THE PHYLOGENETIC ANALYSIS OF PINUS NIGRA ARNOLD SUBSPECIES PALLASIANA VARIETIES WITH RESPECT TO NON-CODING *trn* REGIONS OF CHLOROPLAST GENOME

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ABSTRACT

THE PHYLOGENETIC ANALYSIS OF PINUS NIGRA ARNOLD SUBSPECIES PALLASIANA VARIETIES WITH RESPECT TO NON-CODING *trn* REGIONS OF CHLOROPLAST GENOME

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More than half of the Pinaceae is including in genus *Pinus* covers the large parts of vegetation of northern hemisphere. The Anatolian Black Pine is one of the subspecies of European Black Pine, growing naturally as a widespread mid elevation species of Taurus, western Anatolian and northern Anatolian Mountains of Turkey.

Although it is disputed that there are 5 varieties of Anatolian black pine but three of these are well recognized. These are *Pinus nigra* subsp. *pallasiana* var. *pallasiana*, *Pinus nigra* Arnold subsp. *pallasiana* var. *pyramidata* (pyrimidal black pine) and *Pinus nigra* Arnold subsp. *pallasiana* var. s*eneriana.*

To determine the genetic relationship between Anatolian black pine and its well recognized varieties, 3 different taxa of Anatolian black pine (well recognized varieties) were sampled in the natural range of species and non-coding *trn* regions of chloroplast DNA (cpDNA) were sequenced to assess the genetic structure of the species. Three sectors of *trn* region were examined.

Analysis was assessed with using MEGA version 4.0 and Arlequin 2.000 softwares.

Considering genetic diversity of three Anatolian black pine taxa with respect to *trn* regions and parsimonic sites, the result showed that *P. nigra* subsp *pallasiana* var *seneriana* was more polymorphic than other two taxa. Also, the most distant taxon that show differences in *trn* sequences when compared to other taxa was *P. nigra* subp *pallasiana* var *pyramidata*.

The constructed phylogenetic tree showed that individuals of *P.nigra* subsp *pallasiana* var *pyramidata* were grouped together. However, other two taxa showed a dispersed allocation in the tree. This result indicates that var *pyramidata* was the most distant taxon.

According to present study, there is no clear speciation between varieties and var *pallasiana*. The differences between them may be a result of mutation which may have occured in the genes coding for growth and form of Anatolian black pine.

Key Words: *Pinus nigra*, *trn*, cpDNA, genetic variance, phylogeny

ÖZ

KLOROPLAST GENOMUNDAKİ KODLANMAYAN *trn* BÖLGELERİNİN KARŞILAŞTIRILMASI YAPILARAK PINUS NIGRA ARNOLD ALT TÜR PALLASIANA VARYETELERİNİN FİLOGENETİK ANALİZİ

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Pinaceae ailesinin yarısından çoğunu kuzey yarımkürenin vejetasyonunun büyük bir bölümünü kapsayan *Pinus* cinsi oluşturmaktadır. *Pinus* cinsi içinde *Pinus nigra* türü Türkiye' nin büyük bölümünde bulunmaktadır. Avrupa karaçamının bir alt türü olan Anadolu karaçamı (*Pinus nigra* Arnold subspecies *pallasiana*) Torosların orta yükseltilerinde, Türkiye' nin batı ve kuzey Anadolu dağlarında doğal olarak yaygındır. Anadolu karaçamının 5 varyetesi bulunmakla birlikte iyi bilinen 3 varyetesi vardır. Bunlar, *Pinus nigra* alttür *pallasiana* var. *pallasiana*, *Pinus nigra* Arnold alttür *pallasiana* var. *pyramidata* (primidal karaçam, "Ehrami Karaçam"), ve *Pinus nigra* Arnold alttür *pallasiana* var. *şeneriana* ("Ebe Karaçamı").

Bu amaçla tür içinde ve kloroplast DNA' nın kodlanmayan trn bölgesi üzerinde 3 farklı takson (iyi bilinen 3 varyete) örneklenmiştir. Bunlar *Pinus nigra* Arnold alttür *pallasiana, Pinus nigra* Arnold alttür *pallasiana* var. *pyramidata* ve *Pinus nigra* Arnold alttür *pallasiana* var. *şeneriana.* Anadolu karaçamı varyete bakımından incelenmiştir. Ayrıca hangi bölgenin daha çeşitli olduğunu belirlemek için 3 tane *trn* bölgesi incelenmiştir. Bu çalışmadaki bütün moleküler analizler MEGA versiyon 4.0 ve Arlequin 2.000 yazılımlarıyla yapılmıştır.

Çalışılan *trn* bölgeleri ve parsimonik bölgelere göre 3 Anadolu karaçamı taksonunun genetik çeşitliliğini göz önüne alırsak, sonuçlar şunu göstermiştir ki *P. nigra* alttür *pallasiana* var *seneriana* diğer iki taksona göre daha polimorfiktir. Ayrıca *trn* zincirlerindeki farklılıklar karşılaştırıldığında en uzak taksonun *P. nigra* subp *pallasiana* var *pyramidata* olduğu gösterilmiştir.

Kurulan filogenetik ağaçta, *P.nigra* subsp *pallasiana* var *pyramidata* bireylerinin bir grup oluşturduğu gözlemlenmiştir. Fakat diğer taksonlar yayılmış bir dağılım göstermiştir. Bu sonuç var *pyramidata*'nın en farklı takson olduğunu göstermektedir.

Yapılan bu çalışmaya göre *P. nigra* alttür *palasiana* var *pallasiana* ile diğer iki varyeteler arasında belirgin bir türleşme yoktur. Farklılıkların

Anadolu karaçamının büyümesi ve şekil almasında görev alan genlerde meydana gelen muhtemel mutasyonların sonucunda olduğu söylenebilir.

Anahtar Kelimeler: *Pinus nigra*, *trn*, cpDNA, genetik çeşitlilik, filogenetik

to my unique family and my love Murat…

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

AMOVA	Analysis of Molecular Variance
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
t-RNA	Transfer Ribonucleic Acid
EDTA	Ethylene diamine tetra acetic acid
VAR	Variety
SUBSP	Subspecies
trnL	Leucine tRNA
trnV	Valine tRNA

CHAPTER 1

INTRODUCTION

1.1. Biology of genus *Pinus*

More than one third of gymnosperm species belong to Pinaceae, the largest family of modern conifers, which is divided into 11 genera with 232 species. More than half of the Pinaceae including in the genus *Pinus* (over 100 species) covers the large parts of northern hemisphere. It has been demonstrated from fossilized cones that the ancestors of *Pinus* had evolved at Crateceous Era. Since the evolution of human being, they have been important components of economic values, biogeochemical processes, hydrological and fire regimes and regional and global climate (Richardson, 1998). Pine forests range from near the Arctic region which has very cold winters and short growing seasons to the tropics where no frost occurs and continue to grow throughout the year (Knight *et al*., 1994).

Pines, like many other conifers, are monopodial and possess large size. The largest pine is *P. lambertiana* reaching height of 75m and 5m in diameter. Many pine species have long lifespan plants and that the known oldest living organisms in the world are *P. aristata* and *P. longaeva* (Richardson, 1998).

Pine species are especially important for human needs. People have met with pines about a million years ago in the Mediterranean region. Since then, different pine species are affected in different regions by different human effects such as altered fire regimes, altered grazing/ browsing regimes, various harvesting/construction activities, land clearance and abandonment, purposeful planting and other manipulations of natural ecosystems, alteration of biotas through species reshuffling, and pollution. Mainly, humans have harvested pines and their products for thousands of years (Richardson *et al*., 2007).

Because of its economic and ecological importance, there is considerable attention given to systematics of pines. Morphology, cytology, crossability, protein electrophoresis and comparison of nuclear and choloroplast ribosomal DNA have been used for the classification of genus (Gaussen, 1993).

In terms of morphology, needle and needle fasicle are used in classificaiton of pine species. The number of needles per fasicle is nearly constant for each pine species and utilized for the characterizaiton of the genus. Moreover, lenght of needles and internal anatomical characters of needles are also useful for systematics of pines. The number and position of resin canals, needle age and environmental factors, the morphology of stomatal complexes and wax deposition on needles are also used for the classification of pine species. Wood anatomy of pines and the cell walls of ray tracheids and ray parenchyma cells have been used for the order of the genus (Richardson 1998).

1.2. Biology of *Pinus nigra* subspecies *pallasiana*

1.2.1. Natural Distribution

Pinus nigra Arnold (European black pine) is native to Europe. Its range extends from longitude 5° E in Spain and Morocco to about 40° E in eastern Turkey; and from latitude 35° N in Morocco and Cyprus to 48° N in northeastern Austria (Critchfield and Little, 1966). Black pine grows widely throughout southern Europe from the eastern half of Spain, southern France, and Italy to Austria; south of the Balkans; south-east Russia in the Crimea and southern Turkey; and on the islands of Cyprus, Sicily, and Corsica, with outliers in Algeria and Morocco (Mirov, 1967) (Figure 1.1).

Figure 1.1. Natural distribution of *Pinus nigra* (Isajev *et al.*, 2004)

The Anatolian Black Pine (*Pinus nigra* Arnold subspecies *pallasiana*) is one of the subspecies of European Black Pine, growing naturally as a widespread mid elevation species of Taurus, western Anatolian and northern Anatolian Mountains of Turkey. The range in elevation varies from 250m to 1550m (Kaya and Temerit, 1994) (Figure 1.2). In Black sea region, it rarely grows on coastal zones; but generally on the elevational range of 400 to 1400m. In the mid – elevation, it forms pure stands, while after 1400 m (up to 1700 m) it makes mixed stands with *P.sylvestris, Abies* spp. and *Quercus* spp.

In western Anatolia, the best stands of Anatolian black pine are formed in Bozüyük, Keles, Dursunbey, Bigadiç, Sındırgı, Demirci, Simav, Emet and Tavşanlı, at Ida Mountains (Kazdağı), Muğla – Yılanlı, Köyceğiz, Fethiye, Gölhisar, Acıpayam and Denizli ranging from 200 to 1400 m in elevation. Starting from the Lakes Region, its distribution is limited up to north (Afyon). In southern Anatolian region; it occurs at 1200 – 1400 m in mixture with some other species, especially with *Juniperus* species (Çengel, 2005).

