THE PHYLOGENETIC ANALYSIS OF *PICEA ORIENTALIS* POPULATIONS FROM NORTHEASTERN TURKEY WITH RESPECT TO NON-CODING *trn* AND *matK* REGIONS OF CHLOROPLAST GENOME

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ABSTRACT

THE PHYLOGENETIC ANALYSIS OF *PICEA ORIENTALIS* POPULATIONS FROM NORTHEASTERN TURKEY WITH RESPECT TO NON-CODING *trn* AND *matK* REGIONS OF CHLOROPLAST GENOME

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The genus *Picea* is located from temperate to Taiga (boreal) regions of northern hemisphere from subtropical to high altitude with 34 species. *Picea orientalis* is endemic to Eastern Black Sea Mountainous region of Turkey and Western Caucasus.

To determine the genetic relatedness within *Picea orientalis* populations, as well as the relationship between other Pinaceae species from database, populations were sampled from 15 different locations within the natural range of species and grouped into 5 depending on several criteria. In order to evaluate the genetic structure of the taxon, non-coding *trn* and *matK* regions of choloroplast DNA (cpDNA) were sequenced.

According to genetic diversity analysis of 15 *Picea orientalis* populations with respect to *trn* and *matK* regions, there is not much variation among populations. Among 3 non-coding *trn* and the *matK* region, there is only one variable site which was parsimony informative.

The results indicated that the populations from Artvin had the highest divergence. In this study, the genetic divergence of *Picea orientalis* from other

Pinaceae species were also observed. According to the results obtained from *trn*V region the studied *Picea orientalis* observed to display a close relationship with *Larix* and distinct from other Pinaceae especially *Pinus* genus. This result is unrepresentative due to the results of other studies.

Moreover, as a result of analysis with *trn*cd-ef region, the studied *Picea orientalis* populations possessed close relationship with species from clade *Picea*. Moreover, based on molecular clock estimations the studied *Picea orientalis* populations had close relationships with the species form Asia. Finally, the relationship of *Picea orientalis* with other *Picea* species were analyzed with respect to *matK* region. The result is consistent with the results of *trn*cd-ef region and also with other studies.

Key Words: *Picea orientalis*, *trn*, *matK*, cpDNA, genetic variance, phylogeny

ÖZ

KLOROPLAST GENOMUNDAKİ KODLANMAYAN *trn* VE *matK* BÖLGELERİNİN KARŞILAŞTIRILMASI YAPILARAK KUZEYDOĞU ANADOLU'DAKİ *PICEA ORIENTALIS* POPULASYONLARININ FİLOGENETİK ANALİZİ

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Picea cinsinin 34 türü kuzey yarımkürenin ılıman bölgeleri ile taiga bölgeleri arasında alt tropik kesimden yüksek kesimlere kadar bir yayılım gösterir. *Picea orientalis* Türkiye' de Doğu Karadeniz Bölgesi'nin dağlık bölgelerinde ve Batı Kafkaslar'da endemik olarak yetişir.

Picea orientalis populasyonları içindeki genetik ilişkiyi belirlemek amacıyla 15 populasyon türün farklı doğal yayılış alanından örneklendi ve belli kriterlere dayanarak beş gruba ayrıldı. Populasyonların genetik yapılarını değerlendirmek amacıyla kloroplast genomundaki kodlanmayan *trn* ve *matK* bölgelerinin DNA dizi analizleri yapıldı.

Onbeş Doğu Ladini (*Picea orientalis*) populasyonun çalışılan *trn* ve *mat*K bölgelerine dayalı olarak ortaya çıkan genetik varyasyon sonuçlarına göre populasyonlar arasında çok belirgin bir farklılık gözlenmemiştir. Üç tane *trn* bölgesi ve *matK* bölgesi arasında sadece bir tane parsimonik olarak bilgi verici varyasyon görülmüştür. Analizlere göre, Artvin'den elde edilen populasyonlar en fazla farklılığı gösteren grup olmuştur.

Doğu ladinin diğer Pinaceae türleriyle ilişkisi de bu çalışmada incelenmiştir. Veri tabanlarından elde edilen Pinaceae türlerinin *trn*V bölgesine göre ilişkisi incelendiğinde *Picea* cinsinin *Larix* cinsiyle yakın olduğu, *Pinus* başta olmak üzere diğer cinslerden daha uzak olduğu gözlenlenmiştir. Bu sonuç yapılan diğer sonuçlara göre sağlıklı sonuçlar vermemiştir.

*trn*cd-ef bölgesine bağlı olarak yapılan analizlerde, Doğu Ladinin *Picea* seksiyonuyla yakınlığı gözlemlenmiştir. Ayrıca molekular saat tahminlerine göre çalışılan doğu ladini populasyonunun Asya'daki türlerle daha yakın ilişki içinde olduğu bulunmuştur. Son olarak *mat*K bölgesiyle yapılan çalışmada *trn*cd-ef bölgesine ve daha önce yapılmış diğer çalışmalarla benzer sonuçlar elde edilmiştir.

Anahtar Kelimeler: *Picea orientalis*, *trn*, *matK*, cpDNA, genetik çeşitlilik, filogenetik

For my family and my wife Aysun with all my love.

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LIST OF ABBREVIATIONS

AMOVA	Analysis of Molecular Variance
DNA	Deoxyribonucleic Acid
cpDNA	Chloroplast DNA
mtDNA	Mitochondrial DNA
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
***trn*L**	trnA coding Leucine carrying tRNA
***trn*L-F**	trnA coding Phenylalanine carrying tRNA
***trn*cd**	trnA (with codes of primer pairs) coding Leucine carrying tRNA
***trn*ef**	trnA (with codes of primer pairs) coding Phenylalanine carrying tRNA
***trn*V**	trnA coding Valine carrying tRNA
***mat*K**	Maturase K
SE	Standard Error
mya	Million Years Ago
trnA	trnL + trnL-F + trnV
RFLP	Restriction Fragment Length Polymorphism

CHAPTER 1

INTRODUCTION

1.1. Biology of Genus *Picea*

Picea is a genus of about 40 species within the family *Pinaceae,* found in Northern temperate and Taiga (boreal) regions of the earth. The genus is related most closely to *Pinus,* but it still differs. However, the genus is highly monophyletic such that making a classification with respect to genus level is not suggestable (Farjon, 1990). According to Farjon (2001), there are 34 spruce species, of which 24 native to Asia, 8 to North America and 2 to Europe.

