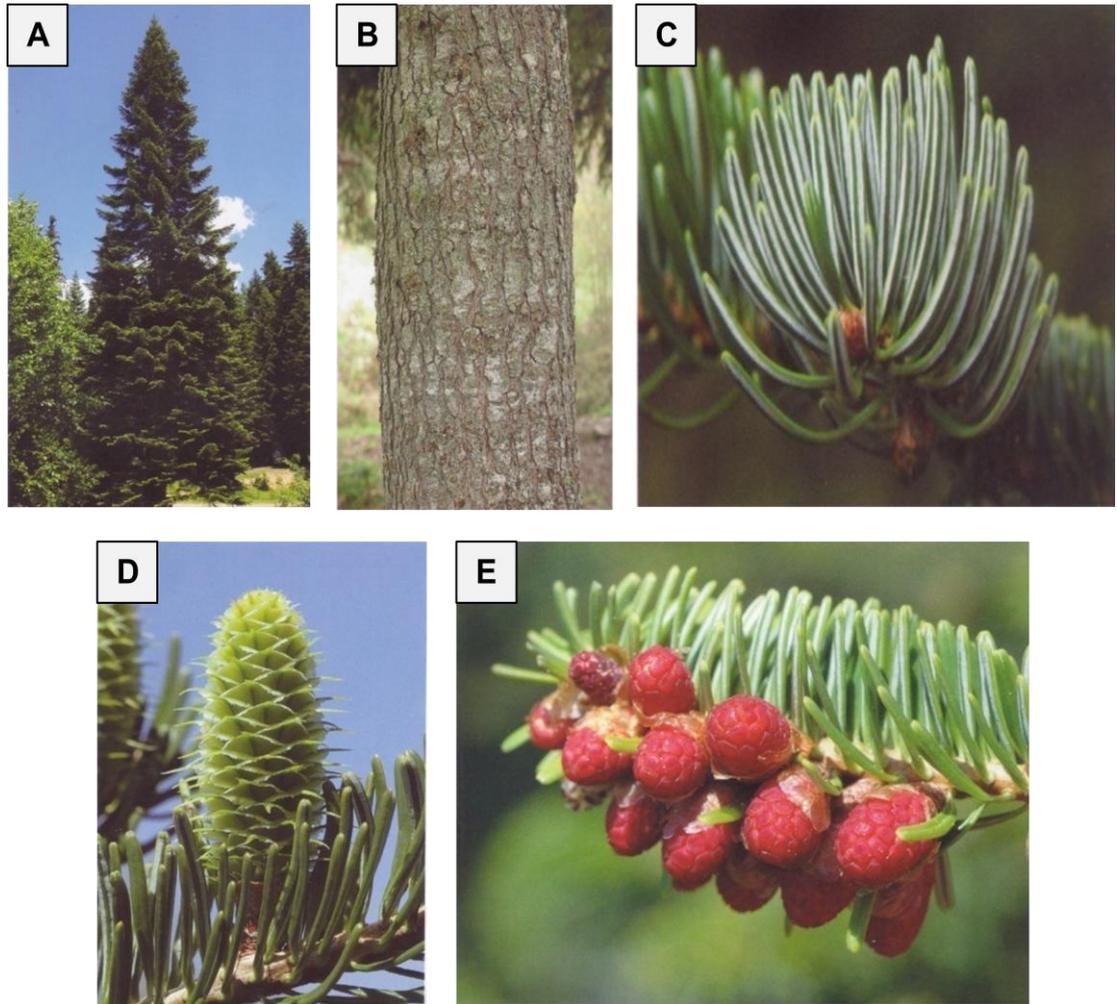
Figure 1.3 General appearance of *A. bornmuelleriana* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (Mamıkoğlu, 2007).

Kazdağı Fir (*A. nordmanniana* subsp*. equi-trojani* (Asch. *et. al* Sint.)) (Figure 1.4) is a narrow endemic species that only occurs in the Kazdağı Mountain in north-west of Turkey at 400 m – 1650 m high (Saatçioğlu, 1969; Vidakovic, 1991). It has a limited distribution covering 3600 ha (Gülbaba *et al.*, 1998). Kazdağı fir naturally occurs and forms stands with different species such as *Quercus* species, Anatolian black pine (*Pinus nigra* Arnold subsp. *pallasiana* (Lamb) Holmboe) and Oriental beech (*Fagus orientalis* Lipsky) (Ata 1975). Because of its growing area and competence of growing faster than other fir

species, is the species is identified as a priority species in breeding programs as a valuable genetic resource (Kaya *et al*., 1997).
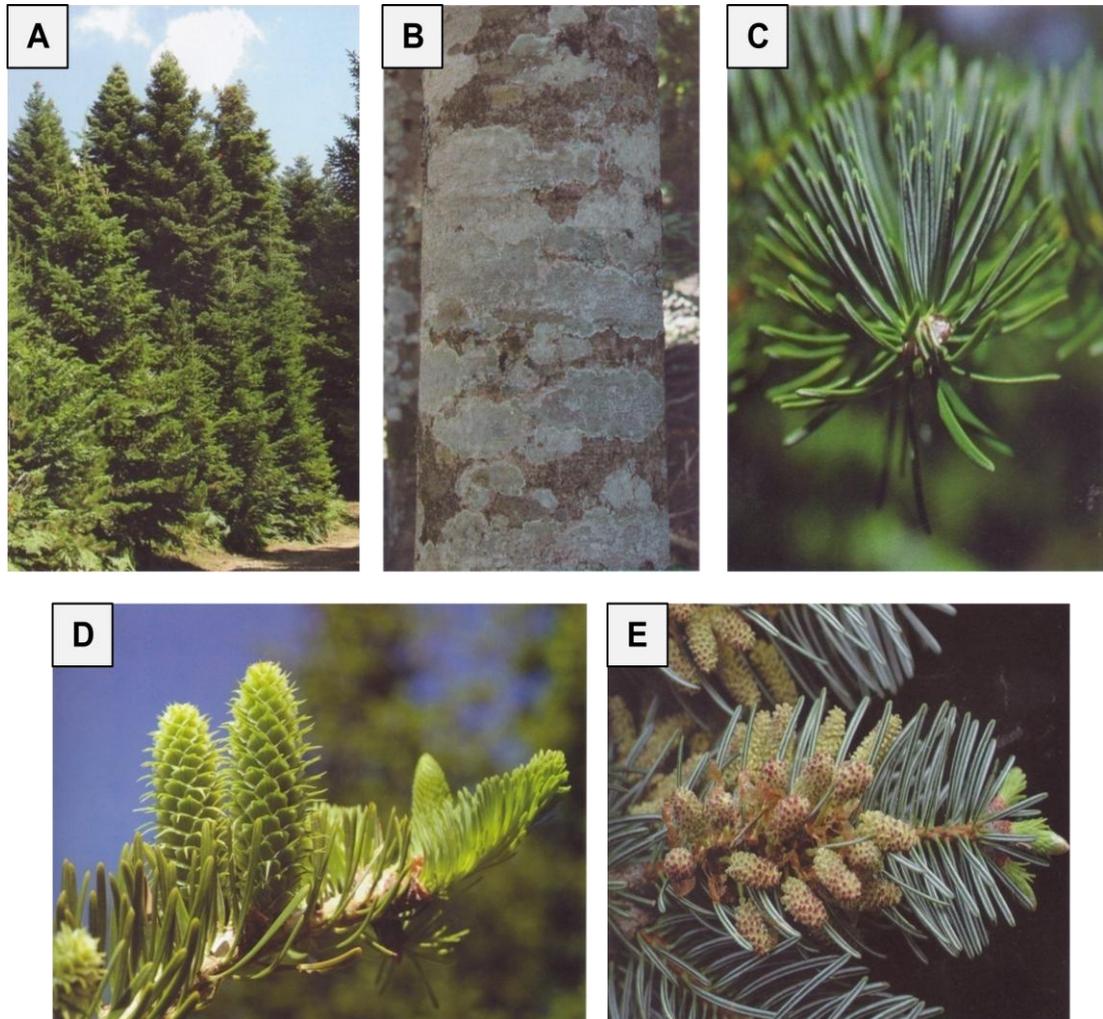
Figure 1.4 General appearance of A. *equi-trojani* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female cone let, E. Male cone (Mamıkoğlu, 2007).

Cilicican Fir (*Abies cilicica*) is naturally found in Middle Anatolia (Figure 1.5), east Taurus Mountains and Amonos Mountains in Turkey at the elevational range of 1150m- 2000m (Saatçioğlu 1976; Kayacik 1980; Mayer and Aksoy 1988). Cilician fir has two subspecies; *i) Abies cilicica* subsp. *isaurica* Coode & Cullen is an endemic species and exists in western Taurus Mountains

(Antalya-Konya), ii) *A. cilicica* (Ant. & Kotschy) Carr. subsp. *cilicica* exists in eastern Taurus Mountain (Mersin, Adana, Kahramanmaraş, Hatay) (Bozkuş 1988). In the Taurus Mountains, Cilicican Firs naturally exist with *Cedrus libani* A.Rich, *Juniperus excelsa* M. Bieb., *J. foetidissima* Willd. and *Pinus nigra* Arnold subsp. *Pallasiana* (Doğan *et al.*,2010).
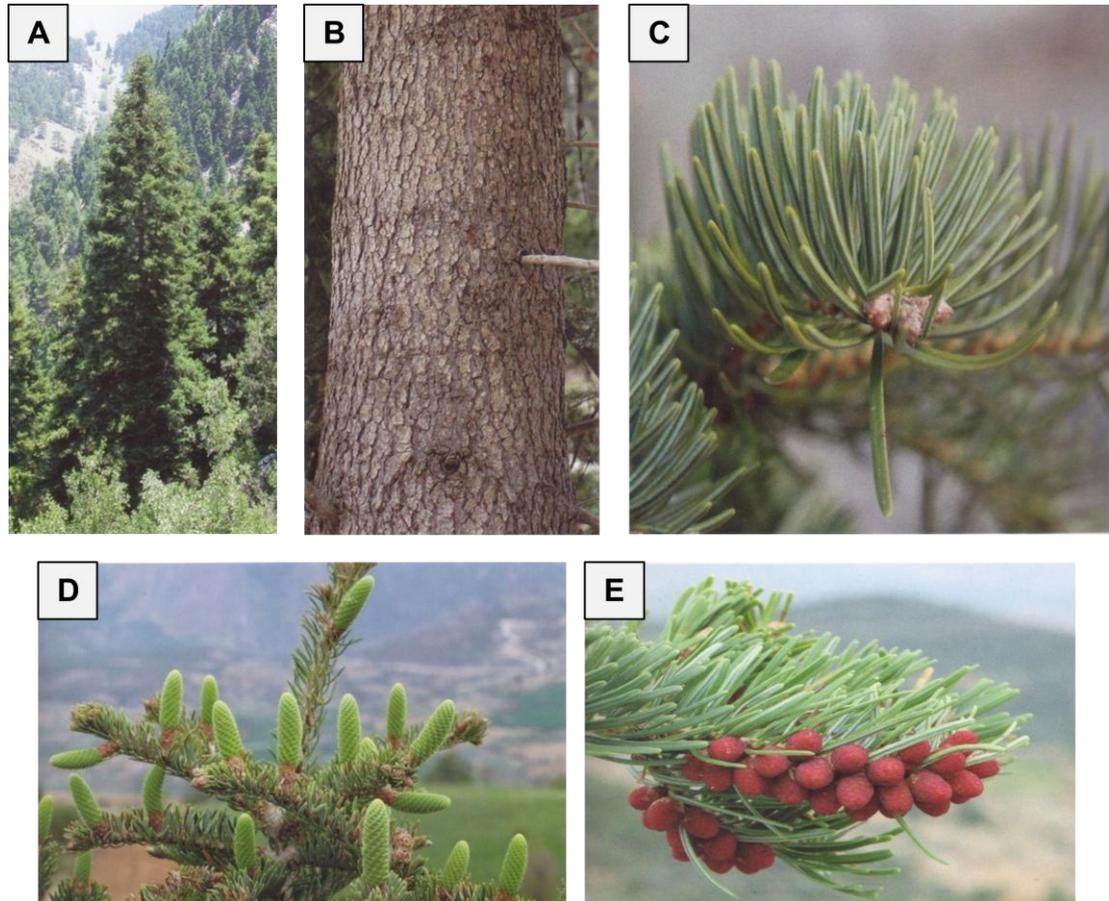
Figure 1.5 General appearance of *A. cilicica* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (Mamıkoğlu, 2007).