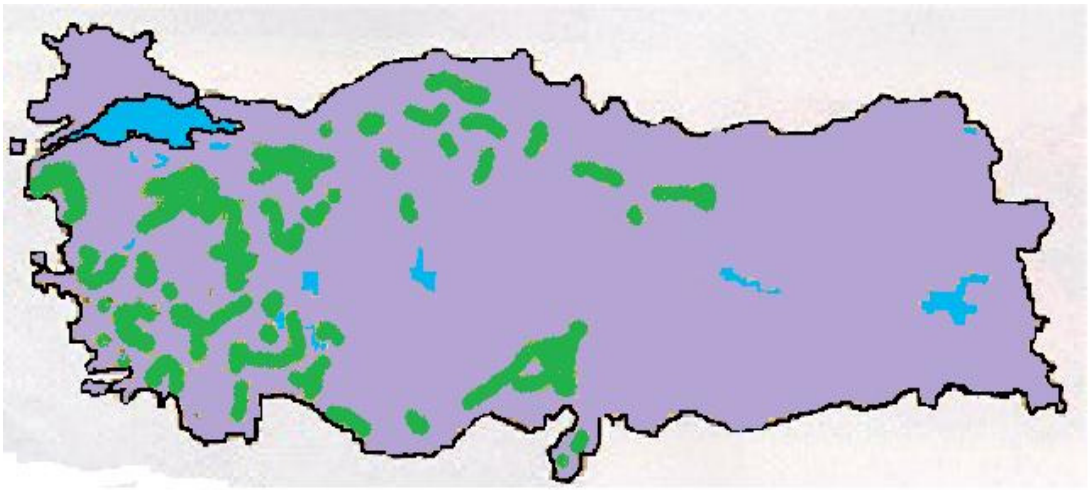


Figure 1. 2. *Pinus nigra* subsp *pallasiana* distribution in Turkey

1.2.2. Taxonomy

The taxonomy of European black pine is complicated. Thus, the taxonomy of the species has occupied botanists for years although no satisfactory classification has been reached yet (Yaltırık, 1993). Moreover, according to Gaussen *et al*. (1993) European black pine is highly variable and divided into geographical variants which are often not clearly separable. Besides geographical distribution, some leaf characteristics such as length, thickness, color and rows of hypodermal cells etc are also considered as additional traits in classification of species.

Pinus nigra Arnold (black pine) belongs to Phylum Pinophyta, Class Pinopsida, Order Pinales, Family Pinaceae, and Genus *Pinus*. It has also common names like European black pine, Austrian pine, Crimean pine (www.conifers.org, *Pinus nigra*, January 2009).

European black pine taxa have been described by many authors under different names which caused a further confusion for the agreement on its nomenclature (Vidakovic, 1991). There are intermediate groups between groups such that black pine has been divided into six subspecies which are subsp. *pallasiana*, subsp. *fenzlii*, subsp. *dalmatica*, subsp. *nigra*, and subsp. *laricio* and subsp. *salzmanii* (Schwarz, 1938; Kaya *et al.,* 1985).

Turkish populations of *Pinus nigra* were classified as var. *pallasiana* Schneid. Anatolian black pine (syns: var. *caramanica* (Loudon) Rehd., *P. pallasiana* D. Don) by Kayacık (1980) and as subsp. *nigra* var. *caramanica* (Loudon) Rehder by Frankis (Güner *et al*., 2000). *P. nigra* var. *caramanica* occurs in Turkey, Cyprus and Greece, and may be best told from other varieties of subsp. *nigra* by its cones often being yellower at maturity, but the varieties are only distinguishable on the basis of population means; many individual trees cannot be reliably identified from either var. *nigra* (Austria, Balkans) or var. *pallasiana*.

From these taxonomic classifications formulated by different authors, the following conclusion could be made.

- Taxonomic classifications based on some cone and/or needle characteristics overlap extensively throughout its distribution (Boydak, 2001).

Anatolian black pine has the second most extensive natural distribution area among the pine species native to Turkey. This species covers 2 527 685 hectares (Anon., 1997).

In addition to *P. nigra* subsp *pallasiana* var *pallasiana*, there are also four varieties of Anatolian black pine have been reported by Boydak (2001). These are:

- *Pinus nigra* Arnold subsp. *pallasiana* var. *pyramidata* (Pyrimidal Anatolian black pine, "Ehrami Karaçam" in Turkish). Pyrimidal black pine (var. *pyramidata*) occurs between 980 – 1350 m elevation; within 39°10'07" – 39°39'50" N latitudes and 29°20'05" – 29°52'55" E longitudes; in Kütahya (Tavşanlı, Pullar, Esatlar, Kızık and Vakıf) province (Yücel, 2000).
- *Pinus nigra* Arnold subsp. *pallasiana* var. *seneriana* (Globular-shaped Anatolian black pine, "Ebe Karaçamı" in Turkish). Globular – shaped Anatolian black pine occurs between 800 – 1250 m altitudes, within 38°16'63" – 40°46'03" N latitudes and 28°29'71" – 31°34'14" E longitudes; in Bolu (Çaydurt), Manisa (Alaşehir) and Kütahya (Tavşanlı, Domaniç, Aslanapa, Aydıncık) provinces as individuals or in small groups (Ünaldı, 2005).

- *Pinus nigra* subsp. *pallasiana* var. *yaltırıkiana* Alptekin (large coniferous black pine) shows distribution in Sinop – Boyabat, Karabük – Yenice. In Karabük, Yenice, these black pines have different stem forms and wood characteristics than var *pallasiana* and are named as "Camiyanı Karaçamı" (Sıvacıoğlu, 2007).

Pinus nigra subsp. *pallasiana* var. *columnaris – pendula* Boydak It is distributed in Soğukoluk, Adana, Ballısu, Antalya, Kaleboynu, Kahramanmaraş, Arslanköy, Mersin (Boydak, 2001).

Among these four varieties, first two are well recognized varieties and more common than the last two varieties. Thus, var. *yaltirikiana* and var. *columnaris-pendula* may not be considered as distinct varieties at all.

1.2.3. Ecology

Anatolian black pine grows in a cool to cold temperate climate. Like European black pine, Anatolian black pine is classed as intolerant of shade, and, therefore, must be planted in places where it will receive full sunlight. Although Anatolian black pine often is found on poor, calcareous, sandy, and even pure limestone soils, nevertheless, it requires a deep soil (Elkiey *et al*., 1982). Anatolian black pine can easily adapt to extreme climates and can grow successfully in steppe ecosystems of Anatolia.

1.2.4. Botany

Anatolian black pine is a large tree, growing up to 30 m tall at maturity. The bark is grey to yellow-brown, and is widely split by flaking fissures into scaly plates, becoming increasingly fissured with age. The leaves ("needles") are in fascicles of two, dark green, and 8–20 cm long (Figure 1.3). In general, the ovulate and pollen cones appear from May to June. The mature seed cones are 5–10 cm long, with rounded scales; they ripen from green to pale yellow-buff in about 18 months after pollination from September to November. The winged-seeds are wind-dispersed when the cones open from December to April.

Sexual maturity is reached at 15–40 years; large seed crops are produced at 2–5 year intervals. It is moderately fast growing (30–70 cm/year) and usually has

a rounded conic form, becoming irregular with age; it is fairly long lived, with some trees probably over 500 years old (Yaltırık, 2000).

Anatolian black pine is monoecious, with staminate and ovulate strobili born separately on the same tree. Staminate strobili, clustered at the base of new shoots, mostly on older lateral branches in the lower crown, are cylindrical, short-stalked, bright yellow, about 2 cm long with numerous scales, and contain pollen in great quantity (Richardson, 1998).

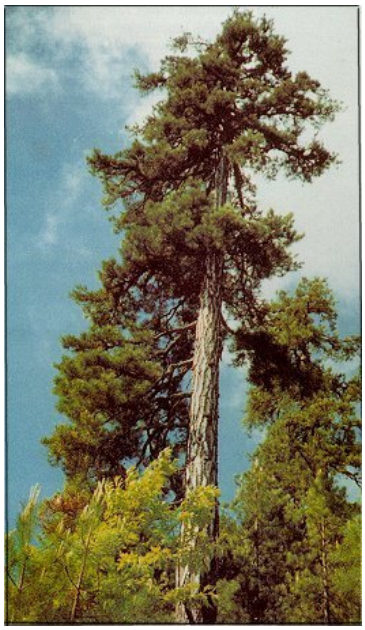
Globular – shaped Anatolian black pine is a compact tree with multiple branches (Yücel, 1997). It is up to 6 – 10 m in height, branching densely from the base. Generally it does not have a main stem, instead it has many sub – stems. Needles are in groups of two, bunching at the shoot tips like a rosette, bright green; 5 – 11 cm long. Cone number, seed formation ability and fertility are less compared to Anatolian black pine (Figure 1.4).

Pyrimidal Anatolian black pine is a tree up to 20 m in height and 50 – 55 cm in diameter. It has a pyrimidal shape, which is maintained throughout its life cycle. Needles are in groups of two, dark green; 5 – 13 cm long; usually straight or curved (Figure 1.5).

Pinus nigra subsp. *pallasiana* var. *yaltirikiana* has the main property of the fact that almost all cross section surface is composed of wood extracts and this wood extract becomes darker in color and does not exude the resin (Sıvacıoğlu, 2007). Its cones are much bigger than those of var *pallasiana* (Alptekin, 1986). Its vigorous wood is in color of whitish-redish-yellowish; however its pith is in color of red and its annual rings are distinct. Tracheid cells are square shaped in spring wood but circular shaped in summer wood (Gündüz *et al*., 2007).

Pinus nigra subsp. *pallasiana* var. *columnaris – pendula* differs from other known varieties in its shorter, thinner and pendant branches of nearly equal length or long pendant branches with an obtuse angle forming narrower and compact and columnar habit (Figure 1.6) (Boydak, 2001).

A



B

C

D

Figure 1.3. General appearance of Anatolian black pine and some of its features (Photo from General Directory of Forestry, University of Connecticut and FTSTBRD archives) A. General appearance, B. Trunk of mature tree, C. One-year old female conelet, D. Male cone

Figure 1. 4. A view of *P. nigra* subsp. *pallasiana* var *seneriana* at Derbent, Konya (Photo: Z. Kaya)

Figure 1. 5. A view of clonal seed orchard *P. nigra* subsp *pallasiana* var *pyramidata* from Tavsanlı, Vakıfköy (Photo: M: Boydak)

Figure 1. 6. A view of *P. nigra* subsp *pallasiana* var. *columnaris pendula* at Andırın, Kaleboynu, Kırksuderesi (Kahramanmaraş) (Photo: M. Boydak)

1.3. Genetic Variation and Molecular Markers

Climatically and topographically diverse and fragmented distribution of black pine evolved through natural selection. Basic and haploid chromosome number are equal to 12 (n=12), two of which are heterobrachial and the others mostly isobrachial (Borzan, 1981). Kaya *et al*. (1985) analyzed the karyotypes of black pine and found that chromosomes XI and XII were especially variable which can be used in taxonomy of European black pine.

There are also isozyme variation studies on Anatolian black pine natural populations. Doğan *et al.* (1998) carried out a study on isozyme based linkage analysis in Anatolian black pine populations sampled from Ida Mountains. Tolun *et al.* (2000) and Çengel *et al*. (2000) also studied isozyme variation in natural populations and reported the existence of high genetic diversity localized within populations.

Moreover, utility of RAPD markers in Anatolian black pine for population genetics was investigated by Kaya and Neale (1993). Results of the study have shown that RAPD markers can be used efficiently in population genetics studies of Anatolian black pine.

1.4. Determination of Genetic Variation

Since the early 1970's, electrophoretic techniques have been used in genetic studies of forest tree populations. These techniques offer a number of advantages over other biochemical or quantitative approaches: (a) genetic inheritance of electrophoreticaIly detectable traits can be easily demonstrated; (b) most isozyme loci are codominant and gene frequencies can be calculated without the necessity of genetic crosses; (c) estimates of genetic variation can be compared directly between populations or between species (Hamrick *et al*., 1979).