Ran *et al.* (2006) noted that the earliest known fossil of *Picea* was a pollen grain from Paleocene (~60 mya) era and the genus was well represented in Eocene (~45 mya) sediments of North America, but it was not common in Asia until the Oligocene (~30 mya) and in Europe until Pliocene (~4 mya, LePage, 2001). Ran *et al.*(2006) also agreed that the species were originated in North America and dispersed through Asia to Europe.

Today, *Picea* species are restricted to subtropical high altitude, temperate and boreal regions of the northern hemisphere. The main location of spruce species is boreal forests where the species are generally located in the Scandinavia, Russia, Alaska, Canada as well as mountains of south and west China and Japan. The southern species of the genus (*P. morrisonicola*) is on Taiwan and there are also some species of *Picea* on Tropic of Cancer at 23° N, but does not reach to Tropic (Taylor *et al.*,1994).

Spruces are evergreen trees with conic to spirlike shape. They are generally 20-60 m tall with gray to reddish brown, thin and scaly bark. Leaves spread radially, persisting up to 10 years and mostly rigid. Pollen cones are single or grouped with yellow to purple color. They shed their pollens in spring. Seed cones are green to purple in color, maturing pale to dark brown in autumn. They usually shed at maturity, born mostly on upper branches. Seeds are winged with 5 to 10 cotyledons (Taylor, 1993; Yaltirik and Efe, 2000).

Its timber with long fibers is not heavy, but strong. Moreover, it is odorless and rarely resinous. Hence, the valuable spruce timber is used in pulp and paper production, furniture industry and production of musical instruments (Yaltirik and Efe, 2000).

1.2. Biology of *Picea orientalis*

1.2.1. Natural Distribution

Picea orientalis **(**Oriental Spruce) is a species with limited natural distribution; found only in northeastern Turkey and western Georgia. It ranges from northeastern Anatolia coasts and mountains as well as the Caucasus mountains in 40°23'-43°50' N and 37° 40'-44° 13' E (Kayacık, 1960) (Figure 1.1). It forms either pure or mixed forests with *Fagus orientalis*, *Abies nordmanniana* and *Pinus sylvestris*. It is usually located in 1200 – 2400 m altitutes of Black Sea mountains (Yüksel, 1998; Yaltirik and Efe, 2000) (Figure 1.2).

Oriental spruce covers, as either pure or mixed stands, about 300 000 ha forest land accounting for 1.4% of total forest land in Turkey (General Directorate of Forestry, 2009). It ranges from Posof Basin in east with Çoruh Valley and Yusufeli to Trabzon. It also forms pure stands in Şavşat, Ardanuç, Meydancık and Veliköy (Atalay, 1983). *Picea orientalis* is also located in Artvin, Rize, Giresun, Kars, Erzurum and Ordu with either pure or mixed forests (Kayacık, 1965; Saatçioğlu, 1969; Gökmen,1970).

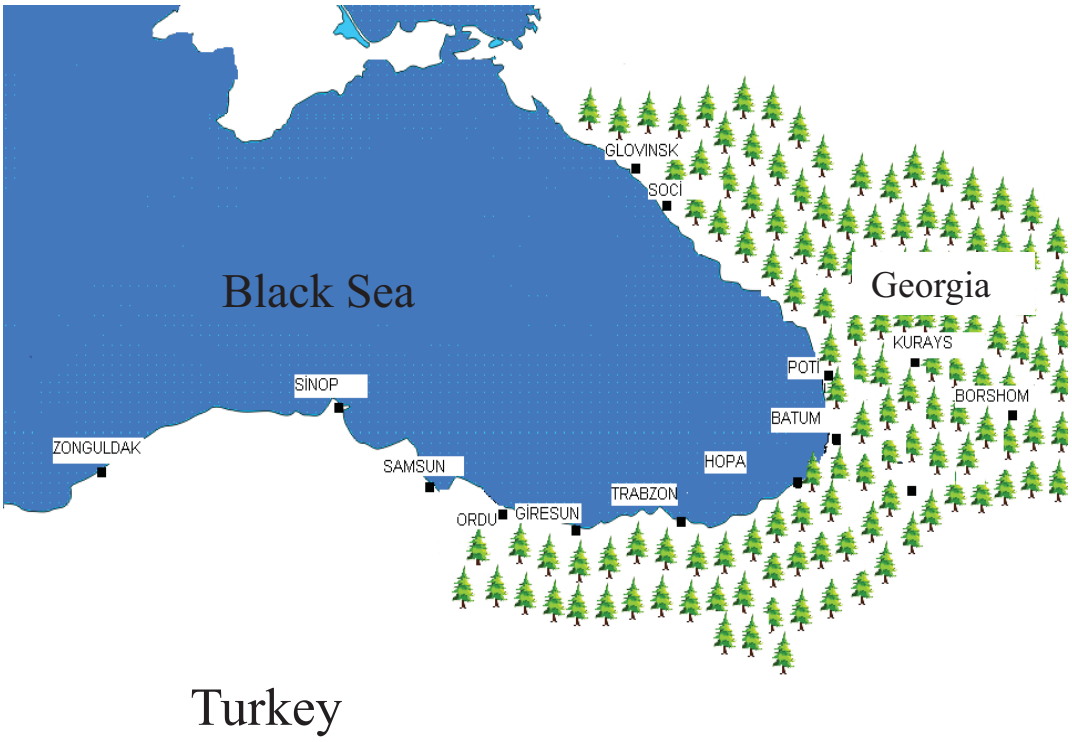


Figure 1.1. Natural distribution of *Picea orientalis*

Figure 1.2. *Picea orientalis* individual from Black Sea Mountains (Photo: F.Temel)

1.2.2. Taxonomy

There is no doubt about monophyly of *Picea* (Wright, 1955; Prager *et al.*, 1976; Frankis, 1988; Price, 1989; Sigurgeirsson and Szmidt, 1993). However, classification in subspecies level still remains a matter of debate (Liu, 1982; Schmidt, 1989; Farjon, 1990 and 2001; Fu *et al.*, 1999). Classification within the genus has been problematic, and no satisfactory phylogeny was worked out despite numerous historical attempts (Wright 1955, Bobrov 1970, Liu 1982, Aldén 1987, Page and Hollands 1987, Rushforth 1987, Schmidt 1989, Farjon 1990, Frankis 1992, Sigurgeirsson and Szmidt 1993).

According to Ran *et al*. (2006), *Picea* is divided into three clades of which *P. orientalis* is included in Clade II with other 13 species ranging from North America to West Asia.