Nordman Fir (*A. nordmanniana* subsp. *nordmanniana* (Stev.) Spach) (Figure 1.6) grows at 1000 -2000 m altitudes in North Eastern Mountains of Turkey (Liu 1971). This fir species often occurs as pure or mixed stands with other species such as Oriental beech (*Fagus orientalis* Lipsky), Oriental spruce

(*Picea orientalis*) and Scots pine (*Pinus sylvestris*) on southern slopes of the North Eastern Mountains in Turkey (Mattfeld 1928; Kayacik 1980).
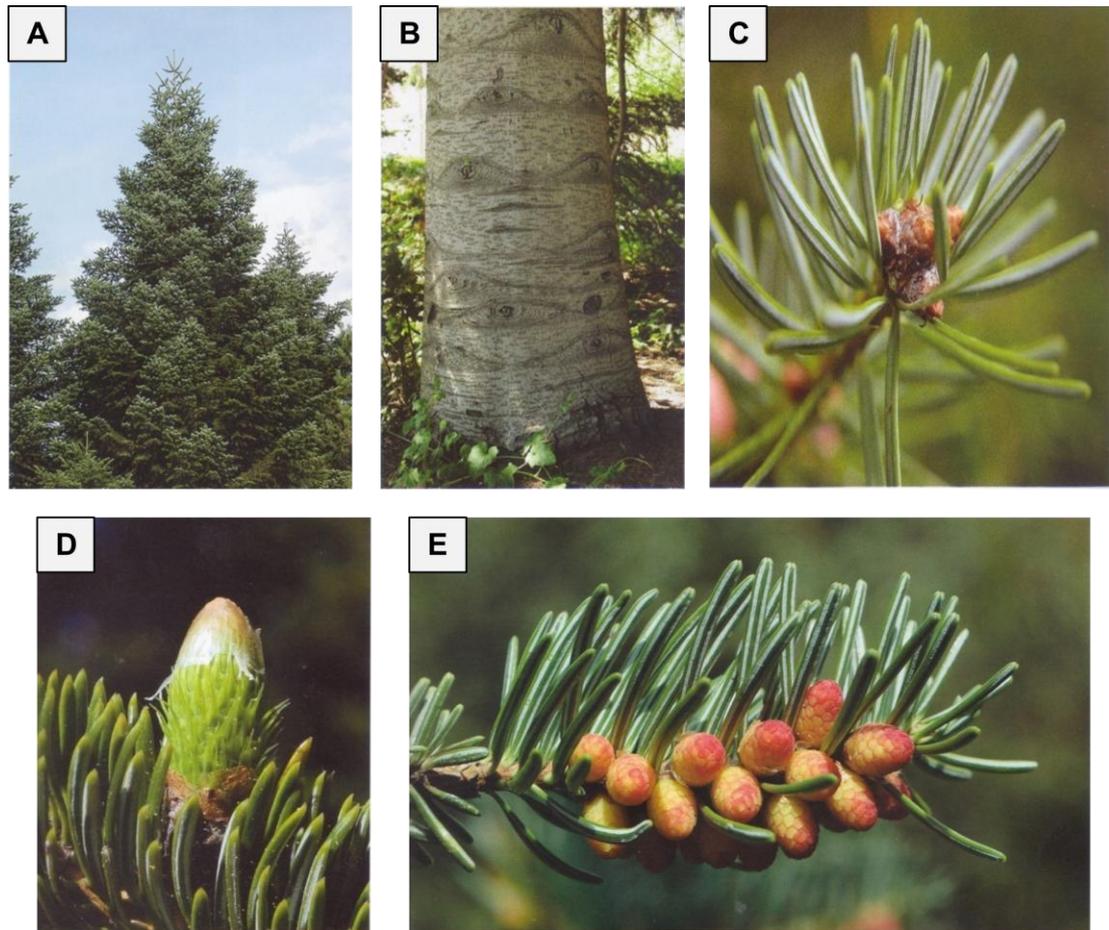
Figure 1.6 General appearance of *A. nordmanniana* and some of its features A. General appearance, B. Trunk of mature tree, C. Needle-like leave, D. One-year old female conelet, E. Male cone (Mamikoğlu, 2007).

Moreover, an exceptional endemic taxon exists in Turkey in Çataldağ area located between Susurluk (Balıkesir) and Mustafa Kemal Paşa (Bursa) at 800 – 1300 m in altitudes (Ata and Merev, 1987). This fir species grows in pure and mixed stands. Although this taxon is morphologically very similar to Kazdağı fir, its pollen characteristics and wood anatomy are different. Moreover, it grows faster than other fir species (Ata and Merev, 1987).

1.2.2. Taxonomy of *Abies* spp. in Turkey

Previous studies used different approaches to classify Turkish Firs. Ata (1975) reported that naturally grown firs around Mediterranean, Europe and Anatolia indicate distinct features with regard to their habitats, external, internal and pollen morphology. For instance, although it was reported by Davis (1965) that Kazdağı fir has buds without resins while the others have resinous buds (Mattfeld 1928; Gökmen 1970; Kayacik 1980).

Davis (1965) reported that *A.cilicica* and *A. nordmanniana* can only be separated from each other via their ripen cone characters. Although *A.nordmanniana* bracts are emitted between the cone scales, *A.cilicica* bracts are not. This feature is also parallel with geography. He reported that *A.cilicia* is only exists in south and *A. nordmanniana* exists only in northern Turkey. Therefore; Uludağ and Kazdağı firs are recognized as a subspecies of Nordman fir.

Davis (1965) also reported that if *A.nordmanniana* subsp.*equitrojani* is intervened between *A.nordmanniana* subsp.*bornmulleriana* and *A.cephalonica* from Greece, all replacement fir series from Caucasus to western Europe might be distinguished*: A. nordmanniana* (subsp. n*ordmanniana,* -subsp. *bornmulleriana,* -subsp. *equi-trojani*) – *A. cephalonica* – *A. borisii-regis* – *A. alba.*

Moreover Davis (1965) indicated that *A.nordmanniana* species were diagnosed according to morphological characteristics. The following key was provided for diagnosis.

1. If leaves are just a little acute; buds are not resinous; young shoots are glabrous, ……………*Abies nordmanniana* subsp. *equi-trojani*

2. If leaves are truncate or emarginate; buds are resinous and / or shoots are hairy
    a) If shoots are hairy; buds are not resinous;…………………....
       …………………………*Abies nordmanniana* subsp. *nordmanniana.*
    b) If shoots are glabrous; buds are resinous;…………………..
       …………………………*Abies nordmanniana* subsp. *bornmulleriana.*

Furthermore Davis (1965) said that two subspecies of *A. cilicica* were identified and they are discriminated by:

1. If buds are not resinous and young shoots are hairy;
   ………………………………………….*Abies cilicica* subsp. *cilicica.*

2. If buds are resinous and young shoots are glabrous;
   ………………………………………*Abies.cilicica* subsp. *isaurica.*

Aytuğ (1959a) reported that four species of Turkish firs have very different via internal features such as vascular rays and cell number. Identifying *Abies* species according to their morphological features is very confusing (Aytuğ 1959a; Ata 1975; Bozkuş and Çoban 2006); therefore; the internal morphological characteristics were studied extensively by many researchers (Aytuğ 1959a; Ata 1975; Ata and Merev 1987). For instance, according to Aytuğ (1959a), four fir species are very different from each other especially in terms of cell number and maximum length of vascular rays.

1.2.3. Botany of Turkish Firs

Most of fir species are morphologically alike. They are evergreen, erect, pyramidal and narrow. Except, *Abies nordmanniana*, all fir species have drooped branches. The crown of the tree is vertical. They are resistant to winds and storms. Amount of resinous of buds are changed from species to

species. Genarally fir's needles are 2-3 cm long, green and with 2 white bands underneath and their cones are cylindrical and 12-15 cm in length.

Abies nordmanniana grows about 60 m tall in the Caucasus Mountains. It is conical with a spire-like crown. Even though main branches are horizontal, lower branches are sweeping downwards. Its bark is grayish and smooth when it is young. However it is brownish and rough when it is older. Buds are not resinous, but they are conical-ovate and red-brown. Needles are 2-3 cm long, green and above with 2 white bands underneath; linear and side parallel, their tips are rounded. Cylindrical cones are 14 cm long and tip pointed.

Abies cilicica has a narrow columnar shape with a spire-like crown. Bark is beech grey and buds are not resinous, but ovoid with a conical tip. Leaves are 3 cm long and 2 mm wide and green and with 2 white bands underneath. They have a rounded tip with a tiny notch. Cones are cylindrical with noticible tapper toward tip and 14 cm long-4 cm wide.

Abies bornmulleriana is rounded conical and broad crown. Young bark is grey and smooth though old bark is rough. Different than other Turkish firs, buds are resinous. Leaves are 2.5-3cm long and have rounded tip like other firs. Cones are 12-15 cm long and 4 cm wide and have turret shape (Warren and Johnson, 1988).

1.2.4. Importance of Turkish Firs

Fir species are very valuable because of their high utilization potential and technological features for timber industry. Their woods are used as raw material for cellulose and paper, for furniture industry since they do not have resin canal. In addition, wood of firs is very soft and light colored (Anşin and Özkan, 1997). Uludağ Fir is especially preferred by Turkish timber industry. The features of heat-treated timber of Uludağ Fir offer manyutlity areas for timber industry (Yaltirik and Efe, 2000).

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Abies cilicica resins are traditionally used as an antiseptic, anti-inflammatory, antipyretic, antibacterial and antiviral medicine and as a chewing gum against some stomach diseases (e.g., ulcer), lip-dryness, asthma and curing wounds (Baytop, 1999).

Nordman fir has a great economical value in forest tree industry and marketing. Because of its evergreen, late scattered leaves this fir species is also used as a Christmas tree in north European countries (Nielsen 1993). Moreover, the leaves of Nordmann Fir has an important feature that they can ensure cheap source as biosorbents for toxic metal removal from natural or waste waters (Serencam *et al.,* 2000).