The enzymes that share a common substrate, but differ in electrophoretic mobility is called as isoenzyme (isozymes) which is briefly known as multiple molecular forms of enzymes. When tissue extracts are subject to electrophoresis in various types of gels and submersed in solutions containing enzyme specific stains, isozymes are revealed. Some of the variant electromorphs are encoded by alternate alleles at a single locus, in which case the allelic products are termed as allozymes (Wendel and Weeden, 1989). Allozymes exhibit polymorphism among individuals as well as Mendelian inheritance, codominant expression and complete penetrance and absence of pleitropic and epistatic interactions. Patterns of genetic variation in isozyme level allows rapid assessment of the genetic composition of a population and multilocus identity of individuals.

In recent years, DNA – based genetic markers have been developed such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) based DNA markers (e.g. variable number of tandem repeats (VNTR), amplified restriction fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD)).

By revealing differences in the DNA sequence among individual trees, DNA markers provide the potential to increase genetic gain from tree improvement programmes through DNA fingerprinting of genotypes, parentage testing of superior seed and through the identification of DNA markers associated with traits of economic value in an integrated marker-assisted breeding programme. Differentiation of the genotypes through DNA fingerprinting is now routinely carried out in many conifer breeding programmes as a means of eliminating misidentified individuals in archives and seed orchards. (Walter *et al.,* 1998).

1.5. Transfer Ribonucleic Acid Region of the Choloroplast DNA (cpDNA)

In recent years choloroplast DNA (cpDNA) has provided significant insights in many phylogenetic studies (Palmer *et al.,* 1988). Noncoding sequences tend to evolve faster than coding sequences and thus may provide more informative characters for phylogeny reconstruction (Wang *et al.,* 1999). The region between the *trnL* (UAA) and *trnF* (GAA) and the gene *trn*V (UAC) which codes valine carrying tRNA are particularly suitable due to the succession of conserved *trn* genes and small non-coding regions as well the higher rate of molecular evolution of the single-copy regions (Taberlet *et al*., 1991). 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. Group I introns are characterized by a highly conserved core structure encoding the active site. In plants, the *trnL* intron usually shows sequence conservation in the regions flanking both *trnL* exons, whereas the central part is highly variable. The region between the *trn*L and *trn*F and the region *trn*V are suitable for

evolutionary studies due to the succession of the conserved *trn* genes and several hundred base pairs of non – coding regions, the higher rate of mutations in the single – copy regions and the absence of gene rearrangements among many species (Wolfe *et al*., 1987).

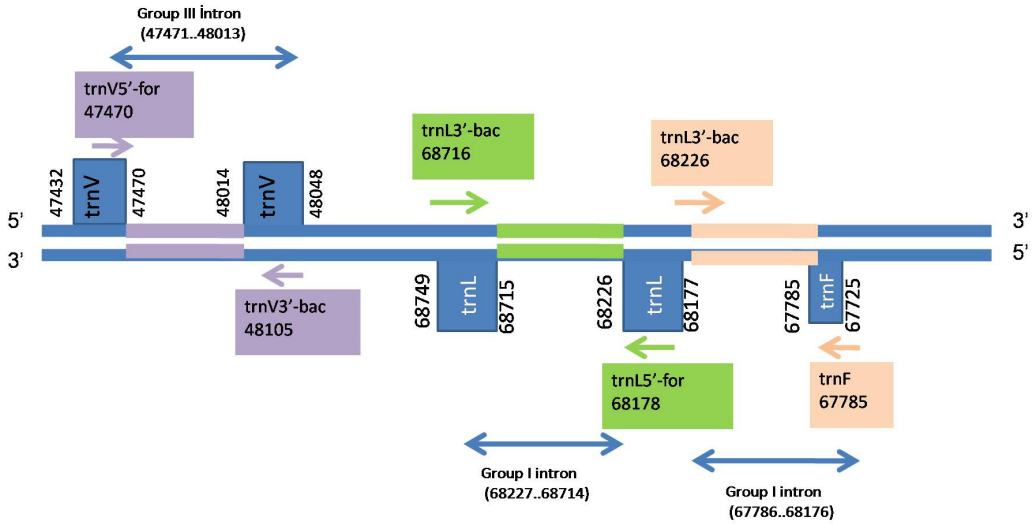


Figure 1. 4. *trn* region of cpDNA (Taberlet *et al.,* 1991; NCBI, NC_0016231; Wakasugi *et al.*, 1994)

In this study, the evolutionary relations among varieties of Anatolian black pine were explored by studying molecular diversity in the non – coding tRNA (*trn*) regions of cpDNA. Three regions within *trn* sequences were used. The first region is between *trn*L5' and *trn*L3' amplified by *trn*c and *trn*d primer set, 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.

1.6. The significance of the study

Anatolian black pine is the Turkish subspecies of the European black pine (Alptekin, 1986). About fifteen geographical variants were observed by Alptekin in his extensive study on Anatolian black pine (1986). He studied 23 characters (cone, seed and needle characteristics) of Anatolian black pine by sampling from 92 populations comprising whole Turkey; 2 populations from Cyprus and Macedonia. In addition, until the study of Alptekin (1986), Anatolian black pine was regarded as var. *caramanica*. For that reason there is no consensus on satisfactory classification of taxonomy for Anatolian black pine. Different publications or different volumes of the same publication (e.g. 1st volume of the Flora of Turkey and East Aegan islands, Davis, 1965) do not agree on its taxonomy.

Furthermore, Anatolian black pine is an economically important tree species in Turkey. Because of its growth characteristics and natural distribution, it is used for the most of the afforestation and reforestation lands available. In the last decade, there are increasing number of studies dealing with the species' genetic diversity by means of quantitative traits (Kaya and Temerit, 1994; Şimşek *et al*., 1995; Üçler and Gülcü, 1999; Velioğlu *et al*., 1999); isozymes variation (Doğan *et al*., 1998; Çengel *et al*., 2000; Çengel, 2005; Tolun *et al*., 2000) and RAPD variation (Kaya and Neale, 1993). Despite limited studies on genetic diversity of Anatolian black pine,there is no molecular systematics study with the species. Thus there is a need to assess systematic states of Anatolian black pine.

The regions of *trn* are particularly suitable for evolutionary studies because of;

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

Thus, the sequence analysis and comparison of *trn* regions of Anatolian black pine varieties could be useful to classify some of the taxonomic problem of the species.

CHAPTER 2

OBJECTIVES OF THE STUDY

The general objective of this study is to determine evolutionary relationships of Anatolian black pine varieties, based on molecular diversity in tRNA region of cpDNA.

The specific objectives of the study are:

1. To estimate molecular diversity of tRNA region in varieties vs. *P. nigra* subsp. *pallasiana*
2. To estimate magnitude of genetic differentiation of varieties of *P.nigra* subsp. *pallasiana*
3. To construct a phylogenetic tree using molecular diversity statistics for *P. nigra* subsp. *pallasiana*, var. *seneriana*, var. *pyramidata* and outgroup.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant Material

Pinus nigra subsp. *pallasiana* (Anatolian black pine) DNA sources were obtained from Forest Tree Seeds and Tree Breeding Research Directorate, Ministry of Environment and Forestry, Ankara where DNA isolation had previously been done by Cengel *et al*. (2005) from seeds. The study materials included seed samples from Anatolian black pine varieties (var. *pyramidata* and var. *seneriana*) and Anatolian black pine var *pallasiana* Dursunbey seed stand (Balıkesir Alaçam – Değirmeneğrek). Seeds of pyrimidal black pine (*P. nigra* subsp. *pallasiana* var. *pyramidata*) had been sampled from seed orchard in Eskişehir (National Seed Orchard Registration Number: 47). While seeds of var. *seneriana* had been sampled from a natural stand located in Bolu – Çaydurt (Table 3.1. and Figure 3.2).

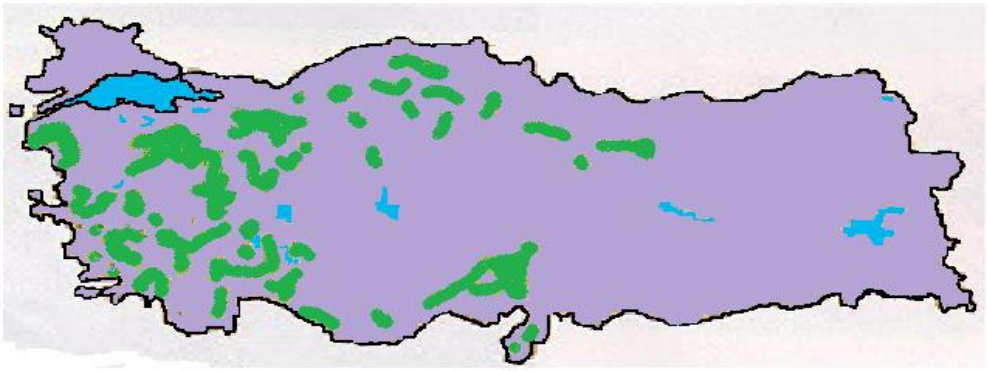


Figure 3.1. Map showing the distribution of Anatolian black pine

Figure 3.2. Map showing study sites (The sites red dots are the regions from where samples were used in this study (Table 3.1)

Table 3. 1 Description of studied Anatolian black pine seed sources/populations

Seed Source	Longitude	Latitude	Type	District – Subdistrict	Number of Trees
P. nigra subsp *pallasiana* var *pallasiana*	28° 34' 10'' E	39° 25' 50'' N	Seed Stand	Alaçam-Değirmeneğrek	20
P.nigra subsp. *pallasiana* var. *pyramidata*	30° 07' 35'' E	39° 49' 20'' N	Seed Orchard	Eskişehir-İnönü	12
P. nigra subsp *pallasiana* var. *seneriana*	31° 45' 00'' E	40 ° 45' 00'' N	Conservation Area	Bolu-Çaydurt	35

3.2. DNA Isolation

DNA isolation from seeds had been previously performed by Çengel (2005). The method was the modification of the methods described by Kreike (1990) and Dellaporta *et al*. (1983). Seeds were soaked in distilled water at 4°C for 24hrs. Seed embryo was excised and removed and then megagametophytes were homogenized in 400 µl extraction buffer I (0.1 M Tris HCl pH: 8.0, 0.1 M EDTA, 0.25 M NaCl) in 1.5 ml Eppendorf tubes. After homogenization, 400 µl extraction buffer II (0.1 M Tris HCl pH: 8.0, 0.1 M EDTA, 0.25 M NaCl, 2% SDS) was added.

Homogenized tissues were kept in a 65°C water bath for 30 – 40 minutes. Then, 250 µl of 5M potassium acetate solution was added to tubes and incubated on ice in refrigerator for at least 60 min. Following the centrifugation at 14 000 rpm at 4°C for 15 min, supernatant was transfered to a new tube and mixed with 500 µl chloroform – octanol (24:1) solution. After 10 min centrifugation the supernatant was transfered to a new tube and 700 µl absolute ethanol/0.3 M sodium acetate solution was added.

The tubes were incubated at -80°C for at least 60 min. After then, 10 min centrifugation was performed, supernatant was poured off and pellet washed twice with cold 70% ethanol. The pellet was dried and re-suspended in 50 µl TE buffer. The DNA samples were stored at -20°C.