Moreover, according to fossil records Clade II evolved in North America and then dispersed to Asia, subsequently becoming extinct in North America. *Picea orientalis* was first discovered by Tournefort on the mountains of north eastern Anatolia in 1717, subsequently Pallas described it as *Pinus picea* in 1785. Some years later Bieberstein (1808) recognized it as a distinct species from *Pinus picea* and resuscitated Tournefort's trivial name “orientalis”. In 1855 Carrére transferred the species to the genus *Picea* (Kayacık, 1955). *Picea orientalis* belongs to Phylum Pinophyta, Class Pinopsida, Order Pinales, Family *Pinaceae*, and Genus *Picea*.

1.2.3. Ecology

Picea orientalis requires high moisture, and tolarates to low temperature at high altitudes (over 1,200 m) where the winters are severe and with heavy snowfalls. It is sensitive to summer drougth and air pollution. It can tolerate shade during its early stages (Yaltırık and Efe, 2000). On the other hand, *Picea orientalis* could be also seen in valleys and low elevations in eastern Black Sea Region where the climate is mild. According to rain and temperature conditions, annual and the mean monthly relative humidity is rather high. Another noticeable feature in the area, particularly the upper regions, is frequent fog events. The sum of cloudy days is rather high; an average of some 160 days each year (Kayacık, 1955). Furthermore, it is important in water production, control of erosion and water regime hence it is a major component in fragile ecosystems in North eastern Turkey (Temel, 2010).

1.2.4. Botany

It is a large evergreen tree growing 30-45 m tall (exceptionally 57 m) and with a trunk diameter of up to 1.5 m.

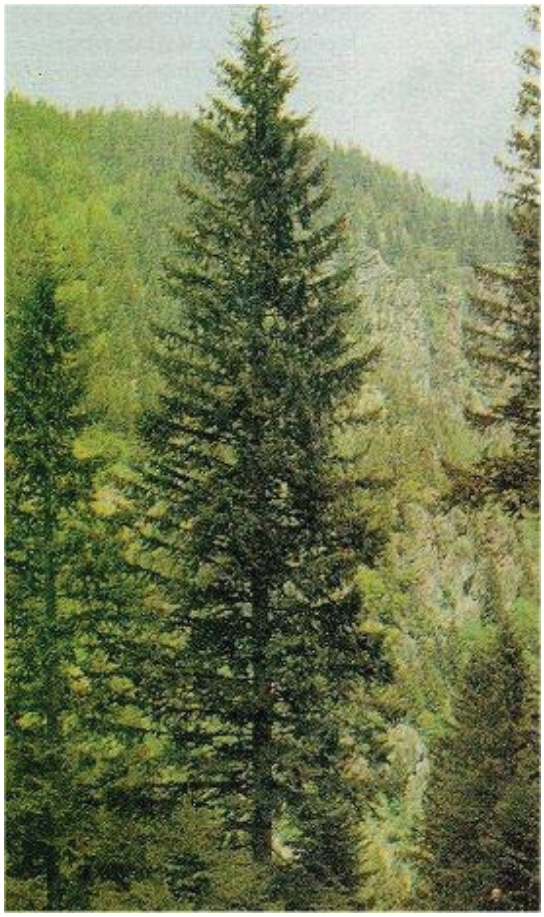
It has a pyramidal shape with dense branchings (Kayacık, 1955; Davis,1965; Anşin, 1988). The shoots are buff-brown and moderately hairy.

The mature trunks are darker and with cracks. The leaves are needle like, the shortest of any spruce, 6-8 mm long, rhombic in cross section, dark green with inconspicuous stomatal lines (Kayacık, 1965; Davis, 1965; Gökmen, 1970; Küçük, 1986; Anşın, 1988) (Figure 1.3).

Male flowers are eliptical with 1 cm length having pink scales with the shape of cones. Female flowers are violet and they form a right angle with the ground before pollination and lean out after pollination. The cones are cylindiric-conic, 5-9 cm long and 1.5 cm width, red to purple when young, maturing dark brown 5-7 months after pollination and have stiff, smoothly rounded scales. Pollination occurs on May. Mature cone scales are opened and the seeds are shed in October. It grows very slowly at first years (Küçük, 1989).

Picea orientalis forms a shallow root system; therefore it is very sensitive to wind as well as snow damage.

A.



B.

C.

D.

Figure 1.3. General appearance *Picea orientalis* and some of its features A. General appearance (Photo: General Directory of Forestry) , B. Trunk of mature tree (Photo: General Directory of Forestry), C. Male conelet (Photo: F.Temel), D. Female cone (Photo: F.Temel)

1.3. Genetic Variation and Molecular Markers

In *Pinaceae*, the chloroplast, mitochondrial and nuclear genomes are paternally, maternally and biparentally inherited, respectively (Stine and Keathley, 1990; Sutton *et al.*, 1991; Hipkins *et al.*, 1994; Mogensen, 1996; Ahuja, 2001). The first molecular phylogeny of spruces were constructed at genus level by using cpDNA-RFLP analysis (Sigurgeirsson and Szmidt, 1993), but relationships of many species were not resolved. As mentioned by the authors, the result of that study may be not very accurate due to limited detection of RFLPs for changes, such as detection of non-homologous characters. A DNA-sequence based phylogeny of the whole genus *Picea* has not been obtained yet due to the shortage of good markers. According to databases there is only one cpDNA genome construction by Cronn *et al.* (2008) of *P. sitchensis*.

In recent years, the combined analysis of multiple genes from one or more genomes has been successfully used in robust reconstructions of complex phylogenies, and thus shed more light on biogeographical histories of many plant groups (Kusumi *et al.*, 2002; Xiang *et al.*, 2005). To resolve interspecific relationships, sequences of nuclear ribosomal DNA internal transcribed spacers (nrDNA ITS) and the chloroplast *trn*T-*trn*F region are most widely used (Wang *et al.*, 1999; Wei and Wang, 2003; Shaw *et al.*, 2005). However, the development of DNA markers in conifers has been difficult due to

(1) a large nuclear genome with highly complex gene families (Kvarnheden *et al.*, 1995; Kinlaw and Neale, 1997; Murray, 1998);

(2) a mitochondrial genome with the slow molecular evolution rate and high level of intraspecific polymorphism (Ahuja, 2001); and

(3) a long nrDNA ITS region, which is too intragenomically variable in length to be used in investigating species phylogenies (Maggini *et al.*, 1998; Wei *et al.*, 2003; Campbell *et al.*, 2005).