1.3. Molecular Markers and cpDNA of Plants

The development of molecular markers, suitable for statistical procedures, and user-friendly computer software that applied these statistical procedures permitted the detection of molecular markers associated with quantitative trait loci (QTL) for complex traits. Marker-assisted selection was then offered as a means of exploiting markers related with QTL to develop improved cultivars (Bernardo, 2008).

Molecular marker techniques in forest genetics and tree breeding have developed rapidly. Several types of markers have been used for population genetic analyses (Neale *et. al.*, 1992).

The molecular markers are supreme to the previous performed markers which are morphological or biochemical markers. Data which are obtained from molecular analyses are based on the DNA sequences. Therefore; they are neutral to environmental conditions. The polymorphisms which are defined via DNA analyses are highly versatile (Neale *et al.*, 1992). In order to study population genetic analyses, molecular markers are the best tools to understand genetic variability within and between tree populations (Aronen 1995).

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There are three main types of DNA sequence data in plants. These are nuclear (nDNA), chloroplast (cpDNA), and mitochondrial (mtDNA) DNAs which are used for genetic studies in plants (Simpson, 2006). The nDNA is not usually utilized in systematic botany due to having mixed and highly repetitive features. Moreover, mtDNA is utilized at species level because there are quick changes in its size, configurations and gene order (Liang, 1997). On the other hand, cpDNA is generally utilized for phylogenetic studies, (Palmer, *et al.*, 1988; Learn, *et al.*, 1992). Typically, chloroplast genomes range in size from 120 to 170 kilobase pairs (kb), and there is a relatively high degree of conservation in size, structure, gene content, and linear order of the genes in land plants (Shaw *et. al.* 2007). The non-coding parts of cpDNA show high frequency of mutations. Therefore, these parts are generally used in evolutionary-relationship analyses (Taberlet *et al.*, 1991). The cpDNA includes first single copy genes and it is relatively efficient component of plant total DNA. Therefore, it is easy to extract and analyze. Furthermore, chloroplast genome is much more conservedthat is very useful in plant systematics and widely used in phylogenetic reconstructions (Liang, 1997).

1.4. Recent Studies in *Abies* spp.

In order to realize the phylogenetic positions and genetic structure of *Abies* species, different molecular markers from different genomes are used. ITS (internal transcribed spacer), *trn* (transfer ribonucleic acid), *mat*K (maturase K), SSRs (Simple sequence repeats), rpoC (RNA polymerase beta prime subunit) and rbcl (ribulose-bisphosphate carboxylase gene) are the most frequently used genes and markers. However, for Turkish *Abies* species, studies are limited. The first studies were accomplished by Miraboğlu (1955, 1957) and Aytuğ (1958). Şimşek (1992), Gülbaba *et al.* (1998), Çiçek *et al.*, (2005), Özer (2000), studied isozyme variations in different *Abies* spp. in Turkey. According to Şimşek (1992), there is not any relationship between Kazdağı Fir and Uludağ Fir. Kazdağı Fir is not a hybrid, but a separate species. Moreover, Gülbaba *et al.* (1998) and Çiçek *et al.*, (2005) studied

Kazdağı Fir and reported that this species is genetically different from other Turkish Firs.

Also, Fady and Conkle (1993) reported that A.alba and *A. bornmulleriana* have genetically related based on 22 isozyme loci. Scaltsoyiannes *et al.* (1999) studied nineteen natural Mediterranean Fir populations, belonging to eight species *(A. alba, A. cephalonica, A. bornmulleriana, A. nordmanniana, A. equi-trojani, A. pinsapo, A. numidica, A. cilicica)* and one natural hybrid (A. x *borisii-regis)* by using starch and polyacrylamide gel electrophoresis. The results indicated new phylogenetic relationships that high genetic similarity was seen both between the Calabrian Fir population and the one from northwest Greece, and between *A. equi-trojani* grown in Asia Minor and the southern Greek populations.

Some researchers studied phlygenetic relationships of *Abies* spp using molecular markers. These studies were done with different molecular markers such as *trn*, *mat*K, *rbcl* regions of cpDNA and tandom repeats, microsatellites of nDNA and cp DNA (Suyama *et al.* 2000; Isoda *et al.* 2000; Kormutak *et al.* 2004; Ziegenhagen *et al.* 2005; Cremer *et al.* 2006; Kaya *et al.* 2008; Liepelt *et al.* 2010). Most of them found that Turkish Fir species are genetically very different from other firs. Nordmann fir is separated from three other fir species in northern parts of Turkey; furthermore, studies point out the possiblity of an ancestral species.

1. 5. *mat*K Gene

There are various studies where *mat*K (maturase K) gene sequence is used in phylogenetic analysis so far. These studies include family, genera and species levels.Previous studies found that the sequences of *mat*K gene in cpDNA are varied among plant species. The *mat*K gene is located in the large single copy region of the chloroplast genome, in the vicinity of one of the inverted repeat regions (Figure 1.7). Mostly, the open reading frame (ORF) of the gene is about 1,500 base pairs (bp) in length (Hilu and Barthet,

2008). In angiosperm phylogenetic studies, the *mat*K gene has become one of the most used chloroplast markers. As *mat*K can easily be co-amplified with the flanking non-coding intron parts, the complete *trnK* intron is increasingly used, expanding the dataset to 2400–2700 bp. Therefore, the utility of this region could be extended to the inter- and intra-species level (Müller and Borsch, 2005a; Wanke et al., 2006).For that reason, *mat*K gene supplies researchers with a simple alternative way of recognizing different plant species (www.science20.com, 2011).
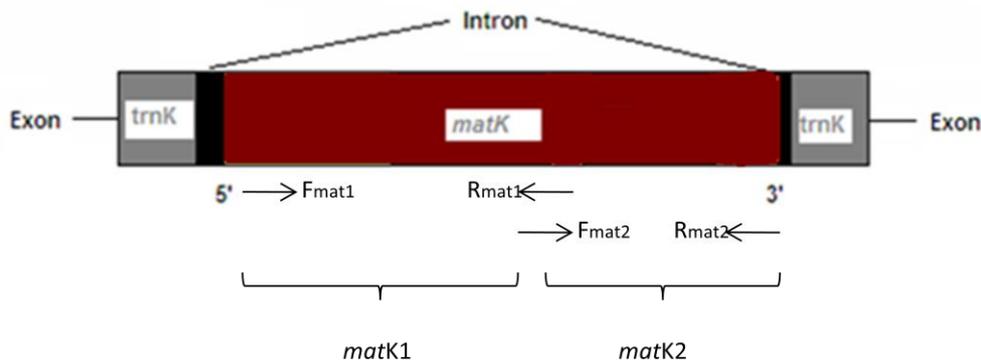
Figure 1.7 Position of *mat*K gene region on chloroplast DNA (Adapted from Li *et.al*.1997).

Rather than any other studied chloroplast genes, the matK was indicated to have higher variation. Although, the variation was slightly higher at the 5ternativ than at the 3' region, approximately even distribution was observed throughout the entire gene. Moreover, the high ratio of transversion (altering from purine to a pyrimidine) or transition in the *mat*K gene may provide high phylogenetic knowledge. Therefore, all of these features make *mat*K gene be useful in molecular systematic studies (Tanaka *et al.*, 1997). In addition to this, due to its evolutionary theme and steps, *mat*K is the most useful gene that used in angiosperm phylogeny (Olmstead and Palmer, 1994; Hilu and Liang, 1997; Hilu *et al.*, 2003). In addition to all, the *mat*K gene evolves roughly three times faster than plastid genes *rbcL* and *atpB* (Hilu et al., 2003)

1.6. Significance of The Study

The taxonomic status of Turkish firs is not clear yet. There are some discrepancies that if *A. equi-trojani* and *A. bornmulleriana* are different species or subspecies of *A. nordmanniana*. Moreover, some researchers claim that A. *equi-trojani* is a hybrid among *A. nordmanniana* and *A. cephalonica* (Liu, 1971). Furthermore, *A. cilica* subsp. *isaurica* is not accepted as subspecies of *A.cilicica.* With taxonomic confusion and wide morphological variation, with the expectation that molecular data which has been generated by this study could provide some additional information   to explain the relationships within *Abies* genus further. Moreover, with the advance of molecular markers, it is expected that molecular data can provide new perspectives in construction of new phylogeny. It is now possible to use DNA markers to solve the problems of conservation genetics and plant taxonomy.

This study has undertaken the challenging phylogenetic issue   of the *Abies* taxa distributed in Turkey. Moreover, the study has aime to shed lighy further on phylogenetic relationships between Turkish firs and other European, American and Asian fir species.

CHAPTER 2

OBJECTIVES OF THE STUDY

The general objective of the study is to explore the phylogenetic relationships among Turkish Firs as well as the relationship between Turkish firs and other fir species by using *mat*K gene region of cpDNA

The specific objectives of the study are:

- To obtain molecular diversity data from *mat*K region of cpDNA that may help to solve taxonomic problems of Turkish Firs,
- To provide information on pattern of *mat*K 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

Open pollinated seeds of six *Abies* taxa (Table 3.1), which were collected from different parts of Turkey by the Forest Tree Seeds and Tree Breeding Research Directorate, Ministry of Environment and Forestry, were utilized in this study (Figures 3,1; 3,2 and 3,3). Seeds were removed from cones and hold in +4°C. These seeds are collected from 20-25 mother trees for every population. All the data that include population's locations, types, altitude, and latitude were provided by the Forest Tree Seeds and Tree Breeding Research Directorate, Ministry of Environment and Forestry and presented on Table 3.2.

Figure 3.1 Fir cone (*A. nordmanniana* subsp. *bornmuelleriana*, Bolu-Kökez) (Photographs provided by the Forest Tree Seeds and Tree Breeding Research Directorate).

Table 3.1 The number of Turkish fir populations included this study.