3.3. DNA Quantification

DNA quantification had been performed by Çengel (2005) with Hoefer DyNA Quant™200 Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA) which is a filter fluorescence photometer with a fixed excitation bandpass source (365nm) and emission bandpass filter (460nm). DNA yields per megagametophyte varied from 500 to 5000 ng. All stock DNA samples were stored at -20°C to be able to use throughout the course of the study. The presence and quality of the DNA was also checked by 0.8% agarose gel electrophoresis.

3.4. t-RNA region of the chloroplast DNA primers

Choloroplast DNA sequence variations are being widely used in phylogenetic studies (Palmer *et al.,* 1988; Learn *et al.,* 1992). The non-coding regions display the highest frequency of mutations and can be efficiently used for evolutionary relationship analysis (Taberlet *et al.,* 1991). One of these coding regions; the t-RNA (*trn*L-*trn*F and *trn*V) regions are the most extensively examined cpDNA fragment due to their wide use in addressing phylogenetic relationships at the levels below family (Taberlet *et al.,* 1991; Kelchner, 2000). This region is composed of the *trn*L gene and a flanking intergenic spacer, i.e. *trn*LF. The *trn*L gene consists of two highly conserved exons that are split by a group I intron, in which both flanks are also quite conservative whereas the central part is highly variable (Bakker *et al.,* 2000). The *trn*V gene consist of an exon that are split by a group III intron. The *trn*L-*trn*F and *trn*V regions exhibit a quite high substitution rate in many plant groups (Bayer and Starr, 1998; Bakker *et al.,* 2000; Mansion and Struwe, 2004). Three sets of primers (*trn*c and *trn*d, *trn*e and *trn*f, *trn*VF and *trn*VR) were used to amplify tRNA region in PCR. The primer sequences for the non coding region of tRNA are as follows:

<u>*trn*L5'-*trn*L3' region:</u>
*trn*c (Forward): 5' CGA AAT CGG TAG ACG CTA CG 3'
*trn*d (Reverse): 5' GGG GAT AGA GGA CTT GA AC 3'

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

<u>*trn*V5'-*trn*V3' region:</u>
*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 Conditions

PCR reactions were performed in a total volume of 50 µL. For the optimization of PCR conditions, different concentrations of template DNA, primer, $MgCl_2$, dNTP were tested (Table 3.2).

Table 3. 2 Tested PCR components and template DNA concentration for amplification of *trn* region chloroplast genome of Anatolian black pine

10X Buffer	$MgCl_2$ (25 mM stock solution)	dNTP(10mM)	Primer pairs (100µM)	*Taq* DNA polymearse	DNA
5.0 µL	6.0 µL	0.5 µL	0.5 µL + 0.5 µL	0.2 µL	2.5 µL
5.0 µL	7.0 µL	0.5 µL	0.5 µL + 0.5 µL	0.2 µL	2.5 µL
5.0 µL	6.0 µL	0.5 µL	1.0 µL + 1.0 µL	0.2 µL	2.5 µL
5.0 µL	7.0 µL	0.5 µL	1.0 µL + 1.0 µL	0.3 µL	3.0 µL
5.0 µL	6.0 µL	1.0 µL	1.0 µL + 1.0 µL	0.2 µL	2.5 µL
5.0 µL	6.0 µL	0.5 µL	0.5 µL + 0.5 µL	0.3 µL	3.0 µL

Optimized PCR conditions for *trn*L5'-*trn*L3' and *trn*V5'-*trn*V3' regions contained 2.5 µL of template DNA (7.5 ng/µL); 1X of 10X buffer (750 mM Tris.HCl pH: 8.8, 200 mM $(NH_4)_2SO_4$; MBI Fermentas, Lithuania); 0.2 µL (1 unit) of *Taq* DNA polymerase (Fermentas, Ontorio, Canada); 0.1mM of dNTP mix (Fermentas, Ontorio, Canada); 3mM $MgCl_2$ and 50 pmol of each primer. For the *trn*L3'-*trn*F region, PCR conditions were optimized as; 2.5 µL template DNA; 1X of 10X buffer; 0.2 µL (1 unit) of *Taq* DNA polymerase (Fermentas, Ontorio, Canada); 0.2 mM of dNTP mix (Fermentas, Ontorio, Canada); 3mM $MgCl_2$ and 100pmole of each primer (Table 3.3). The thermal cycler (Eppendorf-Mastercycler, Eppendorf, Canada) programs were optimized as indicated in table 3.4.

Table 3. 3 Optimized PCR conditions for *trn* region of chloroplast genome of Anatolian black pine

	*trn*cd'		*trn*ef		*trn*V	
PCR contents	**Volume used in PCR (µL)**	**Final Concentration**	**Volume used in PCR (µL)**	**Final Concentration**	**Volume used in PCR (µL)**	**Final Concentration**
PCR Grade Water	34.8	NA	33.8	NA	34.8	NA
10X PCR Buffer	5	1X	5	1X	5	1X
$MgCl_2$ (25mM stock)	6	3 mM	6	3 mM	6	3 mM
dNTP (10mM of each dNTP)	0.5	0.1 mM	1	0.2 mM	0.5	0.1 mM
Forward primer (100µM)	0.5	1 µM	1	2 µM	0.5	1 µM
Reverse primer (100µM)	0.5	1 µM	1	2 µM	0.5	1 µM
Taq DNA polymerase (5u/µL)	0.2	0.02u/µL	0.2	0.02u/µL	0.2	0.02u/µL
DNA	2.5	7.5 ng/µL	2.5	7.5 ng/µL	2.5	7.5 ng/µL
Total Volume	50		50		50	

Table 3. 4 Optimized thermal cycler program used for amplification of *trn* region of chloroplast genome of Anatolian black pine

***trn* regions**	**Temperature (^{o}C)**	**Duration**	**Number of cycles**	**Purpose**
*trn*cd	94	5 minutes	1	Initial denaturation
	94	30 seconds	30	Internal denaturation
	55	30 seconds		Annealing
	72	50 seconds		Extension
	72	5 minutes	1	Final extension
*trn*ef	94	2 minutes	1	Initial denaturation
	94	1 minute	35	Internal denaturation
	60	1 minute		Annealing
	72	2 minutes		Extension
	72	5 minutes	1	Final extension
*trn*V	94	3 minutes	1	Initial denaturation
	94	45 seconds	30	Internal denaturation
	58	50 seconds		Annealing
	72	80 seconds		Extension
	72	5 minutes	1	Final extension

3.6. Data collection and anaylsis of sequence data of *trn* region

3.6.1. Sequencing of PCR products

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 performed before the sequence analysis. Both purification and sequencing reactions were carried out in the Refgen Biotechnology facilities, 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. In sequence analysis, first PCR product was purified and 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.

Table 3. 5 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

Table 3. 6 Thermal cycler program for sequencing

Temperature (^{0}C)	Duration	Number of cycles	Purpose
96	1 minute	1	Initial denaturation
96	10 seconds	25	Denaturation
50	5 seconds		Annealing
60	4 minutes		Extension
4	∞	1	Hold

After thermal cycling for sequencing, precipitation procedure was applied and it was 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 applied:

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.

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. If any incompatibility was present between the two sequences, the sample was neglected from the analysis.

3.7. Collection and analysis of data

Phylogenetic and molecular evolutionary analysis were conducted using MEGA version 4 (Tamura *et al*., 2007) and Arlequin (Schneider *et al*., 2000) softwares. The sequences were pre-processed in FASTA format by aligning "-" to gaps and "N" to the unknown bases between the three regions *trn*cd, *trn*ef and *trn*VFVR of t-RNA. When the analyzed and unprocessed sequences were compared, it was found that the analyzed sequences were shorter than unprocessed sequence data. When automatic sequencing systems are used, the quality of sequencing decreases at the begining and at the end of the sequencing.

The distances between Anatolian black pine varieties were computed by using Kimura (1980) two – parameter test in MEGA version 4.0. Kimura (1980)'s two parameter model corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and the rates of substitution do not vary among sites. Also when computing distances, pair wise deletion method was used in which gaps and missing data are discarded during analysis when necessary.

3.7.1. Population Genetic Structure Inferred by Analysis of Molecular Variance (AMOVA)

The differentiation between Anatolian black pine var *pallasiana* and other two varieties (Anatolian black pine taxa: i.e. *P. nigra* subsp *pallasiana*, var. *pyramidata* and var. *seneriana*) was investigated by an analysis of variance framework, as initially defined by Cockerham (1969, 1973), and extended by others (e.g. Weir and Cockerham, 1984). This is The Analysis of Molecular Variance (AMOVA) approach which was carried out with Arlequin Software (Excoffier et al., 1992).

Formally, in haploid case, it is assumed that the *i*th haplotype frequency vector from the *j*th taxa in the *k*th group is linear equation of the form as follows:

$\mathbf{X_{ijk} = x + a_k + b_{jk} + c_{ijk}}$ (Equation 1)

The vector **x** is the unknown expectation of $\mathbf{X_{ijk}}$, averaged over the whole study. The effects are **a** for group, **b** for the taxa within group, assumed to be additive, random, independent, and to have the associated covariance components, σ_a^2, σ_b^2 and σ_c^2, respectively. The total molecular variance (σ^2) is the sum of the covariance component due to the differences among haplotypes within a taxa (σ_c^2), the covariance components due to the differences among haplotypes in different taxa within a group (varieties), (σ_b^2), and the covariance components due to the differences among the **G** groups (var *pallasiana* vs two other varieties) (σ_a^2).

Fixation index (F_{ST}) is a measure of population variety differentiation based on genetic polymorphism data, such as single nucleotide polymorphisms (SNPs) or microsatellites. It is a special case of F-statistics, the concept developed in the 1920s by Sewall Wright (Hudson *et al.,* 1992). In terms of inbreeding coefficients and coalescent times, this F_{st} can be expressed as

$$F_{ST} = \frac{f_0 - \bar{f}}{1 - \bar{f}} = \frac{\bar{t} - t_0}{\bar{t}} \qquad \text{(Equation 2)}$$

Where f_o is the probability of identity by descent of two different genes drawn from the same population, $\bar{f}$ is the probability of identity by descent of two genes drawn from two different populations, $\bar{t}$ is the mean coalescence time of two genes drawn from the same population. The significance of the fixation indices is tested using a non-parametric permutation approach described in Excoeffier *et al*. (1992), consisting of permuting in haplotypes, individuals or populations, among individuals, taxa or groups of taxa. After each permutation round, all statistics were recomputed to get their null distribution. Depending on the tested statistic and the given hierarchical design, different types of permutations are formed. Under this procedure, the normality assumption usual in analysis of variance tests is no longer necessary, nor is it necessary to assume equality of variance among taxa or groups of taxa. A large number of permutations was carried out to obtain some accuracy on the final probability. The covariance components are used to compute fixation indices, as originally defined by Wright (1951, 1965), in terms of inbreeding coefficients, or later in terms of coalescent times by Slatkin (1991).

All estimations were performed using Arlequin Software (version 2000) (Schneider *et al*., 2000). The AMOVA design and expected mean squares were given in Table 3.7.