Therefore, most molecular phylogenetic studies in conifers at the genus level, were based on chloroplast gene markers (Sigurgeirsson and Szmidt, 1993; Wang *et al.*, 1999, 2005; Kusumi *et al.*, 2000; Wei and Wang, 2003). For that reason, Ran *et al* (2006) reconstructed the molecular phylogeny of *Picea* using sequences of two cpDNA regions *trn*T-*trn*F and *trn*C-*trn*D and has shown great potential in phylogenetic analysis at low taxonomic level (Lee and Wen, 2004; Shaw *et al.*, 2005).

1.4. Determination of Genetic Variation

Due to the extreme utilization of spruce forests for years and excessive reproduction of some bark beetles (*Dendroctonus micans, Ips sexdendatus* and *Ips typographus*), they are liable to irrevocable damages. According to Bern Agreement to which Turkey is a side, *Picea orientalis* forests are in danger such that the social pressure and insect destruction are ever increasing. Hence, in order to reveal the genetic structure of *Picea orientalis* and to protect the forest viability and genetic diversity, gene conservation processes should be implemented. Genetic diversity is essential for adaptation, continuity and evolution of species and forest trees (Müller-Starck *et al.,* 1992). Generally, the species with low genetic diversity are more prone to environmental changes and diseases (Oleksyn *et al.,* 1994). For determination of genetic diversity there are several methods. Morphologic characteristics (Langner, 1953), isoenzyms (Bartels, 1971; Morgante ve Vendramin, 1991; Krutovskii ve Bergmann, 1995; Müller-Starck, 1995; Geburek 1999) and DNA markers (Collignon ve Favre, 2000) were used to detect genetic structures and dispersal of populations. Also, sequence-tagged sites (STS) (Scotti *et al.*, 2000), cpDNA microsattelites (Vendramin *et al.*, 2000) or mtDNA microsattelites (Gugerli *et al.*, 2001, Sperisen *et al.*, 2001) are used. Since nuclear DNA is inherited from both parents, it has some restrictions. Organelle DNA's inherited from one parent are better alternatives in that way.

In order to monitor seed dispersal, mtDNA; and for pollen propagation, cpDNA are good sources (Neale and Sederoff, 1989; Birky, 1995; Vendramin and Ziegenhagen, 1997; Vendramin *et al.*, 2000).

The studies on genetics of *Picea orientalis* are generally in morphological and isoenzyme levels. In Turkey, the study for investigating the variation in growth performaces of *Picea orientalis* was first performed at Belgrad Forest (Istanbul) in 1959. According to this study, the origins from Karanlıkmeşe (Artvin), İkizdere (Rize) and Bicik (Giresun) showed the best development and with respect to their morphology they had desirable properties (Ürgenç *et al.,* 1990). The morphological studies of *Picea orientalis* with populations coming from three elevations (1250, 1450 and 1650 m) which are Atila (Artvin), Meryemana (Trabzon) and Örümcek (Torul, Gümüşhane) showed that seed size, embryo length, seedling weight, number and length of cotyledons, length of hypocotyl and epicotyl are variable within and between sources (Gezer, 1976). Turna (1996) found that *Picea orientalis* populations have vast amount of genetic variation with respect to two enzyme systems (LAP and GOT).

In other spruce species, there are several molecular genetics studies. Maghuly *et al.* (2006) studied the *P. abies* populations by using nuclear, mitochondrial and chloroplast DNAs. According to this study genetic variation within populations is higher than that of among populations as well as higher variations were seen in the populations from higher elevations. Yazdani *et al.* (2003) used SSR markers among seven *P. abies* populations. They reported that polymorphic codominant SSR markers are useful in population studies, seed certification, paternal detection and QTL mapping. Microsattelite markers were used by Wang *et al.* (2005) for detection of genetic variation in *P. asperata*. A'Hara and Cottrell (2004), developed microsatellite markers for *P. sitchensis* by using expressed sequence tags (EST) of *P. glauca.* Rajora *et al.* (2001) developed microsatellites for *P. glauca* and they indicated the

utilizaiton of these markers for other spruce species in genetical, biotechnological, breeding, genome mapping, conservation and restoration.
Even though, it was detected that there are variations within genus, this information is not sufficient to reveal the genetic structure of the *Picea* and to create a program for conserving the genetic diversity of the species within genus.

1.5. Chloroplast DNA (cpDNA)

The nuclear genome is used in systematic botany less frequently, because it has a complex and repetitive characteristic. Because of its rapid changes in its structure, size, configuration, and gene order, the mitochondrial genome is usually used at the species level.
cpDNA, on the other hand, is a relatively abundant component of plant total DNA, thus facilitating extraction and analysis. It also contains single copy genes and has a conservative rate of 2 nucleotide substitution per generation. Therefore, most phylogenetic reconstructions in plant systematics conducted so far is focused on molecular data generated from the cpDNA genes (Liang, 1997).

1.5.1. Transfer Ribonucleic Acid Region of the cpDNA

In recent years cpDNA has provided significant insights in many phylogenetic studies (e.g., Palmer *et al.,* 1988, Learn *et al.,* 1992). Noncoding sequences tend to evolve faster than coding sequences (Taberlet *et al.,* 1991). Thus, it may provide more informative characters for phylogeny reconstruction (Wang *et al.,* 1999). The region between the *trnL* and *trnF,* and the region *trn*V which codes valine carrying *trn*A are particularly suitable due to;

1. the succession of conserved *trn* genes
2. small non-coding regions

3. the higher rate of molecular evolution of the single-copy regions (Taberlet *et al.*, 1991; Kelchner, 2000).

The *trn*L-F region is composed of *trn*L gene and an intergenic spacer which is *trn*L-F. The *trn*L gene consists of two highly conserved exons and is splited 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 is usually conserved in flanking regions of both *trnL* exons, but high variability at the central part. Therefore, the region between the *trn*L and *trn*F and the region *trn*V is found to be highly suitable for evolutionary studies due to;

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

1.5.2. The Maturase Kinase (*matK*) Gene

The chloroplast gene Maturase Kinase (*matK*), which is located within the intron of *trnK* (lysine *trn*A) gene, is an open reading frame (ORF) that encodes a maturase, a protein, used in RNA splicing (Neuhaus and Link, 1987; Wolfe *et al*, 1992; Mort *et al*, 2001).