Studied *Abies* Species in Turkey	Number of Population
Abies nordmanniana subsp. *nordmanniana*	3
Abies nordmanniana subsp. *bornmuelleriana* (endemic)	5
Abies nordmanniana subsp. *equi-trojani* (endemic)	3
Abies cilicica subsp. *isaurica* (endemic)	3
Abies cilicica subsp. *cilicica*	3
Abies x olcayana (endemic)	1
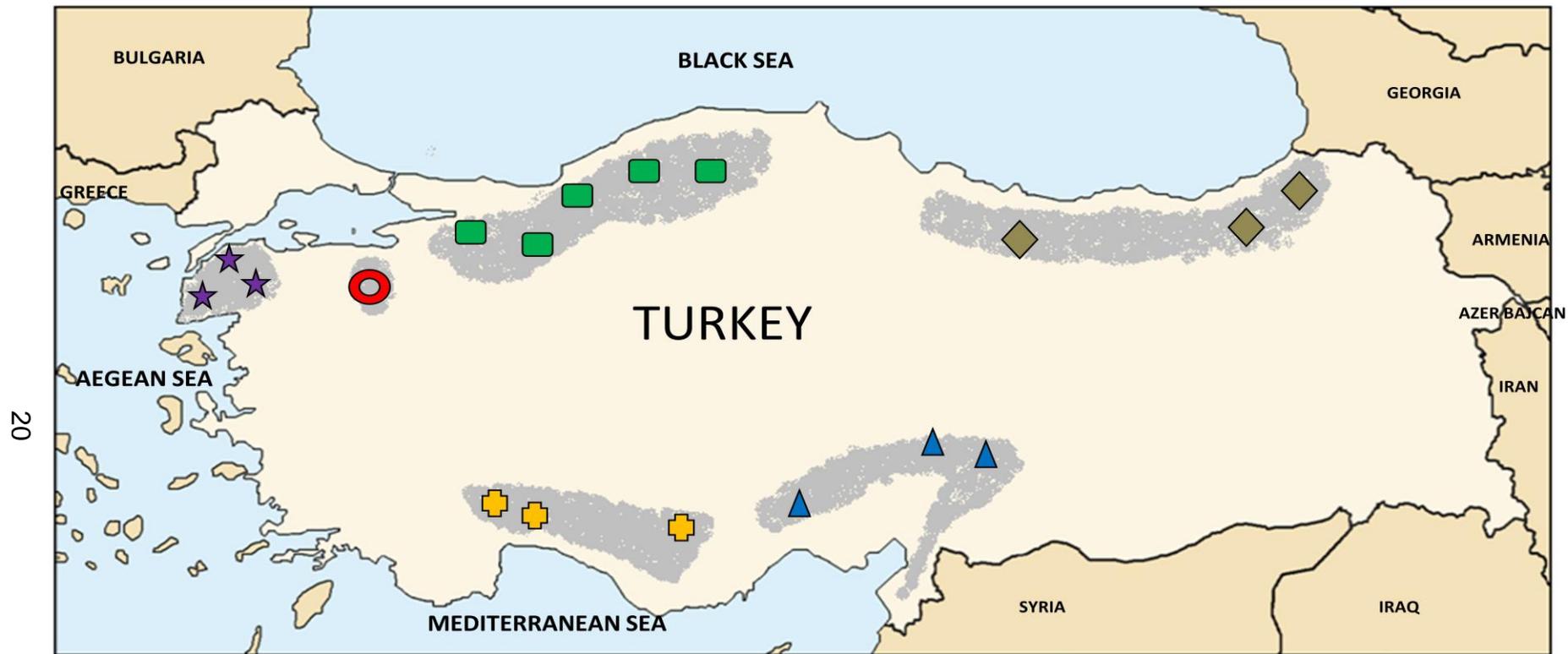
Figure 3.2 **Map showing the distribution of Turkish firs and sampled population sites. (** *Abies cilicica* **sub.sp.** *isaurica,* *Abies cilicica* **subsp.** *cilicica,* *Abies nordmanniana* **subsp.** *bornmulleriana,* *Abies nordmanniana* **subsp.** *equitrojani,* *Abies nordmanniana* **subsp.** *nordmanniana,* *AbiesXolcayana.***)**

Table 3.2 Description of studied Turkish Fir populations

POPULATION CODES	Taxon	Population	Type	Latitude	Longitude	Altitude(m)	Geographic exposure
ANN1	*A. nordmanniana* subsp. *nordmanniana*	Artvin-Ortaköy	Seed Stand	41°16'37"	41°57'47"	1600	N
ANN2	*A. nordmanniana* subsp. *nordmanniana*	Mesudiye-Arpaalan	Seed Stand	40°20'40"	37°51'40"	1850	N-NW
ANN3	*A. nordmanniana* subsp. *nordmanniana*	Artvin-Yayla	Seed Stand	41°13'25"	42°27'20"	1800	N
ANB1	*A. nordmanniana* subsp. *bornmuelleriana*	Uludağ Milli Parkı	Seed Stand	40°07'04"	29°07'35"	1600	N
ANB2	*A. nordmanniana* subsp. *bornmülleriana*	Akyazı-Dokurcun	Seed Stand	40°37'30"	30°51'00"	1300	SW-SE
ANB3	*A. nordmanniana* subsp. *bornmuelleriana*	Bolu-Kökez	Seed Stand	40°39'05"	31°36'56"	1300	N-W
ANB4	*A. nordmanniana* subsp. *bornmuelleriana*	Karabük-Sarıçiçek	Seed Stand	41°20'30"	32°36'24"	1500	N-NE
ANB5	*A. nordmanniana* subsp. *bornmuelleriana*	Bursa-Baraklı	Natural Stand	39°52'03"	28°21'10"	700	NW
ANE1	*A. nordmanniana* subsp. *equi-trojani*	Edremit-Gürgendağı	Seed Stand	39°45'48"	26°57'50"	1300	S
ANE2	*A. nordmanniana* subsp. *equi-trojani*	Kalkım-Eybekli (GKO)	Gene Conservation Forest	39°42'35"	27°07'42"	950	W
ANE3	*A. nordmanniana* subsp. *equi-trojani*	Çan-Çan (GKO)	Gene Conservation Forest	39°56'00"	27°05'33"	750	E
ACI1	*A. cilicica* subsp. *isaurica*	Akseki-Akseki(Kuyucak)	Seed Stand	37°06'51"	31°46'52"	1350	NE
ACI2	*A. cilicica* subsp. *isaurica*	Bucak-Uğurlu	Seed Stand	37°19'52"	30°37'41"	1200	N-W
ACI3	*A. cilicica* subsp. *isaurica*	Bucak-Y.Bademli	Natural Stand	37°19'52"	30°37'41"	1200	N-W
ACC1	*A. cilicica* subsp. *cilicica*	Saimbeyli-Tufanbeyli	Natural Stand	37°19'52"	30°37'41"	1200	N-W
ACC2	*A. cilicica* subsp. *cilicica*	Göksun-Göksun	Natural Stand	37°06'51"	31°46'52"	1350	NE
ACC3	*A. cilicica* subsp. *cilicica*	Tarsus-Cehennemdere	Natural Stand	37°06'51"	31°46'52"	1350	NE
AXO	*Abies x olcayana* (GKO)	MKP-Paşa	Gene Conservation Forest	39°52'03"	28°21'10"	700	NW

3.2. Extraction of DNA from Seeds

For this study, 5 parent trees were selected for each population. About 20-25 seeds per parent tree were used for every population.

For extracting DNA from seeds, Cetyl Trimethyl Ammonium Bromide (CTAB) method developed by Doyle and Doyle in 1990, was applied to embryonic tissues. The steps of DNA extraction were as follows (Figure 3.5);

1. Seeds were soaked in distilled water at 4°C for 48 hours before excising and removing seed coat and endosperm .
2. Then, the megagametophyte of each seed was carefully dissected away and preserved separately at -20°C for potential subsequent analysis.
3. 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. 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.
4. 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, supernatant was transfered to clean microfuge tubes and mixed with 500 μl chloroform – octanol (24:1) solution to denature proteins and inactivate DNAse .
5. 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.
6. The tubes were placed at -80°C for at least 60 min.

7. 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.

8. The pellets were left to dry at room temperature and re-suspended in 50 µl TE buffer to solve DNA.

9. The DNA samples were stored at -20°C. DNA presence was detected by 2% agarose gel electrophoresis (Figure 3.3).

After the DNA extraction, the presence and quality of extracted DNAs were checked by loading sample DNAs into %2 agarose gels. Then, quality checked DNAs were stored at -20°C in eppendorf tubes until they were used (Figure 3.3).
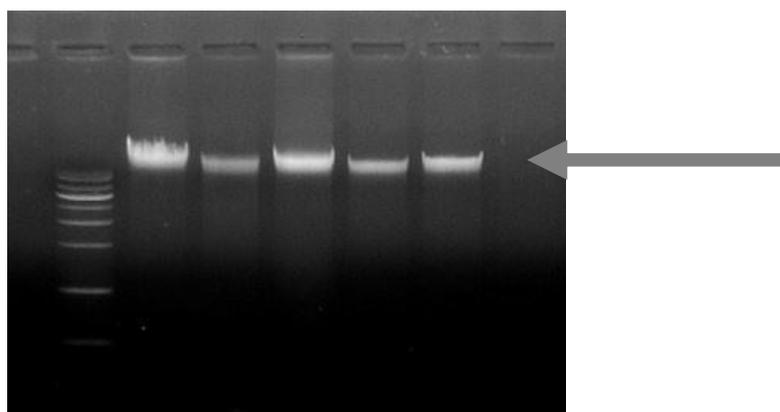
Figure 3.3 The photo showing 2% agarose gel electrophoresis of genomic DNA (pointed with an arrow).

3.3. DNA Quantification

Although the sample size was less than 25 in some populations, generally, about 20 to 25 DNA samples from every population of Turkish firs species were measured with Gene Quant pro RNA/DNA Calculator Spectrophotometer (manufactured by Biochrom Ltd, England, and October 2001).This instrument uses cuvettes. First, cuvette was filled 2 ml of ultra pure water and measurement was recorded and necessary calibration of the

instrument made. After the calibration, 1990 µl pure water and 10 µl DNA sample were put into cuvette for DNA quantification. The results of DNA measurements were given in Table 3.3. The DNA samples with maximum DNA concenrations were used in this study.

Table 3.3   Mean DNA concentrations of Turkis fir species. A- A. nordmanniana subsp. nordmanniana, B- A. nordmanniana subsp. bornmuelleriana, C- A. nordmanniana subsp. equi-trojani, D- A. cilicica subsp. isaurica, E- A. cilicica subsp. cilicica, F- Abies x olcayana

			DNA Concentration ng/µL		
A) *A. nordmanniana* subsp. *Nordmanniana*		Sample Size	Mean±SD*	Minimum	Maximum
Artvin-Ortaköy (ORT)		11	1618±154	634	2480
Mesudiye-Arpaalan (ARP)		25	1269±43	1412	2525
Artvin-Yayla (YYL)		14	1098±54	326	1882
(n=3)	Total	50	1328±216	1098	1618
B) *A. nordmanniana* subsp.*bornmuelleriana*					
Uludağ Milli Parkı (UMP)		20	1167±128	362	3511
Akyazı-Dokurcun (DKC)		21	953±58	199	1756
Bolu-Kökez (KKZ)		20	783±31	217	1683
Karabük-Sarıçiçek (SCK)		21	1815±112	470	3855
Bursa-Baraklı (BRL)		25	871±60	217	1919
(n=5)	Total	107	1118±371	871	1815
C) *A. nordmanniana* subsp. *equi-trojani*					
Edremit-Gürgendağı (GGD)		20	1675±80	271	3041
Kalkım-Eybekli (EYB)		25	1225±30	489	2407
Çan-Çan (CAN)		21	1249±124	253	4272
(n=3)	Total	66	1383±207	1225	1675

Table: 3.3 (Continue)

D) *A. cilicica* subsp. *isaurica*					
Akseki-Akseki (AKS)		25	2665±205	290	4652
Bucak-Uğurlu (UGL)		25	1811±116	597	3801
Bucak-Y.Bademli (BDL)		25	3125±86	1213	5032
(n=3)	Total	75	2534±544	1811	3125
E) *A. cilicica* subsp. *cilicica*					
Saimbeyli-Tufanbeyli (TFB)		25	2625±82	561	4054
Göksun-Göksun (GKS)		25	1849±119	181	4941
Tarsus-Cehennemdere (CHD)		25	2390±50	760	4181
(n=3)	Total	75	2288±325	1849	2625
F) *Abies x olcayana*					
MKP-Paşalar (MKP)					
(n=1)	Total	25	1426±34	652	3095

*SD=Standard deviation

3.4. Selection of *mat*K Primers for The Study

Although many *mat*K primers which were identified from different studies (Suyama et al., 2000, Gadek et al., 1996, Wang et al, 1999, Li et al., 1997), two sets of primers that were designed in Primer3 design program by Gulsoy (2010) were adapted for this study since they yielded the best DNA amplification in the PCR (Polymerase Chain Reaction).