Table 3. 7 Expected AMOVA table for testing variety effect in Anatolian black pine

Source of variation	Degrees of freedom	Sum of Squares	Expected Mean Squares
Among Anatolian black pine taxa group (var. pallasiana vs two other varieties) (G)	G-1(1)	SSD(G)	$n''\sigma_a^2 + n'\sigma_b^2 + \sigma_c^2$
Among Anatolian black pine taxa within group (AT/G)	T-G(2)	SSD(AT/G)	$N\sigma_b^2 + \sigma_c^2$
WithinAnatolian black pine taxa (T/AT)	N-T(37)	SSD(T/AT)	σ_c^2
Total	N-1(40)	SSD(OT)	σ_T^2

SSD(OT) :Total Sum of Squared Deviations
SSD(G) :Sum of Squared Deviations Among Groups of Taxa
SSD(AT/G) :Sum of Squared Deviations Among Individuals of Anatolian Black Pine Within Taxa
SSD(T/AT) :Sum of Squared Deviations Among Taxa, Within Group
G :Number of Varieties in the Structure
T :Total Number of Taxa
N :Total Number of Sequences Involved in the Analysis

3.7.2. Models for Estimating Genetic Distance of Anatolian Black Pine

The evolutionary distance between a pair of sequences usually is measured by the number of nucleotide substitutions occuring between them. Evolutionary distances are fundamental for the study of molecular evolution and are useful for phylogenetic reconstructions and the estimation for divergence times. There are some methods for distance estimation for nucleotide sequences. Further details of these methods and general guidelines for the use of these methods are given by Nei and Kumar (2000).

In addition to distance estimates, also standard errors of estimates were computed using the analytical formulas and the bootstrap method. In nucleotide method, sequences were compared nucleotide-by-nucleotide. p-distance model were chosen in this study. This distance is the proportion (p) of nucleotide sites as which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. It does not make any correction for multiple substitutions at the same site, substitution rate biases (for example, differences in transitional and transversional rates, or differences in evolutionary rates among sites) (Nei and Kumar, 2000).

3.7.3. Estimation of Pairwise Genetic Distances (F_{st}) among Taxa

Estimation of pairwise genetic distances among populations, the pairwise F_{st}' s may be used as genetic distances, with the application of a slight transformation to linearize the distances with the population divergence time (Reynolds *et al.*, 1983; Slatkin, 1995). The pairwise F_{st} values were calculated and given in the form of a matrix. The null distribution of pairwise F_{st} values under the hypothesis of no difference among the populations (varieties) is obtained by permuting haplotypes between the populations.

3.7.4 Construction of Phylogenetic Trees for Anatolian Black Pine Taxa

The phylogenetic tree was constructed by using maximum parsimony method together with bootstrap test analysis (Camin and Sokal, 1965). Parsimony is part of a class of character-based tree estimation methods which use a matrix of discrete phylogenetic characters to infer one or more optimal phylogenetic trees for a set of taxa, commonly a set of species or reproductively-isolated populations of a single species.

The bootstrap test was applied in this study. The bootstrap test, in which the reliability of a given branch pattern is ascertained by examining the frequency of its occurence in a large number of trees, each based on resampled dataset. 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 4.0.

CHAPTER 4

RESULTS

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

Single bands were observed for *trn*cd, *trn*ef and *trn*V in good quality. Optimized PCR conditions for *trn*cd and *trn*V regions contained 2.5 µL of template DNA (7.5 ng/µL); 1X of 10X buffer; 0.2 µL (1 unit) of *Taq* DNA polymerase (Fermentas, Ontorio, Canada); 0.1mM of dNTP mix (Fermentas, Ontorio, Canada); 3mM $MgCl_2$ and 50 pmol of each primer. For *trn*ef region, PCR conditions was optimized as; 2.5 µL template DNA; 1X of 10X buffer; 0.2 µL (1 unit) of *Taq* DNA polymerase (Fermentas, Ontorio, Canada); 0.2 mM of dNTP mix (Fermentas, Ontorio, Canada); 3mM $MgCl_2$ and 100pmole of each primer (Table 3.3). Because of the good quality of the bands, the fragments amplified by all three primers were selected for sequencing (Figure 4.1).

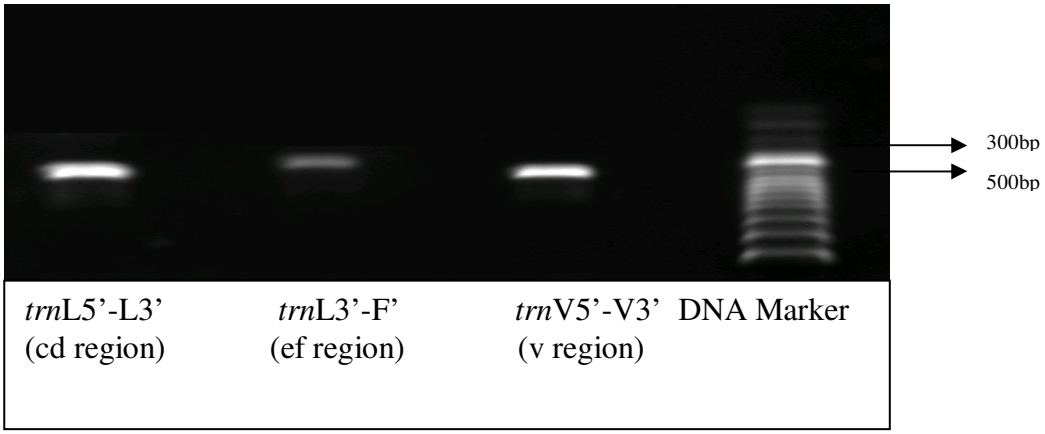


Figure 4.1. Photograph showing the amplified DNA of three regions of tRNA of cpDNA

4.2. Molecular Diversity in the t-RNA Region

In the sequence analysis, the total length is found to be 1345 bp with 35.6% GC content, 26 variable sites, 1255 conserved sites, 15 parsimony – informative sites and 11 singleton sites. There were also 1216 identical pairs, 1 transitional and no transversional pair (Table 4.1). The first region (also called "cd" region) between *trn*L5' and *trn*L3' is about 544 base pairs (bp), the second "ef" region between *trn*L3' and *trn*F is about 492 bp and the third "v" region between *trn*V5' and *trn*V3' is about 553 bp long. Among 544 bp, of "cd" region, GC content was 36.7%. There were 4 variable sites (V), 439 conserved sites, 1 parsimony – informative sites and 3 singleton sites. This region also had 432 identical pairs (ii) and no transitional (si) and transversional pairs (sv) (Table 4.1). The second region (ef region) had 30.9% GC content, 20 variable sites, 338 conserved sites, 9 parsimony – informative sites and 11 singleton sites. Moreover, 341 identical pairs, 1 transitional pairs and 0 transversional pairs were present (Table 4.1).

The third region (v) had 38.8% GC content, 9 variable sites, 468 conserved sites, 0 parsimony – informative sites and 9 singleton sites. There were also 439 identical pairs and no transitional pairs and transversional pairs (Table 4.1).

Table 4. 1 Estimated molecular diversity parameters for *trn*cd, *trn*ef and *trn*V gene region for Anatolian black pine taxa

Molecular Diversity Parameters	**Total**	***trn*L5'-L3' (cd region)**	***trn*L3'-F (ef region)**	***trn*VF5'-VR3' (v region)**
Total sample size	129	43	43	43
Total Length (bp)	1345	544	492	553
GC content (%)	35.6	36.7	30.9	38.8
Conserved sites	1255	439	338	468
Variable sites	26	4	20	9
Singleton sites	11	3	11	9
Parsimony informative sites	15	1	9	0
Identical pairs	1216	432	341	439
Transitional pairs	1	0	1	0
Transversional pairs	0	0	0	0
Usable Site	1229	480	440	456
Polymorphic Site	18	6	33	20
Substutitions	19	3	34	21
Indels	82	6	144	57
Nucleotide Diversity ± S.D. (average over total site)	0.0119 ±0.00606	0.0111 ±0.006	0.0254 ±0.00129	0.0228 ±0.00117

Table 4. 2 Molecular diversity parameters for 3 Anatolian black pine taxa with respect to 3 *trn* regions

	P.nigra* subsp. *pallasiana* var *pallasiana			***P.nigra* subsp. *pallasiana* var. *seneriana***			***P.nigra* subsp. *pallasiana* var. *pyramidata***		
Molecular Diversity Parameters	***Trn*cd**	***trn*ef**	***trn*V**	***trn*cd**	***trn*ef**	***trn*V**	***trn*cd**	***trn*ef**	***trn*V**
Total sample size	10	10	10	24	24	24	7	7	7
Total Length (bp)	544	492	553	544	492	553	544	492	553
GC content (%)	36.6	29.9	39.3	36.6	29.9	39.3	36.6	29.9	39.3
Conserved sites	439	350	467	437	346	472	438	348	473
Variable sites	1	7	2	3	12	5	0	4	2
Singleton sites	1	6	2	3	9	5	0	2	2
Parsimony informative sites	0	1	0	0	3	0	0	2	0
Identical pairs	437	341	450	435	341	435	433	341	442
Transitional pairs	0	1	0	0	1	0	0	1	0
Transversional pairs	0	0	0	0	1	0	0	1	1
Usable Site	417	338	429	434	338	393	413	340	408
Polymorphic Site	9	9	2	4	12	7	14	5	1
Substutitions	1	8	2	2	12	7	0	4	1
Indels	8	2	0	2	1	0	14	1	0
Nucleotide Diversity ± SD (average over total site)	0.0032 ±0.0023	0.0048 ±0.0033	0.0007 ±0.0008	0.0011 ±0.0011	0.0036 ±0.0026	0.0013 ±0.0013	0.0070 ±0.0045	0.0061 ±0.0045	0.0005 ±0.0007

Table 4. 3 Molecular diversity parameters from 3 Anatolian black pine taxa with respect to 3 regions of *trn* combined

	Anatolian Black Pine Taxa		
Molecular Diversity Parameters	***P.nigra* subsp. *pallasiana* var *pallasiana* complete *trn* sequence**	***P.nigra* subsp. *pallasiana* var. *seneriana* complete *trn* sequence**	***P.nigra* subsp. *pallasiana* var. *pyramidata* complete *trn* sequence**
Total sample size	10	24	7
Total Length (bp)	1589	1589	1589
GC content (%)	35.6	35.6	35.6
Conserved sites	1261	1261	1267
Variable sites	2	19	2
Singleton sites	2	17	0
Parsimony informative sites	0	2	2
Identical pairs	1227	1212	1216
Transitional pairs	0	1	1
Transversional pairs	0	0	1
Usable Site	1231	1225	1225
Polymorphic Site	38	78	42
Substutitions	6	17	3
Indels	33	73	39
Nucleotide Diversity ± SD (average over total site)	0.0096±0.0053	0.0123±0.0063	0.0145±0.0086

4.2.1 Molecular Diversity in Anatolian Black Pine

As indicated in Table 4.1, the total length of all samples was 544 bp for *trn*cd region, 492 bp for *trn*ef region, 553 bp for *trn*V region. The total length of the whole region was about 1345 bp. In whole region, 26 variable sites were found. Of these, *trn*cd, *trn*ef and *trn*V had 4, 20 and 9 variable sites, respectively. The overall variable sites were 26. According to Table 4.1, *trn*cd had 1 parsimony site; however *trn*ef and *trn*V had 9 and 0 parsimony sites, respectively. Totally, the number of parsimony sites was 15. Morover, *trn*cd region had 6 polymorphic sites while *trn*ef had 33, and *trn*V had 20 polymorphic sites. The total number of polymorphic sites were 18. According to Table 4.2, *trn*cd region had 1, 3 and 0 variable sites in *P.nigra* subsp *pallasiana* var *pallasiana*, var., *seneriana* and var. *pyramidata*, respectively. The *trn*ef region had 7 in *P.nigra* subsp. *pallasiana* var *pallasiana*, 12 in var. *seneriana* and 4 in var. *pyramidata*. While these were 2, 5 and 2 in *P.nigra* subsp *pallasiana* var *pallasiana*, var. *seneriana* and var. *pyramidata*, respectively for *trn*V region. Considering parsimony sites, *trn*ef had only the parsimony sites as 1, 3 and 2 for the taxa *P.nigra* subsp *pallasiana* var *pallasiana*, var. *seneriana* and var. *pyramidata*, respectively. The polymorphic sites ranged from 2 in *trn*V to 9 in both region in *P. nigra* subsp *pallasiana* var *pallasiana.* The number of polymorphic sites in var *seneriana* were 4, 12 and 7, while they were 14, 5 and 1 in var. *pyramidata* for the regions of *trn*cd, *trn*ef and *trn*V region, respectively. With respect to nucleotide diversities, var *pyramidata* was the most diverse taxa for the regions *trn*cd (0.0069) and *trn*ef (0.0062). For the *trn*V region, var *seneriana* was the most diverse one (0.00131).