The *trn* Lysine (UUU) gene (*trn*K) possesses a group II intron which encodes the *matK* (Hausner *et al*, 2006). Group II introns are self-splicing RNAs and mobile elements which are found in eubacteria, archea and the organelles of fungi, plants, and algae (Bonen and Vogel, 2001; Lambowitz and Zimmerly, 2004; Hausner *et al*, 2006). Because of its encoding function, the *trn*K intron differs from typical group II introns (Hausner *et al.*, 2006).

matK gene has been used as an indicator for the construction of plant phylogenies due to rapid evolution of the ORF's (e.g., Hilu and Liang, 1997; Kelchner, 2002; Hausner *et al*, 2006). There are various studies in which *matK* gene sequence is used in phylogenetic analysis so far. These studies include family, genera and species levels. In the study concerning the family Saxifragaceae, it has been denoted that the gene *matK* evolves approximately three times faster than *rbcL* (RuBisCo Large subunit) (Johnson and Soltis, 1994, 1995; Johnson *et al.*, 1996), the most common cpDNA gene used in phylogenetic analysis (Chase *et al.*, 1993).

In addition, the *matK* gene sequences have been used in Polemoniaceae (Steele and Vilgalys, 1994), Orchidaceae tribe Vandeae (Jarrell and Clegg, 1995), Myrtaceae (Gadek *et al*, 1996), Poaceae (Liang and Hilu 1996), Apiaceae (Plunkett *et al.* 1996), and flowering plants in general (Hilu and Liang, 1997). Among Pinaceae, there are also many studies with *matK* such that for *Picea* species several phylogenetic studies were carried out using this region (Germano and Klein, 1999; Wang *et al.,* 1999, 2000; Quinn *et al*., 2002; Germano *et al.*, 2003; Liu and Wang, 2004; Gernandt *et al*., 2005; Bouille and Bousquet, 2007; Fazekas *et al*., 2008; Ran *et al.,*2010). The *matK* was shown to have higher variation than any other studied chloroplast genes. However, the variation was slightly higher at the 5' region than that at the 3' region. Nevertheless, there is approximate even distribution observed throughout the entire gene. In addition, the high proportion of transversion (a change from purine to a pyrimidine, or vice versa, is a transversion) in the *matK* gene might provide high phylogenetic information. These factors underscore the usefulness of the matK gene in systematic studies and suggest that comparative sequencing of *matK* may be appropriate for phylogenetic reconstruction at subfamily, family, genera and species levels (Tanaka *et al*., 1997).

1.6. The Significance of Study

Picea orientalis is an economically important species and has a potential of desired hereditary features. There is very little genetic knowledge or ongoing research on this species.

According to fossil evidence, although the species had a wide spread once from North America through Asia, its natural distribution is now limited to a small area in northeastern Turkey and Caucasia Mountains. For that reason it is a relict-endemic species with restricted natural distribution.

Moreover, natural distribution area is reduced from day to day due to anthrophogenic factors. Besides, *Picea orientalis* constitutes a crucially important forest ecosystem and it is necessary to develop an effective conservation strategy.

Since little is known about phylogenetic relationships of *Picea* species, especially the geographically restricted species (LePage, 2001), the origin and biogeography of *Picea* are still debated (Ran *et al.,* 2006). For that purpose, the genetic structure of *Picea orientalis* populations urgently needs to be investigated for conservation purposes. For species with limited genetic information, it is often assumed that genetic variation follows geographic and ecological variation. (Alan and Kaya, 2003). This study can help to eloborate such assumptions. For this reason, determination of genetic variation of *Picea orientalis* at population level is of great importance.

Moreover, the *trn* and *matK* regions are particularly suitable for evolutionary studies because of;

- The succession of conserved *trn* and *matK* 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* and *matK* regions of *Picea orientalis* populations could be useful for not only providing information on molecular diversity and its patterns in *Picea orientalis* populations, but also molecular phylogeny of the species and its evolutionary relationship with other spruce species.

CHAPTER 2

OBJECTIVES OF THE STUDY

The general objective of this study is to determine the magnitude and pattern of molecular diversity in *Picea orientalis* populations and molecular phylogeny of the species and its evolutionary relationships with other spruces species with the use of sequence data from *trn*A and *matK* regions of cpDNA.

The specific objectives of the study are:

1) To estimate molecular diversity and evolutionary divergence in *Picea orientalis* with respect to *trn*A and *matK* regions of cpDNA.

2) To construct a molecular phylogenetic tree using DNA sequence data from *trn*A and *matK* regions of cpDNA from populations of *Picea orientalis* representing whole range of its distribution in Turkey.

3) To estimate evolutionary divergence of *Picea orientalis* and its divergence time from other Pinaceae and spruce species with respect to *trn*A and *matK* regions of cpDNA.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant Material

In this study, seeds were collected from 15 natural *Picea orientalis* populations for an earlier TÜBİTAK (The Scientific and Technological Research Council of Turkey) Project (Project No:103O092) named the Genecology of *Picea orientalis* (Oriental Spruce) in Turkey were used (Table 3.1 and Figure 3.1). The criteria were followed in selecting sampled populations and trees within populations (Alptekin 1986):

1- Populations are natural,
2- Dominant and adult trees within each population,
3- Trees without any detected stem disturbance or disease,
4- The distance between sampled trees were at least 150 m,
5- Elevational range of sampled trees is no greater than 300 m within each population,
6- The trees without of any special effect.

These criteria were complied for all populations except for populations 7, 11 and 12. These populations were collected from 2-3 different places. Hence, the 4th and 5th criteria are different for these populations. A total of 15 populations were sampled from five geographic regions.

Information on the sampled populations is provided in Table 3.1.

Table 3.1. Description of the *Picea orientalis* populations for the study

Population no.	Population Code	Regional Forestry Directorate	Forest Management Directorate	Forest Management Unit(s)	Latitude (N)	Longitude (E)	Elevation (m)	Number of Families Used in this Study
1	OG-OC	Giresun	Ordu	Çambaşı	40° 40' 39"	37° 55' 51"	1850	4
2	OG-OG	Giresun	Ordu	Gölköy	40° 39' 43"	37° 42' 19"	763	3
3	OG-TA	Giresun	Tirebolu	Akılbaba	40° 40' 50"	38° 53' 15"	2184	4
4	TR-MH	Trabzon	Maçka	Hamsiköy	40° 43' 30"	39° 39' 10"	1300	4
5	TR-TO	Trabzon	Torul	Örümcek	40° 40' 02"	38° 59' 60"	602	3
6	TR-PC	Trabzon	Pazar	Çamlıhemşin	41° 21' 52"	41° 20' 33"	700	2
7	AB-MT	Artvin	Merkez	Tütüncüler, Taşlıca, Atila	41° 9' 24"	41° 39' 55"	520	4
8	AB-SA	Artvin	Artvin	Saçinka ve Artvin	41° 6' 21"	41° 28' 57"	1750	5
9	AB-BB	Artvin	Borçka	Balcı	41° 21' 35"	41° 47' 49"	600	5
10	AY-YA	Artvin	Yusufeli	Altıparmak	40° 57' 47"	41° 23' 16"	1250	4
11	AY-AA	Artvin	Ardanuç	Ardanuç, Tepedüzü ve Ovacık	41° 4' 52"	42° 3' 56"	500	3
12	AŞ-SY	Artvin	Şavşat	Şavşat, Yayla	41° 12' 47"	42° 22' 21"	1800	4
13	AŞ-SM	Artvin	Şavşat	Meydancık	41° 27' 10"	42° 10' 44"	1410	3
14	AŞ-SV	Artvin	Şavşat	Veliköy (SS)	41° 18' 37"	42° 26' 47"	1370	4
15	AŞ-AP	Erzurum	Ardahan	Posof	41° 29'09"	42° 45' 24"	1200	1