The primer sequences are:
*mat*K 1-Forward Primer (Fmat1) : 5' GATCCTGTATCTTTTGCCAGGA 3'
　　　　Reverse Primer (Rmat1) : 5' GAACCTTTCGTCGCTGGAT 3'
*mat*K 2-Forward Primer (Fmat2) : 5' CTTTCGGGACGACAATAATC 3'
　　　　Reverse Primer (Rmat2) : 5' CGAGCTTCTGTTCCTCGTT 3'

3.5. Optimization of PCR (Polymerase Chain Reaction) Conditions for Turkish Firs

25 µl of reaction volume which included MgCl$_2$, dNTP (deoxyribonucleotide triphosphate) mixture, primers, and template DNA's was used for the optimization of PCR conditions. In Table 3.4, for *mat*K1 and Table 3.5 for *mat*K2, the tested reaction mixture combinations were shown, respectively. The optimized conditions were indicated in bold characters in these tables.

Table 3.4 Tested reaction mixtures for matK1 for optimization of PCR conditions

#	dH2O (µl)	10X Buffer	MgCl2 (50mM)	dNTP (mM)	Primer Pairs (10 µM)	*Taq* DNA polymearse (5u/µl)	DNA (5ng/µl)	PCR result
1	14.5µl	3.0µl	2.0µl	2.0µl	1.0µl + 1.0µl	0.30µl	1µl	No amplification
2	13.5 µl	3.0µl	2.0µl	2.0µl	1.50 µl + 1.50µl	0.30µl	1µl	No amplification
3	**14.20µl**	**3.0µl**	**2.5µl**	**2.0µl**	**1.0µl + 1.0µl**	**0.30µl**	**1µl**	**Good Amplification**
4	10.50µl	3.0µl	3.0µl	2.0µl	2.50µl + 2.50µl	0.30µl	1µl	No amplification
5	12.50µl	3.0µl	3.0µl	2.0µl	1.50µl + 1.50µl	0.30µl	1µl	No amplification
6	16.50µl	3.0µl	2.0µl	1.0µl	0.50µl + 0.50µl	0.30µl	1µl	No amplification
7	15.50µl	3.0µl	3.0µl	2.0µl	1.0µl + 1.0µl	0.30µl	1µl	No amplification

Table 3.5 Tested reaction mixtures for matK2 for optimization of PCR conditions

#	dH2O (µl)	10X Buffer (Fermentas, Ontorio, Canada)	MgCl2 (50mM) (Fermentas, Ontorio, Canada)	dNTP (mM)	Primer Pairs (10 µM)	*Taq* DNA polymearse (5u/ µl)	DNA (5ng/ µl)	PCR results
1	16.80µl	3.0µl	1.5µl	1.0µl	0.75µl + 0.75µl	0.20µl	1µl	No amplification
2	15.80µl	3.0µl	1.75µl	1.25µl	1.0µl + 1.0µl	0.20µl	1µl	No amplification
3	14.80µl	3.0µl	2.0µl	1.5µl	1.25µl + 1.25µl	0.20µl	1µl	No amplification
4	13.80µl	3.0µl	2.25µl	1.75µl	1.50µl + 1.50µl	0.20µl	1µl	No amplification
5	**12.80µl**	**3.0µl**	**2.50µl**	**2.0µl**	**1.75µl + 1.75µl**	**0.20µl**	**1µl**	**Good amplification**
6	11.80µl	3.0µl	2.75µl	2.25µl	2.0µl + 2.0µl	0.20µl	1µl	No amplification
7	14.30µl	3.0µl	1.25µl	1.5µl	2.25µl + 2.25µl	0.20µl	1µl	No amplification
8	14.30µl	3.0µl	1.0µl	1.0µl	2.50µl + 2.50µl	0.20µl	1µl	No amplification

Because of 50µL PCR reaction volume is required for sequence analysis, the *mat*K 1 and *mat*K2 reaction mixtures which were optimum for PCR amplification were adjusted to 50µL volume (Table 3.6). The adjusted reaction mixtures were used again for PCR in the thermal cycler (Eppendorf-Mastercycler, Eppendorf, Canada) machines to get the *mat*K sequences. The PCR cycling programmes were tested and the optimized one was given in Table 3.7. Approximately 5µl of each PCR product were uploaded to %1.0 agarose gel with 5µg ethidium bromide in order to see PCR products as the DNA band. The electrophorases was carried out with 1XTBE buffer and run

at 90 volts for 30 minutes. Then, the gel product was visiualized via gel imaging system (Vilbor Lourmat, France).

Table 3.6 Optimized PCR conditions for *mat*K region of chloroplast genome of Turkish Firs

PCR contents	matK1		matK2	
	Volume used in PCR (µL)	Final Concentration	Volume used in PCR (µL)	Final Concentration
PCR Grade Water	28.4	NA	25.6	NA
10X PCR Buffer	6.0	1X	6.0	1X
MgCl2 (50mM stock)	5.0	2.5 mM	5.0	2.5 mM
dNTP (10mM of each dNTP)	4.0	0.4 mM	4.0	0.4 mM
Forward primer (10µM)	2.0	1 µM	3.5	4 µM
Reverse primer (100µM)	2.0	1 µM	3.5	4 µM
Taq DNA polymerase (5u/µL)	0.6	0.5u	0.4	0.5u
DNA	2	10 ng/µL	2	10 ng/µL
Total Volume	50		50	

Table 3.7 Optimized thermal cycler program used for amplification of *mat*K region of chloroplast genome of Turkish Firs

matK regions	Temperature (°C)	Duration	Number of Cycle	Purpose
matK1	94	5 minutes	1	Initial denaturation
	94	1 minutes	30	Internal denaturation
	60	1 minutes		Annealing
	72	2 minutes		Extension
	72	3 minutes	1	Final Extension
matK2	94	3 minutes	1	Initial denaturation
	94	1 minutes	30	Internal denaturation
	62	1 minutes		Annealing
	72	2 minutes		Extension
	72	7 minutes	1	Final Extension

3.6. Sequencing *mat*K Region of The Chloroplast DNA

PCR products that have amplified *mat*K region of cpDNA were stored at -20°C. For sequencing the *mat*K region, both forward and reverse primers were applied. Before analysing the sequences, PCR purification process was performed. All purification and sequencing of samples were done by Refgen Biotechnology, METU Teknokent, Ankara. In sequence analysis, ABI 310 Genetic Analyser User's Manual was followed and 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.

With the help of 4 primers, *mat*K region was amplified as two parts. Chromotogram data of these parts were aligned visually by the help of Finch TV Version 1.4.0 developed by the Geopiza Research Team (Patterson *et al.*, 2004-2006). After collecting all data, both forward and reverse sequences of each sample were aligned in Multiple Alignment (algorithm) and the accuracy of bases were controlled manually or with a computer program. If there is any incoherence between the two sequences, the sample has been disregarded from the analysis or replaced with the new sequences.

3.7. Collection and Analysis of Data

Molecular diversity parameter-estimations and phylogenetic analyses were conducted by using MEGA 5 (Molecular Evolutionary Genetics Analysis Version 5) (Kumar *et.al.*2008). The sequences were converted into Fasta format and the gaps were indicated with "-". The "N" was put for the unreadable bases. It was found that, the analysed sequences were shorter than unprocessed sequence, because the beginning and the end of the sequences were not clear enough for analyses so the both ends were trimmed. The unreliability of these parts is the reason for shortening. The quality of sequencing decreases dramatically at the begining and at the end of the sequencing by using automatic sequencing systems.

Both matK1 and *mat*K2 regions of *Abies* spp., which were studied from worldwide, were searched from NCBI (National Center for Biotechnology Information, 2011) database. Therefore, both *Abies* species that are found in Turkey and in the world were analysed comparatively in order to explain the relationships of *Abies* spp with respect to *mat*K region in cpDNA.

The *Abies* species and their gene bank accession numbers of the *mat*K sequences, which were available from the NCBI database, were as follows (Table 3.8).

Table 3.8 *Abies* species and their gene bank accession numbers of the matK sequences in NCBI data bank.

Species name	Gene bank accession numbers
Abies alba	HQ619823
Abies hidalgensis	EU269026.1
Abies bracteata	AF456365.1
Abies holophylla	AF143441.1
Abies firma	AF143436.1
Abies numidica	AB019864.1
Abies veitchii	AB029669.1
Abies sibirica	AB029668.1
Abies sachalinensis	AB029667.1
Abies nephrolepis	AB029666.1
Abies mariesii	AB029665.1
Abies lasiocarpa	AB029664.1
Abies koreana	AB029663.1
Abies homolepis	AB029662.1
Abies fraseri	AB029660.1
Abies fargesii	AB029658.1
Abies fabri	AB029657.1

3.8. Estimation of Molecular Diversity Parameters and Construction of Phylogenetic Trees for Turkish Fir Taxa

Based on similarities and differences in genetic and physical features of species, evolutionary interrelationships of species could be revealed by constructing phylogenetic trees. Especially for genetic similarity and differences, phylogenetic trees were drawn according to some parameters. Conserved sites, variable sites, parsimony informative sites and singleton sites are the parameters that were used while drawing the phylogenetic trees. Based on these parameters tree drawing programs could draw the trees with different methods.

There are two types of phlogenetic trees that could be constructed. The first one is rooted tree which is believed that there is a common ancestor. It is shown with a unique node. The second type of phylogenetic tree is unrooted tree which the ancestor of the species is not important. Unrooted tree indicates the relationships like leaf nodes without an ancestor. Generally unrooted trees are made from rooted ones by simplfying the root part.

In bioinformatic techniques, neighbor joining (NJ) method is a bottom-up clustering method for the creation of phenetic trees.This method is created by Naruya Saitou and Masatoshi Nei (1987). It is usually used for trees based on DNA or protein sequence data. The algorithm of the method is to construct the topology of a tree. Moreover, the NJ method provides not only the topology, but also 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 starting with a starlike tree. Therefore, 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.

In this study the phylogenetic tree was constructed by MEGA software version 5 (Kumar *et. al.* 2008).