Among three *trn* regions the highest nucleotide diversity was observed in *trn*ef region with the values of 0.0048, 0.0036 and 0.0062 in *P.nigra* subsp *pallasiana* var *pallasiana*, var *seneriana* and var *pyramidata*, respectively.

As indicated in Table 4.3, the number of variable and polymorphic sites were the highest in var *seneriana* with the value of 19 and 78, respectively.The parsimony sites were low and ranged from 0 in *P.nigra* subsp *pallasiana* var *pallasiana*, to 2 in both var *seneriana* and var *pyramidata*. Considering nucleotide diversity, var *seneriana* (0.01231) and var *pyramidata* (0.01448) had similar diversity values.

4.3. Molecular Variances Among Anatolian Black Pine Taxa

AMOVA analysis with 3 Anatolian black pine taxa was performed. About 99% of total molecular variance was among the taxa and about 1% total molecular variance was within taxa, with respect to all three trn regions. (Table 4.4). When whole *trn* region is considered, 98.49% total molecular variance was among taxa and 1.60% total molecular variance was within taxa (Table 4.5).

AMOVA analysis among taxa was carried out by grouping them as variety *pallasiana* vs other two varieties. One of the groups composed of 10 individuals from subsp. *pallasiana* var *pallasiana*, while the second group was formed with 2 other varieties (var. *seneriana* with 24 trees and var. *pyramidata* with 7 trees). There was little variation among groups. However, the great proportion of total molecular variance (95.70%) was due to among taxa within groups (Table 4.6).

Table 4. 4 AMOVA results for Anatolian black pine taxa with respect to 3 *trn* regions

Source of variation	d.f			Sum of squares			Variance components			Percentage of total variation		
	***trn*cd**	***trn*ef**	***trn*V**	***trn*cd**	***trn*ef**	***trn*V**	***trn*cd**	***trn*ef**	***trn*V**	***trn*cd**	***trn*ef**	***trn*V**
Among taxa	2	2	2	4753.903	3548.858	4414.522	141.79	122.75	142.84	99.57	99.41	99.86
Among trees within taxa	51	46	48	31.74	33.550	9.498	0.62	0.72	0.19	0.43	0.59	0.14
Total	53	48	50	5092.98	3592.48	4424.020	145.70	123.48	143.04	100	100	100

45

Table 4. 5 AMOVA results for Anatolian black pine with regarding to whole *trn* region

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation
Among taxa	2	9994.058	449.52	98.39
Within taxa	37	271.09	7.32	1.61
Total	39	10265.15	456.22	100

Table 4. 6 AMOVA results with respect to varieties vs normal Anatolian black pine

Source of variation	Degrees of freedom	Sum of Squares	Variance Components	Percentage of total variation
Among Anatolian black pine groups (var *pallasiana* vs other two varieties)	1	5656.42	12.50	2.71
Among Anatolian black pine taxa within group	2	4964.04	440.89	95.70
Among trees within Anatolian black pine taxa	37	271.09	7.32	1.59
Total	40	10891.56	460.72	100

4.3.1. Average Diversity in Anatolian Black Pine Taxa

The average diversity among trees (genotypes) within Anatolian black pine taxa were computed. The most diverse taxa was *P. nigra* subsp *pallasiana* var *pyramidata*. This var. *pyramidata* was also genetically the most distant one from *P. nigra* subsp *pallasiana* var *pallasiana*(Table 4.7).

Table 4. 7 Average genetic distance within populations of Anatolian black pine

Complete *trn* region				
Average diversity within taxa of Anatolian black pine		**Genetic distance between taxa of Anatolian black pine**		
		P. nigra* subsp *pallasiana* var *pallasiana	***P. nigra* subsp *pallasiana* var *seneriana***	***P. nigra* subsp *pallasiana* var *pyramidata***
P. nigra* subsp *pallasiana* var *pallasiana	0.0004 (±0.0002)			
P. nigra* subsp *pallasiana* var *seneriana	0.0005 (±0.0002)	0.0004 (±0.0002)		
P. nigra* subsp *pallasiana* var *pyramidata	0.0009 (±0.0006)	0.0011 (±0.0006)	0.0011 (±0.0006)	

4.4 Genetic Differences of among *Pinus* species as well as among Anatolian varieties of *P.nigra* Based on F_{st} Values

Pairwise F_{st} values among Anatolian black pine taxa by using 43 *trn* sequences were estimated and given in Table 4.8. If Fst is equal to zero, compared taxa do not have any difference. F_{st} value between *P.nigra* subsp *pallasiana* var *pallasiana* and *P. nigra* subsp *pallasiana* var *seneriana* was 0.0077; F_{st} value between *P.nigra* subsp *pallasiana* var *pallasiana* and *P.nigra* subsp *pallasiana* var *pyramidata* was 0.0080 and F_{st} value between *P.nigra* subsp *pallasiana* var *seneriana* and *P.nigra* subsp *pallasiana* var *pyramidata* was 0.0088. The F_{st} values between Anatolian black pine taxa and outgroup (*P. sylvestris*) showed considerable high values ranging from 0.5900 to 0.8523.

Table 4. 8 Pairwise comparison of Fst values among Anatolian black pine varieties

Taxonomic units	PNPA	PNSE	PNPY	PS_out
***P.nigra* subsp *pallasiana* var *pallasiana* (PNPA)**	---			
***P.nigra* subsp *pallasiana* var *şeneriana* (PNSE)**	0.0077	---		
***P.nigra* subsp *pallasiana* var *pyramidata* (PNPY)**	0.0080	0.0088	---	
***P. sylvestris* outgroup (PS_out)**	0.5900	0.8261	0.8523	---

4.5 Phylogenetic Trees

Phylogenetic tree was constructed by including *P.nigra* as outgroup from Japan along Anatolian black pine 2 taxa (Figure 4.2). Although the constructed tree for Anatolian black pine did not reveal any clear pattern, it appears that those taxa labeled as var. *seneriana* and var. *pyramidata* varieties were somewhat grouped in different clusters. However, sequences from *P.nigra* subsp *pallasiana* var *pallasiana* and other variaties were mixed in formed clusters, not presenting a firm grouping by taxa.

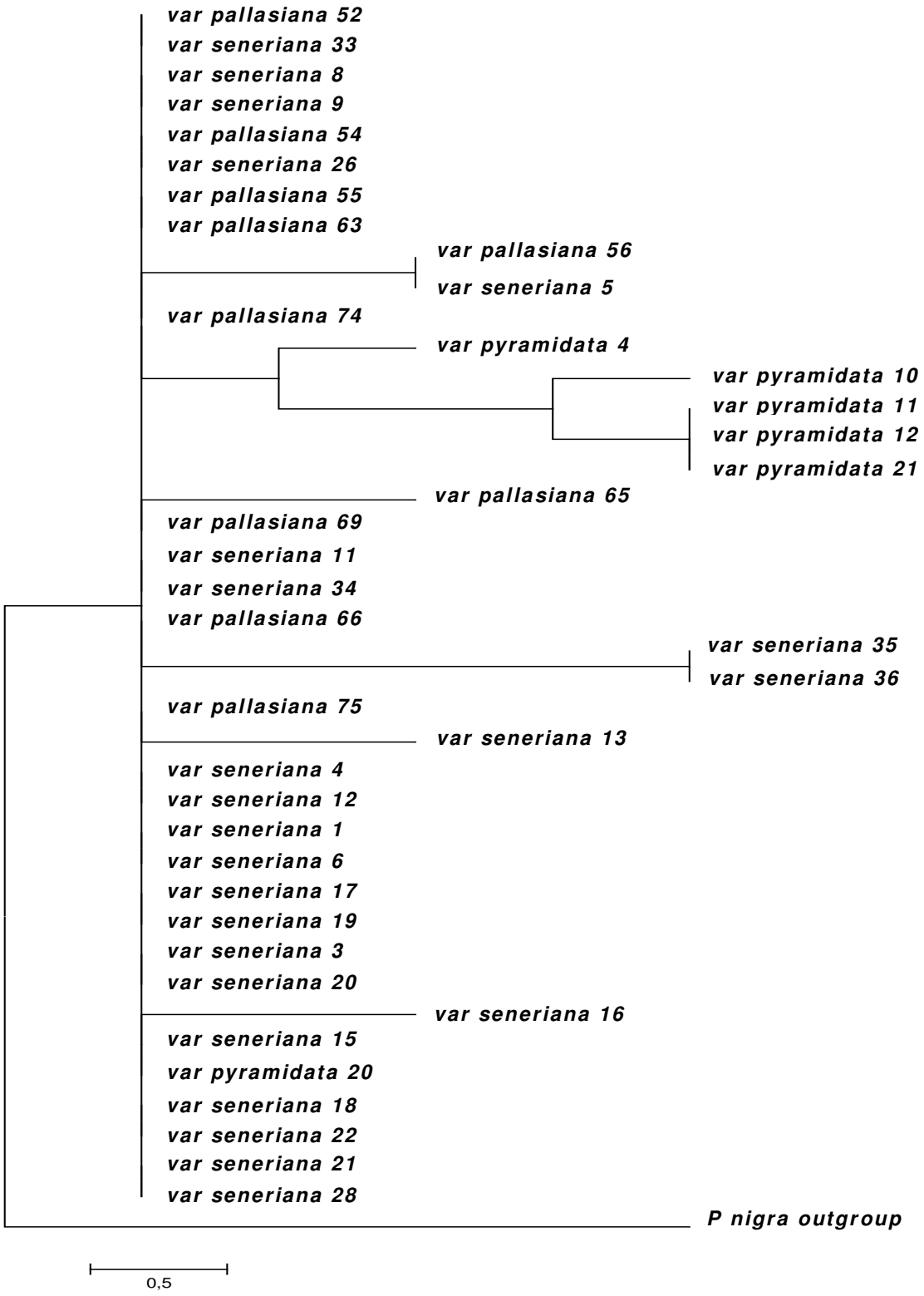


Figure 4.2. The phylogenetic tree constructed by 3 Anatolian black pine taxa and 1 *P. nigra* species as outgroup (the values above and below branches are the bootstrap values)