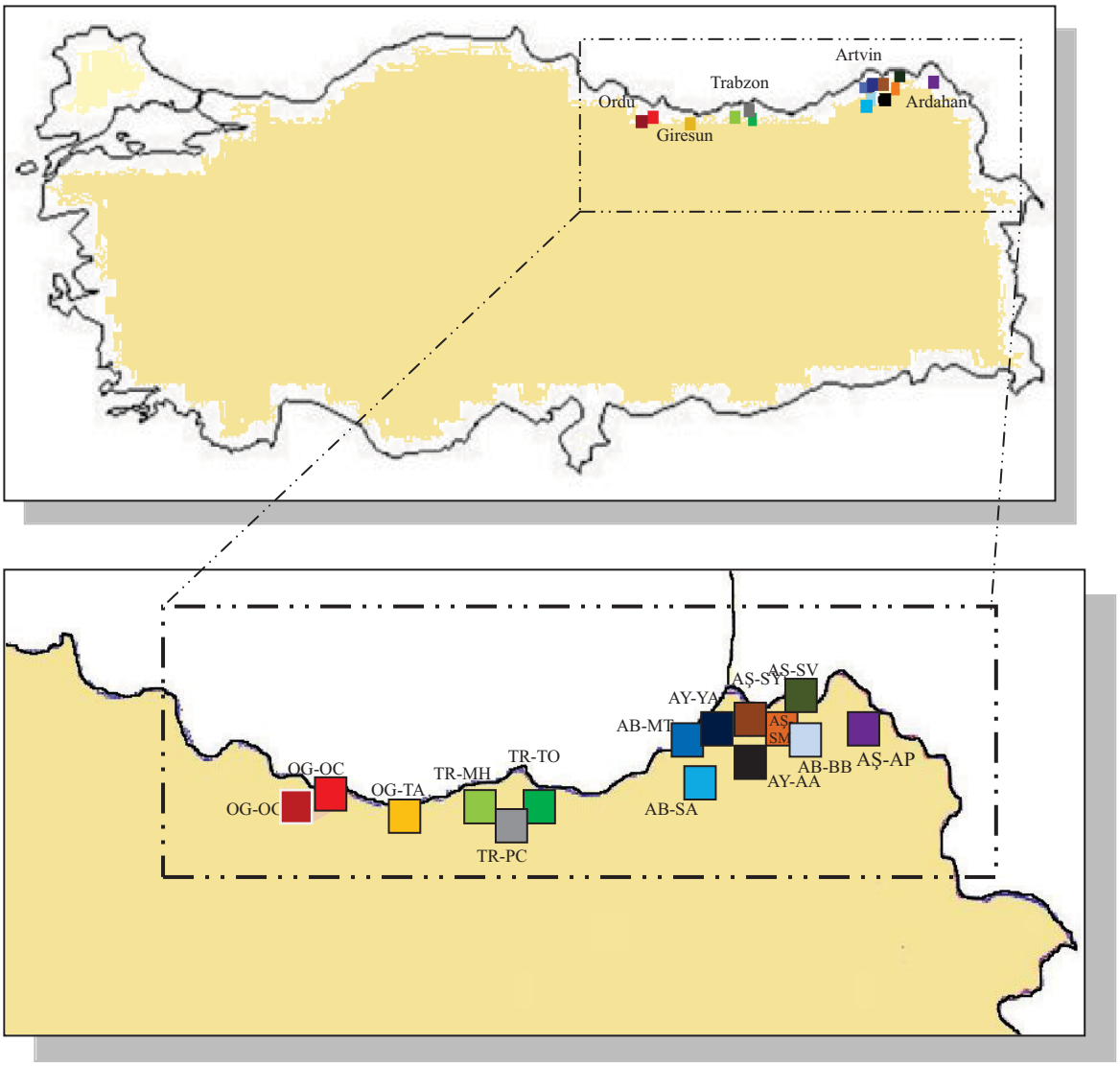


Figure 3.1. Locations of the *Picea orientalis* populations included in the present study (For the legend Table 3.1)

3.2. DNA Extraction

Picea orientalis seeds were collected in the field by Temel at 2006. The seeds were stored at 4°C. Seedling needle tissues were obtained by growing the 8-10 seeds for each in a climatic room (Temperature: 17°C, Light Period=24hrs) for 15 days. Total cellular DNA was isolated using modified 2XCTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). DNA extraction from seedling tissues using CTAB (Cetyl Trimethyl Ammonium Bromide, Chemical composition is in Appendix A) method was carried out for all 15 populations. First, 2-3 seedlings of each population were crushed by the help of liquid nitrogen and put it in the ependorf tubes. Then, the procedure given in Table 3.2 was performed and repeated for each of leaf samples.

Table 3.2. CTAB protocol for DNA extraction

• Physical grinding by 1000 µl CTAB
• Add 100 µl Beta Mercapthoethanol for removal of S-S bonds of proteins
• Incubate for about 1 hour at 65°C in water bath.
• Centrifuge 13000 rpm for 15 minutes
• Transfer supernatant into new tubes and remove pellet
• Add 500 µl 24:1 Chloroform: octanol to supernatant
• Centrifuge 13000 rpm for 15 minutes
• Transfer supernatant into new tubes and remove pellet
• Add 500 µl 2-propanol to supernatant
• Place the tubes –80°C at least for 1 hour (or -20°C overnight)
• Centrifuge 13000 rpm for 15 min
• Remove the supernatant, wash pellet 500 µl, 70 % EtOH (Ethanol)twice.
• Dry the content of the tubes
• Dissolve pellet with 50 µl TE (Tris-EDTA) buffer
• Store DNA samples at -20°C

3.3. DNA Qualification

All stock DNA samples were stored at -20^{o}C until use throughout the course of the study. The presence and quality of the DNA was also checked by running in 0.8% agarose gel electrophoresis.