CHAPTER 4

RESULTS

4.1. Amplification of the *mat*K Region of the Chloroplast DNA in Turkish firs

The selected primers for the *mat*K regions of cpDNA of Turkish firs yielded clear single and good quality bands. The fragments which were amplified by the primer pairs of *mat*K1 and *mat*K2 were chosen for sequencing (Figure 4.1).
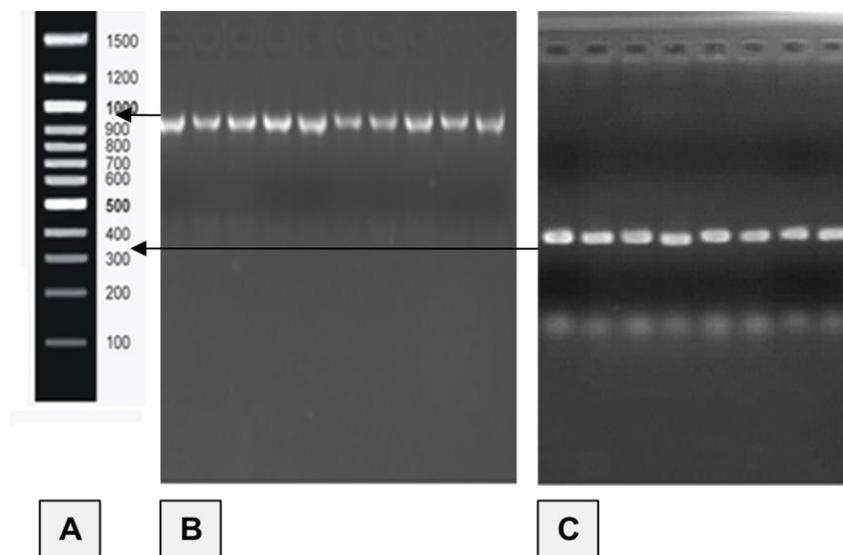
Figure 4.1 Photograph showing the amplified DNA of two *mat*K regions of cpDNA. A is the DNA ladder, B is *mat*K1 with 913bp and C is *mat*K2 with 302bp.

4.2. Sequencing *mat*K Region of the Chloroplast DNA of Turkish Firs

Sequencing procedure was described in chapter 3 and according to this procedure sequencing of *mat*K region was performed. Furthermore, the purified sequence products were run on %2 polyacrylamide gel (Visible Genetics Automated Sequencing System). The quality of the sequence data was high. Therefore, there were no problems of determination of the DNA bases from the chromatogram data (see Appendix A)

4.3. Molecular Diversity and Phylogenetic Analysis in the *mat*K Region

4.3.1. Molecular Diversity Among *Abies* Species With Respect to *mat*K Region

Generally, the length of the *mat*K region is around 1500-1800bp. In this study, partial *mat*K region was sequenced. In the beginning, it was expected that the primers, using in this study, would cover the sequenced of both *mat*K1 and *mat*K2 spacer regions. However, the amplified region from both *mat*K regions was 1215bp in length. The sequenced *mat*K region was partial in Turkish Firs since the sequenced length is less than the expected size of 1500bp.

In the sequence analyses, total length of partial *mat*K region is found to be 1215bp with %35.2 GC content. According to the results, there is no difference among *mat*K sequences of Turkish firs. All 1215 bp site is conserved and there were no variable sites, parsimony – informative sites and singleton sites. The first part of *mat*K sequence (*mat*K1) was 913bp long while the *mat*K2 was 302bp long in this study. The GC content of the first part is 38.7% and the GC content of the second part of *mat*K region is 36% (Table 4.1).

Table 4.1 Estimated molecular diversity parameters for *matK* gene region for Turkish Firs.

Molecular diversity parameters	Total	*matK 1*	*matK2*
Total Length (bp)	1215	913	302
GC content (%)	35,2	38,7	36
Conserved sites	1215	913	302
Variable sites	0	0	0
Singleton sites	0	0	0
Parsimony informative sites	0	0	0

4.3.2. Phylogenetic Analysis of Fir Species in The World Based on *mat*K1 Region

In the world, there are 51 *Abies* species. However, there are only 17 species in which *mat*K1 regions were studied. The sequences for the *mat*K1 region were obtained from the NCBI databank in order to compare the phylogenetic relationships between Turkish firs and the other fir species of the World. With the sequences from databank, molecular diversity parameters were comparatively obtained and provided Table 4.2.

The sequence lengths were not equal because there are only a few studies with complete *mat*K region while the others had partial *mat*K sequences. Therefore, while analysing the sequences overlapped sequences were used. After analysing the overlapped sequences, it was found that for *mat*K1 region which was 615 bp in length, GC content of these species was 34.5. Moreover, conserved sites were 607 and variable sites were 8. There were 3 singleton sites and 5 parsimony informative sites. The, transition/transvertion bias (R) was 3.279.

Table 4.2 Sequence characteristics of *mat*K1 region of *Abies* species in the world

Molecular diversity parameters	All studied *Abies* species in the world *(matK1* region*)*
Total Length (bp)	615
GC content (%)	34.5
Conserved sites	607
Variable sites	8
Singleton sites	3
Parsimony informative sites	5
Transitional pairs	21.68
Transversional pairs	78.32
Transition / TransversionBias(R)	3.279

When Turkish fir species compared to other species of which sequences were getting from NCBI database, it is clearly seen that Turkish firs are separated from most of other fir species according to partial *mat*K1 region. In Table 4.3 some features of the compared sequences were shown. Although Turkish firs are well separated from other fir species (*A.bracteate, A.fargesii, A.lasiocarpa, A.fraseri, A.hidalgensis, A.alba, A.numidica, A.holophylla, A.nephrolepis, A.homolepis, A.vetchii, A.sachalinensis, A.fabri, A.sibirica, A.firma, A.mariesii, A.koreana*) in the World, European *Abies* species seemed to be closer to Turkish firs based on partial *mat*K1 region. There were only 3 singleton sites between Turkish firs and European firs.

Table 4.3. Sequence characteristics of partial *matK 1* region of *Abies* species in the world

	Turkish Firs	North America+ Turkey	Europe + Turkey	Asia+ Turkey
	Turkish Firs	*A.bracteata A.lasiocarpa A.fraseri A.hidalgensis Turkish Firs*	*A.alba A.numidica Turkish Firs*	*A.holophylla A.nephrolepis A.homolepis A.veitchii A.sachalinensis A.fabri A. sibirica A.firma A.mariesii A.fargesii A.koreana Turkish Firs*
Total Length (bp)	913	615	615	615
GC content (%)	36	34.6	34.5	34.4
Conserved sites	913	610	612	608
Variable sites	none	5	3	7
Singleton sites	none	1	3	5
Parsimony informative sites	none	4	none	2
Transitional pairs	33.33	33.48	64.56	10.53
Transversional pairs	66.67	66.52	35.44	89.47
Transition / TransversionBias(R)	0.461	1.807	0.502	7.707

When Turkish fir species compared to other species with respect to *mat*K2 regions, it was clearly seen that Turkish firs were separated from other fir species. However, in NCBI database, complete *mat*K regions were available for only 3 *Abies* species (*A.veitchii, A.firma and A.holophylla* from Asia) were studied. In Table 4.3, some features of the compared sequences were shown. The overlapped sequences were 252 bp in length. Based on these sequences there were 2 variable sites, of 1 singleton and 1 parsimony informative sites.

Table 4.4 Sequence characteristics of partial *matK 2* region of *Abies* species in the world

	Turkish Firs	Asia+ Turkey			
		A.holophylla	*A.veitchii is*	*A.firma*	Turkish Firs
Total Length (bp)	302	252			
GC content (%)	36	38.4			
Conserved sites	302	250			
Variable sites	0	2			
Singleton sites	0	1			
Parsimony informative sites	0	1			

4.4. Phylogenetic Trees

4.4.1. Phylogenetic Tree of Turkish firs Based on *mat*K Region

Genetic distances among all samples of Tukish firs were computed using Mega 5.0 computer software. Since there was no diversity among samples of a given spcies, therefore, the phylogenetic tree was constructed by choosing only one representative individual from species. The phylogenetic tree which was constructed via neighbor joining method was given in figure 4.2. The bootstrap values and branch lengths were provided on the tree. In order to see the separation clearly, *Tsuga diversifolia* was used as outgroup from the Pinaceae family (genebank accession number is EF395589.1). All Turkish firs with the partial *mat*K region produced a single cluster as expected.
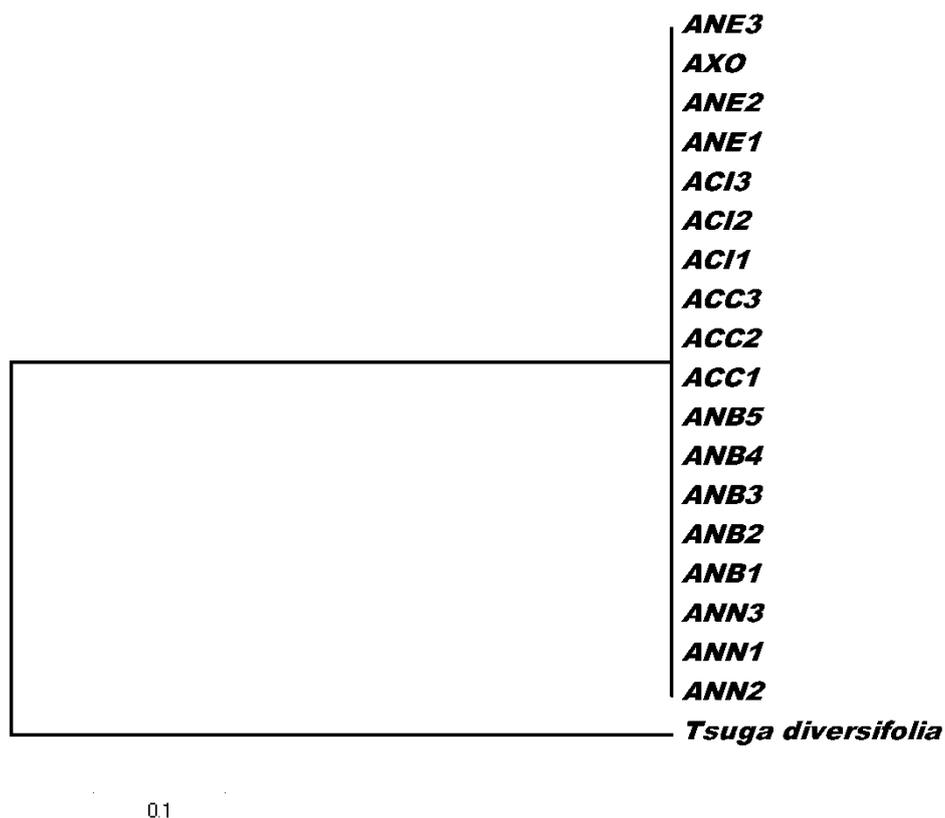
Figure 4.2 Phylogenetic tree was constructed by using neighbor joining method for *Abies* spp. in Turkey

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4.4.2. Phylogenetic Tree of Fir Species in the World Based on *mat*K Region

The phlogenetic tree in Figure 4.3 that constructed via neighbor joining method with bootstrap test analyses indicated the divergence of fir species in the world based on partial *mat*K1 region. According to this tree, it was seen that *Abies* species were divided into 3 major clades and Turkish firs formed a monophylogenetic group with one European species (*A.numidica*). Furthermore other fir species remained in other main branches.

Except Turkish firs, in the phylogenetic tree, there were 2 other main clades. The first main clade was composed of mostly Asian firs, except *A.lasiocarpa* and *A.fraseri,* from North America. In other main clade, *A.mariesii* , from Japan, formed one sub-clade and *A.hidalgensis, A.bracteata,* from North America and *A.alba,* from Europe, formed second sub-clade; however they remained in the same branch.