CHAPTER 5

DISCUSSION

5.1. Molecular Diversity in Anatolian Black Pine

In this study, because of indels (insertion and deletion of bases) cpDNA *trn* region was about 1394bp. The length of *trn*cd region was 544bp in length which was in the range of the reported lengths (Wakasugi *et al*., 1992). However, the previous studies reported that this region ranges from 448 bp to 520 bp in seed plants (Stech *et al*.,2003). The length of *trn*ef region was 492 bp in length. In the study performed by Ferri *et al.* (2008) it was 464bp in length and according to Lopez *et al*. it was about 400 bp in length as a result of several studies (2001, 2002, 2006). Moreover, sequencing showed a length of 471 bp for the loblolly pine, 468 bp for shortleaf pine and 467 bp for slash pine (Chen *et al*., 2002). The DNA sequence of the cpDNA spacer region between the *trn*L and *trn*F genes was determined for 18 *Abies* species. This region varied in length from 403bp to 455bp among species (Isoda *et al*., 2000). The length of *trn*V region was 553bp. According to the several studies, the length of the region is about 540bp (Wang *et al*., 1999; Ann *et al*., 2006; Eckert *et al.*, 2006; Lopez *et al*., 2001). Moreover, the *trnV* intron in *P. thunbergii* is 543 bp long, lying between positions 47471 and 48013 (Wakasugi *et al*., 1994). Because *trn* region is shown high variation, it could be possible that *trn* region is different in length (because of indels).

In the sequence analysis, the entire *trn* region had 35.6% GC content, 26 variable sites, 1255 conserved sites, 15 parsimony – informative sites and 11 singleton sites. There were also 1216 identical pairs, 1 transitional pair and 0 transversional pair. The *trn* region of *Picea* species had 187 variable sites and 74 parsimony informative characters; moreover, 6 indels occured (Ran *et al*., 2006). The total nucletide diversity was 0.009622, 0.012313 and 0.014476 for *P.nigra* subsp *pallasiana*, var *seneriana* and var *pyramidata*, respectively. The total nucleotide diversity was 0.04023, 0.01229, 0.01215, 0.00748, and 0.00542 of *Taxus, Amentotaxus, Cephalotaxus,* and *Torreya*, respectively (Hao *et al*., 2009). The results suggest that different portions of the *trn*L-F region had different evolutionary patterns and might not share the same evolutionary history.

The aligned *trn*L-*trn*F region contained 20 variable sites and 9 parsimony informative sites. In *Larix* species there were 18 variable nucleotide sites, of which 44 are parsimony-informative (Wei et al., 2002). However, other *trn* regions did not show such a variability. The reason can br due to the young evolutionary history of *trn*ef region.

Considering 3 Anatolian black pine taxa for entire *trn* region, *P.nigra* subsp *pallasiana* var *seneriana* had 19 variable sites, 17 singleton sites, 78 polymorphic sites, 17 substitutions and 73 indels which are the highest numbers among 3 Anatolian black pine taxa. These results indicates that *P.nigra* subsp *pallasiana* var *seneriana* was the most polymorphic taxa. According to Sıvacıoğlu and Ayan (2007) and Ünaldı (2005), this endemic variety of the Anatolian Black Pine is indeed genetically rich taxa and should be paid attention to its conservation.

5.2. Partition of Total Molecular Variation in Anatolian Black Pine Taxa

According to AMOVA analysis for all individuals grouped according to their varieties and their located taxa, the percentage of variation within taxa were 1.59 and 1.61 according to varieties and taxa, respectively. These results indicates that there is no substantial differentiation between varieties and normal Anatolian black pines. However, the large portion of the total variance in *trn* region was due to among taxa indicating that at least one of these three taxa such as var. *pyramidata* showing divergence from others. Nevertheless, tihs divergence is not substantiate the speciation among varieties. This issue could be further studied by including sampling the normal and varieites in the same location in their natural settings.

5.3. Average Diversity within Anatolian Black Pine Taxa

Average diversity for entire *trn* region was computed for 3 Anatolian black pine taxa. The result indicated that *P.nigra* subsp *pallasiana* var *pyrmidata* was the most divergent taxon with respect to genetic diversity within taxon as well as divergence from taxa.

Considering 3 *trn* region seperately the result becomes more meaningful such that in *trn*cd the diversity is 0 for *P.nigra* subsp *pallasiana* var *pyramidata*; however, in *trn*ef region the diversity is considerably high in var *pyramidata* and somewhat high in *trn*V. This indicates that the diversity of var *pyramidata* is the result of the divergence of mainly *trn*ef region; however, other regions seem to be highly conserved. When average diversity computed for whole studied taxa of Anatolian black pine is considered, again the *trn*ef region seems to be the most polymorphic region (0.00348 for normal taxa and 0.00372 for variety taxa).

5.4. Genetic Differences of among *Pinus* species as well as among Anatolian taxa of *P.nigra* Based on Fst Values

While comparing the pairwise Fst values among Anatolian black pine varieties, the most differentiation was observed between *P.nigra* subsp *pallasiana* var *pyramidata* and var. *seneriana* with the value of 0.0088. This indicates that although there was no considerable divergence between Anatolian black pine taxa, var *pyramidata* is the most diverse taxon.

5.5 The Constructed Phylogenetic Trees by MEGA 4.0

From the constructed phylogenetic tree (Figure 4.2), it was shown that Anatolian black pine taxa formed three branches with bootstrap values 67, 50 and 92 meaning that those topologies are just phylogenetically informative. In the the branch having a bootstrap value of 50, 5 individuals of *P.nigra* subsp *pallasiana* var *pyramidata* were grouped together. This purposes that there were considerable genetic variation between var *seneriana* and other two taxa when *trn* region is used as a molecular evolutionary tool.

Trees 35 and 36 formed a cluster with a relatively high bootstrap value, 92 which suggests that these trees have significantly seperated from the other taxa of Anatolian black pine phylogenetically.

CHAPTER 6

CONCLUSION

The main purpose of this study was to obtain genetic data that will help to solve taxonomic status of 3 Anatolian black pine taxa (*P.nigra* subsp *pallasiana*, *P.nigra* subsp *pallasiana* var *seneriana* and *P.nigra* subsp *pallasiana* var *pyramidata*) at variety and species levels by means of studying *trn* gene region of cpDNA.

Anatolian black pine *trn* gene was found to be 1345 bp in length. Three different *trn* regions (*trn*cd, *trn*ef and *trn*V), as well as whole *trn* region comparative sequence analysis indicated that *trn*ef region was found to be more variable than other regions of *trn*.

Comparison of the genetic diversity of 3 Anatolian black pine taxa with respect to *trn* region and parsimonic sites showed that *P. nigra* subsp *pallasiana* var *seneriana* were more polymorphic than other two taxa. Also, the most distant taxon that show differences in *trn* sequences when compared to other taxa was *P. nigra* subp *pallasiana* var *pyramidata*.

The constructed phylogenetic tree showed that individuals of *P.nigra* subsp *pallasiana* var *pyramidata* were grouped together. However, other two taxa showed a dispersed allocation in the tree. This result indicates that var *pyramidata* was the most distant taxon.

The result of the present study indicated that there is no clear speciation or genetic divergence of varieties from the normal Anatolian black pine. Although it has to be further data and studies are needed, these varieties seem to be evolved as a result of mutation which may have occured in the genes coding for growth and form of Anatolian black pine.