3.4. Primer Designs for *trn*A and *matK* Regions

In this study, the evolutionary relations within *Picea orientalis* populations were explored by studying molecular diversity in the non–coding *trn*A (*trn)* and *matK* 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 was amplified by *trn*e and *trn*f primers. The last region that lies between *trn*V5' and *trn*V3' was amplified by *trn*VF and *trn*VR primer set (Taberlet *et al*.,1991).

The *trn*L-F region is composed of the *trn*L gene and a flanking intergenic spacer. 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 primer sequences for the non coding region of *trn*A were provided in Table 3.3.

For *mat*K region 2 primer pairs were used as *mat*K1 and *mat*K2 regions. The gene is 1548 bp in length in *Picea orientalis* and is embedded within a 2.5 kb group II intron that interrupts the two *trnK* exons (Cronn *et al.*, 2008) (Figure 3.3).

In this study, two primer pairs whose nucleotide compositions ranging from 18 to 21 nucleotides were designed according to Primer 3 version 0.4.0 (Rozen and Skaletsky, 2000) both with C-code and web interface by Gulsoy and Gulsoy in 2010 (Table 3.3). Moreover, the validity of the primers were checked by using primerBlast of NCBI.

Table 3.3 The list of primers used for PCR amplification of *trn* and *mat*K regions of cpDNA

Marker	Region	Primer Name	Primer Sequence	Length (base pairs)
trn	***trn*L5'-*trn*L3'**	*trn*c (Forward)	5' CGA AAT CGG TAG ACG CTA CG 3'	20
		*trn*d (Reverse)	5' GGG GAT AGA GGA CTT GA AC 3'	19
	***trn*L3'-*trn*F**	*trn*e (Forward)	5' GGT TCA AGT CCC TCT ATC CC 3'	20
		*trn*f(Reverse)	5' ATT TGA ACT GGT GAC ACG AG 3'	20
	***trn*V5'-*trn*V3'**	*trn*V5(Forward)	5' GTA GAG CAC CTC GTT TAC AC 3'	20
		*trn*V3(Reverse)	5' CTC GAA CCG TAG ACC TTC TC 3'	20
***mat*K**	**First Region**	*mat*1MAF (Forward)	5' GAT AAT GTA TCT CCT GCC GA 3'	20
		*mat*1MAR (Reverse)	5' GAT ACC TAA TCG TCT GG AT 3'	19
	Second Region	*mat*2MAF (Forward)	5' CTT TCG CGA CGA CCA TAA TT 3'	20
		*mat*2MAR (Reverse)	5' CGA ACT TCT CTT CGT TG TT 3'	19

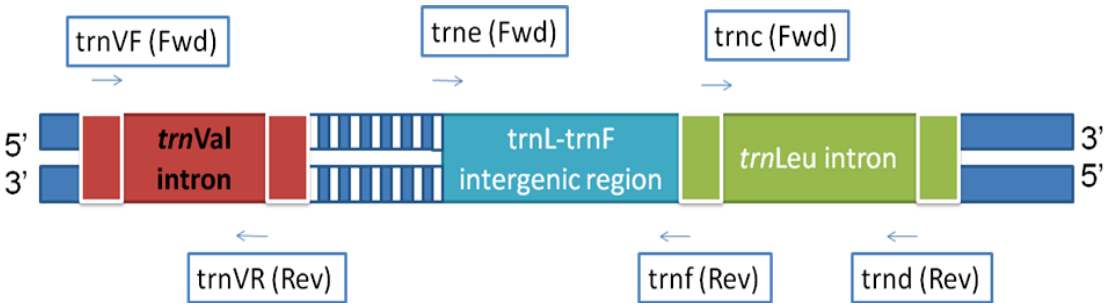


Figure 3.2. Relative positions of *trn* primers

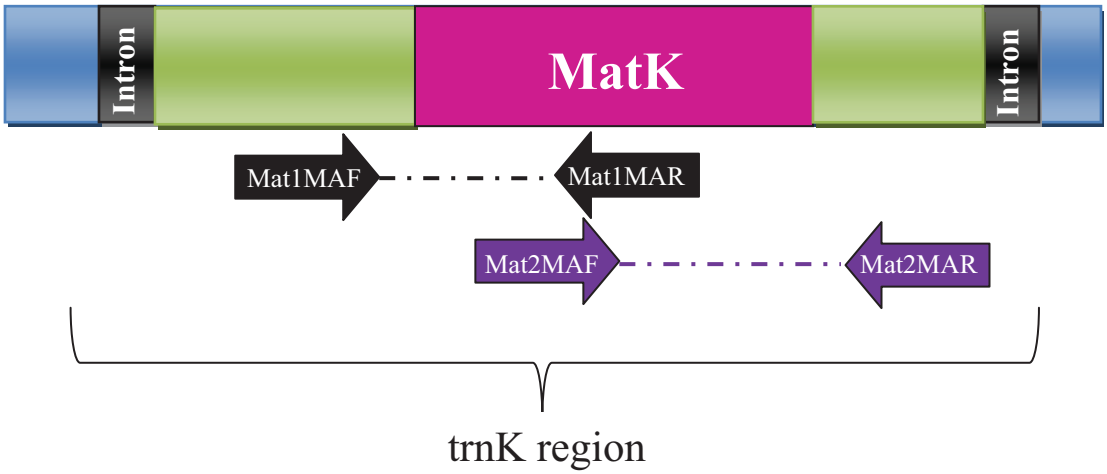


Figure 3.3. Relative positions of *mat*K primers used in PCR amplifications of *Picea orientalis*

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 (Tables 3.4 and 3.5).