According to partial *mat*K2 region, different phylogenetic tree (Figure 4.4) was constructed by using neighbour joining method. In this tree, it is clearly seen that Turkish firs were well separated from other Asian fir species, *A.veitchii, A.firma and A.holophylla.*

Farjon (1990) and Liu (1971) were classified firs differently than our phylogenetic classifications based on morphologic features (Table 4.4). According to the differences between classifications, our phylogenetic tree indicated that the distributions of Firs in the world are not important for grouping of firs in the same branch.
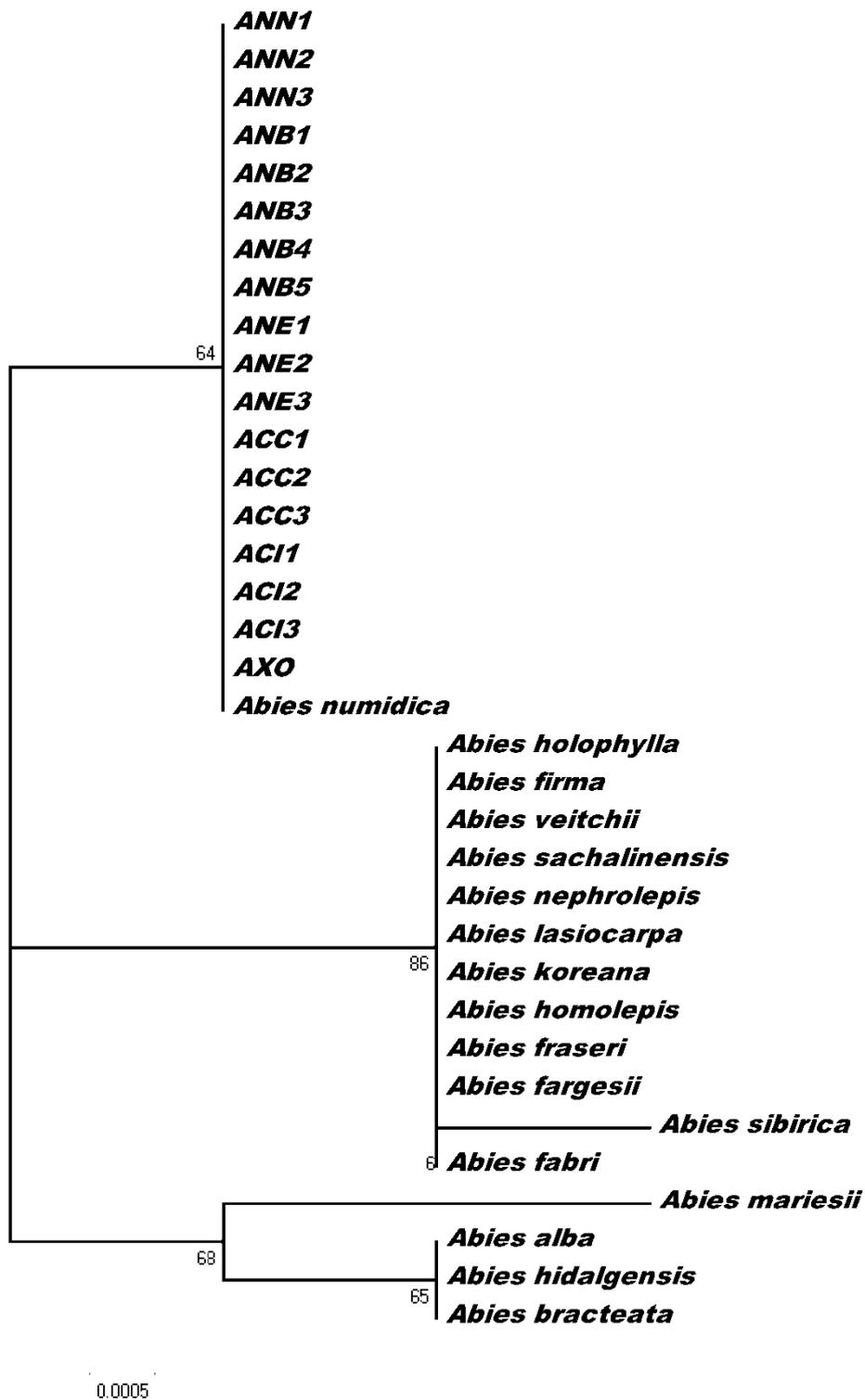
Figure 4.3 Phylogenetic tree was constructed by using neighbour joining method for *Abies* spp. based on *mat*K1 region.

```
                                                                                ACI3
                                                                                AXO
                                                                                ACI2
                                                                                ACI1
                                                                                ACC3
                                                                                ACC2
                                                                                ACC1
                                                                                ANE3
                                                                     64         ANE2
                                                                                ANE1
                                                                                ANB5
                                                                                ANB4
                                                                                ANB3
                                                                                ANB2
                                                                                ANB1
                                                                                ANN3
                                                                                ANN2
                                                                                ANN1
                                                                    Abies holopylla
        29  Abies veitchii
            Abies firma

            0.001
```
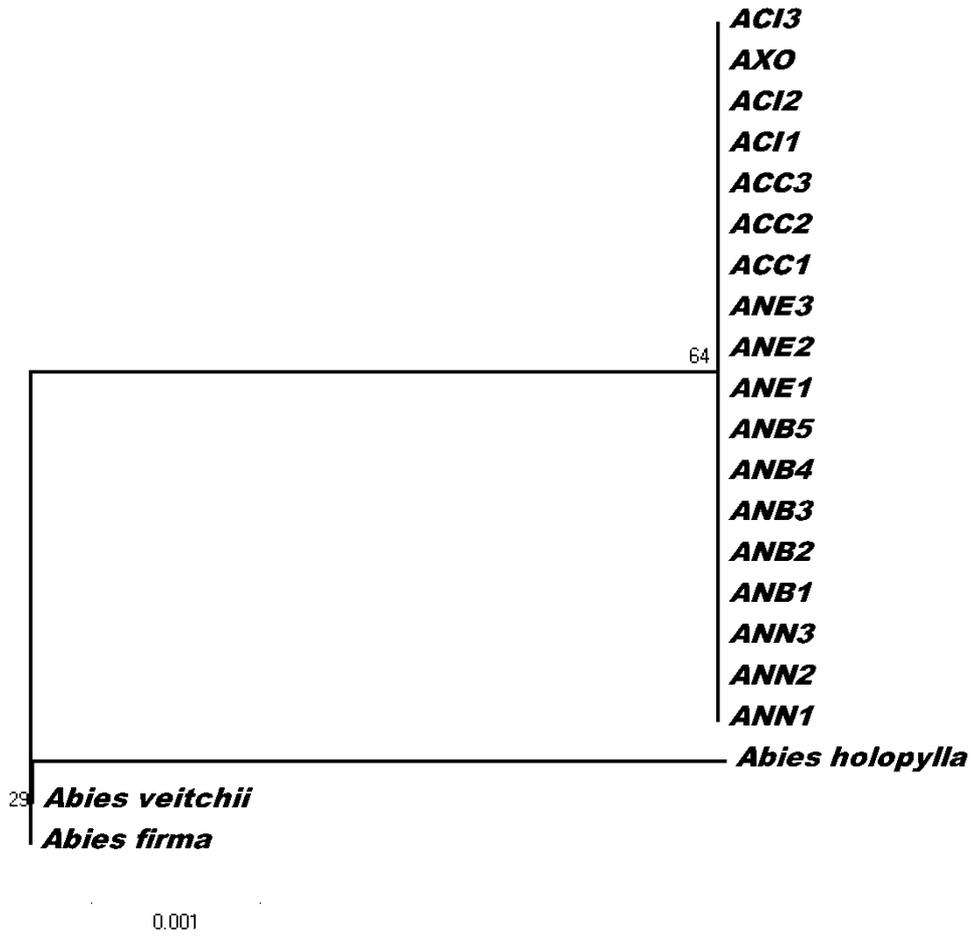
Figure 4.4 Phylogenetic tree was constructed by using neighbour joining method for *Abies* spp. based on *matK2* region

Table 4.5 Materials used in the phylogenetic analysis of *Abies* (Suyama *et. al.*2000)

Species	Section by Liu (1971)	Section by Farjon(1990)	Distribution
A. nordmanniana subsp. *nordmanniana*	*Abies*	*Abies*	Turkey
A.nordmanniana subsp. *bornmuelleriana* (E)			
A. nordmanniana subsp. *equi-trojani* (E)			
A.cilicica subsp. *isaurica* (E)			
A. cilicica subsp. *cilicica*			
Abies x olcayana			
Abies alba	*Abies*	*Abies*	Europe
Abies fargesii	*Elateopsis*	*Pseudopicea*	Asia
Abies fabri	*Elateopsis*	*Pseudopicea*	Asia
Abies holopylla	*Homolepides*	*Momi*	Asia
Abies homolepis	*Homolepides*	*Momi*	Asia
Abies firma	*Momi*	*Momi*	Asia
Abies sibirica	*Pichta*	*Balsamae*	Asia
Abies sachalinensis	*Elate*	*Balsamae*	Asia
Abies veitchii	*Elate*	*Balsamae*	Asia
Abies koreana	*Elate*	*Balsamae*	Asia
Abies nephrolepis	*Elate*	Balsamae	Asia
Abies fraseri	*Balsamae*	*Balsamae*	North America
Abies lasiocarpa	*Balsamae*	*Balsamae*	North America
Abies maressii	*Homolephides*	*Amabilis*	Japan
Abies numidica	*Piceaster*	*Piceaster*	Europe

*E: Endemic

CHAPTER 5

DISCUSSION

5.1. Molecular Diversity in the *mat*K Region

Due to the indels (insertion and deletion of bases), *mat*K region of Turkish firs was obtained partially as 1215 bp in this study. For *mat*K region this length is shorter than it was reported previously by others. For instance, Wang *et.al* (2000) found that *mat*K region was 1551bp in length on Pinecea family. However, Suyuma *et. al.* (2000) reported a partial *mat*K region with 615bp in length. Due to different length of matK region, higher variation is possible.

In the sequence analyses, for Turkish Firs, total length of partial *mat*K region is found to be 1215bp with %36 GC content. According to the results, there was no difference between sequences of Turkish firs, so there are no variable sites, conserved sites, parsimony – informative sites and singleton sites.

Although there was no divergence between Turkish firs, phylogenetic positions of Turkish firs, according to partial *mat*K region, was shown in this study. Based on *mat*K1 region, Turkish firs were closer to European Firs; however, they were distant to Asian and North American firs. Furthermore, according to matK2 region, Turkish firs were distant to Asian fir species.

5.2. The constructed Phylogenetic Trees by Mega 5.0

5.2.1. Phylogeny of the Genus *Abies*

Although many taxonomists have suggested different classification of *Abies*, the most recent classification was done by Liu (1971) and Farjon and Rushforth(1989) and Farjon (1990). Due to the difficulties of classifying *Abies*, the classifications differ in many aspects.

In this study, the *mat*K region of the cpDNA was used in order to determine the phylogenetic relationships of the 6 *Abies* species (*A.nordmannianan subsp.nordmanniana, A.nordmanniana subsp. bornmulleriana, A.nordmanniana subsp.equi-trojani, A.cilicica subsp.isaurica, A. cilicica subsp. cilicica and AbiesX olcayana*) in Turkey. However, by using neighbour joining method together with bootstrap test analyses, it was apparent from the phlogenetic tree that all Turkish firs were formed a monophyletic group with no sequence divergence. This indicates that *mat*K region has been well conserved among Turkish Firs, suggesting the presence of single ancestral chloroplast lineage. Results of this study were supported Şimşek (1992). Şimşek reported that there were not any difference between *A.nordmannianan subsp.nordmanniana* populations and other Turkish Firs.