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APPENDIX A

AN EXAMPLE OF CHROMOTOGRAM DATA

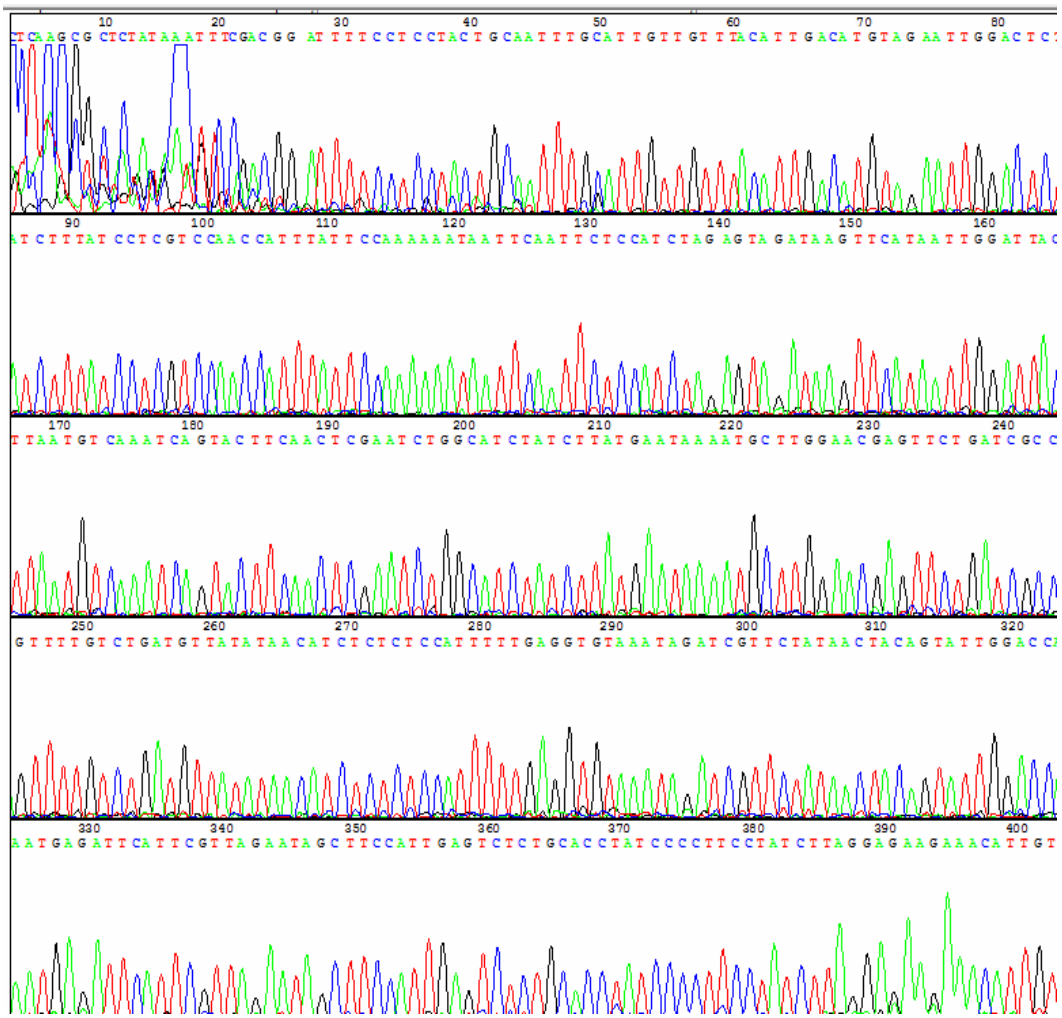

Figure A.1. An Example of Chromotogram Data

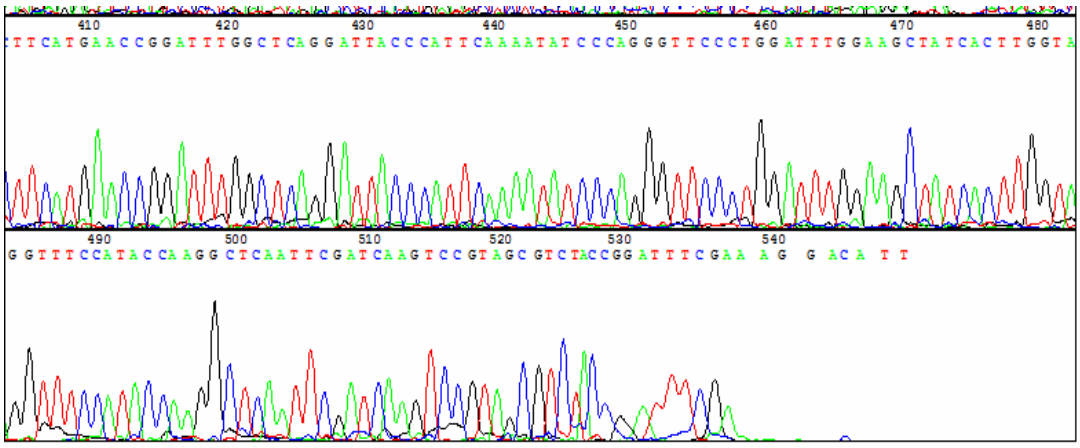

Figure A. 1. Continued

APPENDIX B

AN EXAMPLE OF MEGA DATA FILE

#var pallasiana54 for *trn*L5'-L3'
ACGGATTTTCCTCCTACTGCATTTGCATTGTTGTTTACATTGACATGTAGA
ATTGGACTCTATCTTTATCCTCGTCCAACCATTTATTCCAAAAAATAATTC
AATTCTCCATCTAGAGTAGATAAGTTCATAATTGGATTACTTAATGTCAA
ATCAGTACTTCAACTCGAATCTGGCATCTATCTTATGAATAAAATGCTTG
GAACGAGTTCTGATCGCCAGTTTTGTCTGATGTTATATAACATCTCTCTCC
ATTTTTGAGGTGTAAATAGATCGTTCTATAACTACAGTATTGGACCAAAT
GAGATTCATTCGTTAGAATAGCTTCCATTGAGTCTCTGCACCTATCCCCTT
CCTATCTTAGGAGAAGAAACATTGTCTTCATGAACCGGATTTGGCTCAGG
ATTACCCATTCAAAATATCCCAGGGTTCCCTGGA
#var pallasiana54 for *trn* L3'-F'
TCCATTGGTTCGAATCCATTCTAATTTCTCGATTCTTTTACCTCGCTATTTT
TTTTTTTCATGAAGAGAAGAAATTAGAACATGAATCTTTTCATCCATCTTA
TGACCAGTTG
AGTTGATCTGTTAATAAGCTGATCATATGATCAATTGATTTTGTGATATAT
GATTTACATAGATTAGATCATTTTGAAATTATTCAATTGCAGTCCATTTTT
ATCATATTAGTGACTTCCAGATCGAAAATAATAAAGATCATTCTAAAAAC
TAGTAAAAATACCTTTTTACTTCTTTTTAGTTGACACAAGTTAAAACCCTG
TACCAGGATGATCCACAGGGAA
#var pallasiana54 for *trn*VF5'-VR3'
TTTCGGGAGAGTTTATCGATTCGTCCGATCCACGAAATAGATTCTATGTG
AAATAGTCTTACTCTATAAATTTGTTTCTCTGGGGAACAATAGCATGACA
AAGATTAAGTTCGATCTGATTCGAATTACGGATCTAATTGATATGGTCAA
TCCCAGCTCTGTTCAATGCCAGGCATAATGAGTATAATACGGGGACCTCA
AAATAGATTCTTTTCGCTCTATGAACTTTTAGGTGTATGAAGTGTCATATT
TTACTTTTGGAGCGATAGAAGAGACTCTATTTGAGTCAATCTATGCCCGA
GCAAGGCAGACCTACGTCAAAAAAACCTTTTGAATAACTTTGGGATTGCT
TCCGAAGGGTAAGAATTTGGAGCACACGGAGCCATATTAGTATCTTACCG
GAAAGAGGAGAATGGCAGACTAACCGATCTTTCCATCAGTTAATGAAAG
AGCCCAATGCGAGAAAATGCATG

APPENDIX C

AN EXAMPLE OF ARLEQUIN SEQUENCE DATA

[Profile]

Title="*trn*cd gene"

NbSamples=41
GenotypicData=0
DataType=DNA
LocusSeparator=NONE
MissingData='?'

[Data]

[[Samples]]

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SampleSize=10
SampleData= {
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AATTGGACTCTATCTTTATCCTCGTCCAACCATTTATTCCAAAAAATAATT
CAATTCTCCATCTAGAGTAGATAAGTTCATAATTGGATTACTTAATGTCA
AATCAGTACTTCAACTCGAATCTGGCATCTATCTTATGAATAAAATGCTT
GGAACGAGTTCTGATCGCCAGTTTTGTCTGATGTTATATAACATCTCTCTC
CATTTTTGAGGTGTAAATAGATCGTTCTATAACTACAGTATTGGACCAAA
TGAGATTCATTCGTTAGAATAGCTTCCATTGAGTCTCTGCACCTATCCCCT
TCCTATCTTAGGAGAAGAAACATTGTCTTCATGAACCGGATTTGGCTCAG
GATTACCCATTCAAAATATCCCAGGGTTCCCTGGATTTGG
}
…

SampleName="POP02cdVAR_SENERIANA"
SampleSize=24
SampleData= {
var seneriana1 1 ----
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CATGTAGAATTGGACTCTATCTTTATCCTCGTCCAACCATTTATTCCAAAA
AATAATTCAATTCTCCATCTAGAGTAGATAAGTTCATAATTGGATTACTT
AATGTCAAATCAGTACTTCAACTCGAATCTGGCATCTATCTTATGAATAA
AATGCTTGGAACGAGTTCTGATCGCCAGTTTTGTCTGATGTTATATAACA
TCTCTCTCCATTTTTGAGGTGTAAATAGATCGTTCTATAACTACAGTATTG

GACCAAATGAGATTCATTCGTTAGAATAGCTTCCATTGAGTCTCTGCACC
TATCCCCTTCCTATCTTAGGAGAAGAAACATTGTCTTCA
}
…

SampleName="POP03cdVAR_PYRAMIDATA"
SampleSize=6
SampleData= {
Var pyramidata1 1 ----
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AATTGGACTCTATCTTTATCCTCGTCCAACCATTTATTCCAAAAAATAATT
CAATTCTCCATCTAGAGTAGATAAGTTCATAATTGGATTACTTAATGTCA
AATCAGTACTTCAACTCGAATCTGGCATCTATCTTATGAATAAAATGCTT
GGAACGAGTTCTGATCGCCAGTTTTGTCTGATGTTATATAACATCTCTCTC
CATTTTTGAGGTGTAAATAGATCGTTCTATAACTACAGTATTGGACCAAA
TGAGATTCATTCGTTAGAATAGCTTCCATTGAGTCTCTGCACCTATCCCCT
TCCTATCTTAGGAGAAGAAACATTGTCTTCATGAACCGGATTTGGCTCAG
GATTACCCATTCAAAATATCCCAGGGTTCCCTGG
}
… and other individuals are included as sampled above for 3 *trn* regions

[[Structure]]

StructureName="3 populations and 1 outgroup"
NbGroups=1
#3 populations
Group= {
"POP01cdVAR_PALLASIANA"
"POP02cdVAR_SENERIANA"
"POP03cdVAR_PYRAMIDATA"
"P.nigra"

}

APPENDIX D

AVERAGE GENETIC DISTANCE WITHIN POPULATIONS OF ANATOLIAN BLACK PINE WITH REGARDING 3 trn REGIONS

***trn* L5'-L3'**				
Average diversity within taxa of Anatolian black pine		**Average diversity between taxa of Anatolian black pine**		
		P. nigra subsp pallasiana var pallasiana	**P. nigra subsp pallasiana var şeneriana**	**P. nigra subsp pallasiana var pyramidata**
P. nigra subsp pallasiana var pallasiana	0.00030 (±0.00030)			
P. nigra subsp pallasiana var şeneriana	0.00018 (±0.00018)	0.00023 (±0.00021)		
P. nigra subsp pallasiana var pyramidata	0.00000 (±0.00000)	0.00015 (±0.00014)	0.00009 (±0.00008)	
***trn* L3'-F'**				
Average diversity within taxa of Anatolian black pine		**Average diversity between taxa of Anatolian black pine**		
		P. nigra subsp pallasiana var pallasiana	**P. nigra subsp pallasiana var şeneriana**	**P. nigra subsp pallasiana var pyramidata**
P. nigra subsp pallasiana var pallasiana	0.00348 (±0.00138)			
P. nigra subsp pallasiana var şeneriana	0.00264 (±0.00089)	0.00305 (±0.00091)		
P. nigra subsp pallasiana var pyramidata	0.00573 (±0.00284)	0.00585 (±0.00235)	0.00541 (±0.00227)	
***trn* V5'-V3'**				
Average diversity within taxa of Anatolian black pine		**Average diversity between taxa of Anatolian black pine**		
		P. nigra subsp pallasiana var pallasiana	**P. nigra subsp pallasiana var şeneriana**	**P. nigra subsp pallasiana var pyramidata**
P. nigra subsp pallasiana var pallasiana	0.00071 (±0.00048)			
P. nigra subsp pallasiana var şeneriana	0.00038 (±0.00026)	0.00055 (±0.00028)		
P. nigra subsp pallasiana var pyramidata	0.00100 (±0.00069)	0.00086 (±0.00041)	0.00069 (±0.00036)	

APPENDIX E

AVERAGE DIVERSITY COMPUTED AMONG TAXA OF VARIETIES OF ANATOLIAN BLACK PINE

***trn* L5'-L3'**			
Average diversity among taxa with varieties		**Average diversity between varieties**	
		Normal	**Variety**
Normal	0.00030 (±0.00029)		
Variety	0.00013 (±0.00012)	0.00021 (±0.00020)	
***trn* L3'-F'**			
Average diversity among taxa with varieties		**Average diversity between varieties**	
		Normal	**Variety**
Normal	0.00348 (±0.00131)		
Variety	0.00372 (±0.00108)	0.00364 (±0.00101)	
***trn* V5'-V3'**			
Average diversity among taxa with varieties		**Average diversity between varieties**	
		Normal	**Variety**
Normal	0.00071 (±0.00048)		
Variety	0.00056 (±0.00027)	0.00063 (±0.00027)	
Total *trn* region			
Average genetic distance among taxa with varieties		**Average distance between varieties**	
		Normal	**Variety**
Normal	0.00035 (±0.00023)		
Variety	0.00073 (±0.00030)	0.00055 (±0.00020)	