5.2.2 Phylogeny of Turkish *Abies* and Closely Related Species

All the available sequences of *mat*K regions of *Abies* species in the world were acquired from NCBI database (2011) and analyzed to determine the phylogenetic position of Turkish firs species within the genus.

The conventional system which was proposed by Liu (1971) and Farjon (1990) is shown in Table 4.4. One of the most important results is that the placement of Turkish fir species is in a single clade. The *mat*K region data supported this that Turkish firs are forming single clade and very closely related with European *Abies* species. Fady and Conkle (1993) reported that

A.alba seemed genetically closer to *A. nordmanniana* subsp.*bornmulleriana* based on 22 loci using horizontal starch gel electrophesis. In our phylogenetic tree, *A.numidica* from Europe were formed in the same clade with Turkish firs, so that our results confirm Fady and Conkle (1993) that both Turkish firs and European firs have similar genetic background.

Furthermore, Scaltsoyiannes *et.al.* (1999) reported that 19 natural Mediterranean fir populations which belong to 8 species (*A.alba*, *A.cephalonica, A.bornmulleriana, A.nordmanniana, A. equitrojani, A.pinsapo, A.numidica, A.cilicica)* and one natural hybrid *(A.X borisii-regis)*, had some similarities. They reported that high genetic similarity was observed between calabrian Fir population and north-west Grecee as well as between *A. cephalonica* found in southern Greece and *A.equitrojani* in Asia Minor.

Suyuma *et. al.* (2000) reported that according to *rbcl* sequences, *A.nordmanniana*, *A.numidica, A.pinsapo, A.nebrodensis, and A.alba* consisted in one branch with no variation. However, based on *mat*K region those species (*A.nordmanniana, A.numidica, A.nebrodensis and A.alba*) were stayed in different branches that showed in Figure 4.3

Moreover, Ziegenhagen *et.al.* (2005) reported that Western Mediterranean species were different that Eastern Mediterranean Firs. They indicated that *A.bornmulleriana* and *A.equitrojani* had similar haplotypes with *A.nordmanniana* and *A. cephalonica* according to the usage of primers nad5-4 intron region of mtDNA. Our results also supported this that there is high genetic similarity among *Abies* species in Turkey.

There were other studies dealing with firs. Parducci and Szmidt (1999) found that genome of cpDNA was highly variable in their PCR-RFLP analyses in European Firs. Moreover, Isoda *et. al* (2000) reported that *A.alba* and *A.nordmanniana* were possessed TRT(Tandem repeat type): CB, and according to TRT, these species were different from other 16 *Abies* species. Due to lack of genetic variability in the *mat*K region of Turkish Firs in our

study, our results did not support these studies based on *mat*K region of *Abies* species.

Moreover, Kaya *et al.*(2008) reported that according to genetic similarity and distance values of Turkish Firs, those species were genetically well differentiated. In addition to Kaya *et al.* (2008), Hansen *et al.*(2005) concluded that 15 *Abies nordmanniana* populations which are originating from Caucasian region, were genotyped for 3 chloroplast microsatellites also one mitochondrial marker. As a result, they said that although mitochondrial marker indicated no variation, chloplast microsatellites were highly variable. In addition to these studies, Liepelt *et al.* (2010) also concluded that western Mediterranean *A.pinsapo* and *A.numidica* were definitely separated from each other according to chloroplast DNA markers.

Consequently, based on the DNA types (cpDNA, mtDNA, nDNA) or type of markers (*rbcl, matK, trn, ITS, SSR*, etc.) and length of useable sequenced regions, the different results were obtained in each study. Therefore, our results were different than other studies.

CHAPTER 6

CONCLUSION

The main goal of this study was to obtain gain genetic data from *mat*K gene region of cpDNA of Turkish firs to provide additional information to clarify the taxonomic status of Turkish Firs.

Partial *mat*K gene of Turkish Fir species was found to be about 1215bp in length in this study. Turkish Fir taxa had identical sequences where there were no variable sites. This is the first study using *matK* gene for Turkish Firs. Furthermore, it was revealed that this region is not suitable for differentiation of Turkish fir species.

Although there were no variable sites in *mat*K region of Turkish Firs, the phylogenetic trees constructed with the *mat*K sequences from all available fir species revealed that results were compatible with Liu (1971) and Farjon (1990)'s classification. They placed the Turkish Fir species in section *Abies*. However, the results further indicated that Turkish Firs differed greatly from other fir species in the world, but closer to Europen Firs.

Consequently, it can be said that *mat*K region of Turkish Fir species is highly conserved. Therefore, the sequence data of *mat*K region of Turkish firs did not help to resolve the classification problems of *Abies* species in Turkey, but it may be useful for phylogenetic analysis of the genus *Abies*. For better resolving data for classification of *Abies* species, more informative regions of cpDNA and nDNA should be studied in the future.

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APPENDIXES

Appendix A

An Example of Chromotogram Data
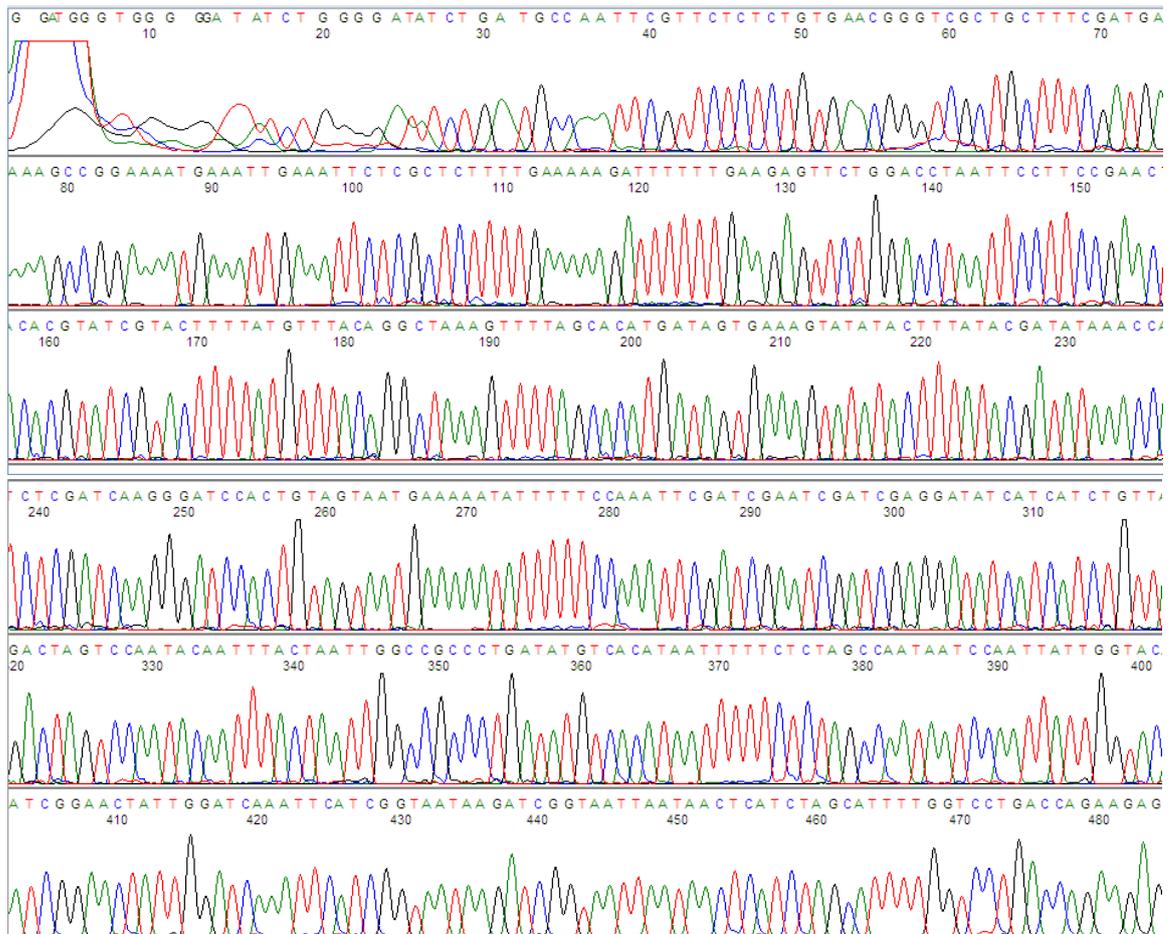
Figure A.1 An Example of Chromotogram Data

Appendix B

An Example of *mat*K Sequence

A.nordmanniana sub.sp. *nordmanniana*

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TTCGTTCTCTCTGTGAaCGGGTCGCTGCTTTCGATGAAAAAGCCGGAAA
ATGAAATTGAAATTCTCGCTCTTTTGAAAAAGATTTTTTGAAGAGTTCTG
GACCTAATTCCTTCCGAACTACACGTATCGTACTTTTATGTTTACAGGCT
AAAGTTTTAGCACATGATAGTGAAAGTATATACTTTATACGATATAAACCA
TCTCGATCAAGGGATCCACTGTAGTAATGAAAAATATTTTTCCAAATTCG
ATCGAATCGATCGAGGATATCATCATCTGTTAGACTAGTCCAATACAATT
TACTAATTGGCCGCCCTGATATGTCACATAATTTTTCTCTAGCCAATAAT
CCAATTATTGGTACAATCGGAACTATTGGATCAAATTCATCGGTAATAAG
ATCGGTAATTAATAACTCATCTAGCATTTTGGTCCTGACCAGAAGAGGTT
TCATCCGAACCCTCAGAAAATAACCTAAAGAAGAAGAACAATTCTTGGAT
AATTGATGAGAACATACCCTATATGGTTCGGACCAAAGATGGAAATAAAA
TTGCCGAAAAATTGGAAGATGATATCTACATTTTTTCACTAGGAGATGAG
TACCCTTTATAGCTATAATAGATCTTTCTGCATATCTAACATAGTGAATTC
TAGGATCCTTCAACGACCAGATACTTTTCAGTGAATTTCGACGATAATTT
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AGAATGGATTCACATCCATAGACATAATGATTCCACAGGAACAGCAAGA
ATCTTGTATTTACTCTCGGGTCGATAATAATTGATTTTTGTAAATTATCTG
GACTATTTCGATANNNNNCAATTGAACGACCGAGGAATTTCCTGATAGA
TCCGAAGCGAAGAGCATTGGACAAGATAGAGAAAAAAATATATATATCC
GGATATTAAGATTCTATATTAAATTCAACAGATTTACTACCCAAGGATCTC
ATTCGTCATGAGGAAGAACGAAAATCTTTTATCCTGGCGACCGATCGCT
CTCCTGACTCATGGGATAAATCAACCTGGTCAGTACACTATTTATCTTAC
ACATTTGTATCGAGTACTTAGGACTCATTGTGAGTGTATAAATCCCTGAT
CTACTCAGGGAAAACCTCCCGA
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