Table 3. 4 Tested PCR components and template DNA concentration for amplification of *trn* region chloroplast genome of *Picea orientalis* (All units are in µL). The bold combinations are the optimum conditions

A)Tested PCR conditions for *trn*V5-V3 and *trn*cd primers

10X Buffer	$MgCl_2$ (25 mM stock solution)	dNTP (10 mM each)	Primer pairs (100µM)	*Taq* DNA polymearse	DNA
5.0	**5.0**	**1.0**	**0.5 + 0.5**	**0.5**	**2.0**
5.0	**6.0**	**2.0**	**1.0 + 1.0**	**0.5**	**2.0**
5.0	5.0	2.0	1.0 + 1.0	0.5	2.0
5.0	6.0	1.0	0.5 + 0.5	0.5	2.0
5.0	6.0	2.0	1.0 + 1.0	0.5	4.0
5.0	5.0	1.0	2.0 + 2.0	0.5	2.0

B)Tested PCR conditions for *trn*ef primers

10X Buffer	$MgCl_2$ (25 mM stock solution)	dNTP(10 mM each)	Primer pairs (100µM)	*Taq* DNA polymearse	DNA
5.0	**5.0**	**1.0**	**2.0 + 2.0**	**0.5**	**2.0**
5.0	4.0	1.0	2.0 + 2.0	0.5	2.0
5.0	6.0	1.0	2.0 + 2.0	0.5	2.0
5.0	5.0	2.0	1.0 + 1.0	0.5	2.0
5.0	5.0	1.0	1.0 + 1.0	0.5	2.0
5.0	4.0	1.0	1.0 + 1.0	0.5	2.0
5.0	6.0	2.0	2.0 + 2.0	0.5	2.0
5.0	6.0	2.0	3.0 + 3.0	0.5	2.0
5.0	5.0	2.0	3.0 + 3.0	0.5	2.0
5.0	6.0	3.0	3.0 + 3.0	0.5	2.0

Table 3.5 Tested PCR components and template DNA concentration for amplification of *mat*K region chloroplast genome of Picea orientalis (All units are in μL) The bold combinations are the optimum conditions

10X Buffer	$MgCl_2$ (25 mM stock solution)	dNTP(10 mM each)	Primer pairs (10μM)	*Taq* DNA polymearse	DNA
6.0	**4.0**	**4.0**	**2.0 + 2.0**	**0.8**	**4.0**
6.0	4.0	4.0	3.0 + 3.0	0.8	5.0
6.0	6.0	4.0	5.0 + 5.0	0.8	6.0
6.0	6.0	4.0	3.0 + 3.0	0.8	4.0
6.0	4.0	2.0	1.0 + 1.0	0.8	4.0
6.0	6.0	4.0	2.0 + 2.0	0.8	6.0

Optimized PCR conditions for *trn*cd and *trn*V5V3 primers contained 2.0 μL of template DNA (7.5 ng/μL). In the mixture for *trn*cd primer containing PCR product, there were 1X of 10X buffer (750 mM Tris.HCl pH: 8.8, 200 mM $(NH_4)_2SO_4$; MBI Fermentas, Lithuania); 0.5 μL (1 unit) of *Taq* DNA polymerase (Fermentas, Ontorio, Canada); 0.2 mM of dNTP mix (Fermentas, Ontorio, Canada); 2.5 mM $MgCl_2$ and 50 pmol of each primer. For *trn*V5V3 primer, 1X of 10X buffer; 0.5 μL (1 unit) of *Taq* DNA polymerase; 0.4 mM of dNTP mix; 3.0 mM $MgCl_2$ and 100 pmol of each primer. For the *trn*ef primer the PCR conditions were optimized as; 2.0 μL template DNA; 1X of 10X buffer; 0.5 μL (1 unit) of *Taq* DNA polymerase; 0.2 mM of dNTP mix; 2.5 mM $MgCl_2$ and 200 pmole of each primer.

Finally, the optimized PCR conditions were the same for both *mat*K1 and *mat*K2 regions with 4.0 μL template DNA (15ng/μL); 1.2X of 10X buffer; 0.8 μL (1 unit) of *Taq* DNA polymerase; 0.8 mM of dNTP mix; 2.0 mM $MgCl_2$ and 20 pmole of each primer (Table 3.6). The reaction mixtures were prepared in thin-walled 0.2 mL Eppendorf tubes and optimized on a thermocycler (Eppendorf- Mastercycler, Eppendorf, Canada, and Techne-genius Thermocycler, Techne, USA) (Tabel 3.7).

Table 3.6 Optimized PCR conditions for *trn*A and *mat*K regions of chloroplast genome of *Picea orientalis*

	***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	35,5	NA	32.5	NA	32,5	NA
10X PCR Buffer	5	1X	5	1X	5	1X
$MgCl_2$ (25mM stock)	5	2.5 mM	5	2.5 mM	6	3 mM
dNTP (10mM of each)	1	0.2 mM	1	0.2 mM	2	0.4 mM
Forward primer (100µM)	0.5	1 µM	2	4 µM	1	2 µM
Reverse primer (100µM)	0.5	1 µM	2	4 µM	1	2 µM
***Taq* DNA polymerase (5u/µL)**	0.5	0.05u/µL	0.5	0.05u/µL	0.5	0.05u/µL
DNA	2	7.5 ng/µL	2	7.5 ng/µL	2	7.5 ng/µL
Total Volume	50		50		50	

Table 3.6 (Cont.) Optimized PCR conditions for *trn*A and *matK* regions of chloroplast genome of *Picea orientalis*

***mat*K**		
PCR Contents	Volume Used in PCR (µL)	Final Concentration
PCR Grade Water	27,2	NA
10X PCR Buffer	6	1.2 X
$MgCl_2$ (25mM stock)	4	3 mM
dNTP (10mM of each dNTP)	4	0.8 mM
Forward primer (10 µM)	2	0.4 µM
Reverse primer (10 µM)	2	0.4 µM
***Taq* DNA polymerase (5u/µL)**	0.8	0.08 u/µL
DNA	4	15 ng/µL
Total Volume	50	

Table 3.7 Optimized thermal cycler program used for amplification of *trn* and *mat*K region of chloroplast genome of *Picea orientalis*

Primers	**Temperature (^{o}C)**	**Duration**	**Number of cycles**	**Purpose**
***trn* primers**	95	1 minute	1	Initial denaturation
	94	30 seconds	30	Internal denaturation
	55	30 seconds		Annealing
	72	50 seconds		Extension
	72	5 minutes	1	Final extension
matK1	94	5 minutes	1	Initial denaturation
	94	1 minute	30	Internal denaturation
	60	1 minute		Annealing
	72	2 minutes		Extension
	72	3 minutes	1	Final extension
matK2	94	3 minutes	1	Initial denaturation
	94	1 minute	30	Internal denaturation
	58	1 minute		Annealing
	72	2 minutes		Extension
	72	7 minutes	1	Final extension

About 5µL of the PCR mixture were visualised in 1.0 % agarose gels. Gels were run in 1XTBE (Tris, Boric Acid, EDTA) buffer at about 90 volts for half an hour. After electrophoresis, DNA bands were stained with 5µg/ml ethidium bromide and visualized under UV light. The gels were also photographed and digitialized by using a gel imaging system (Vilbor Lourmat, France). After checking the good band quality, remaining 45µL mixture was stocked for sequencing analysis.

3.6. Data Collection and Anaylsis of Sequence Data of *trn* and *mat*K Regions

3.6.1. Sequencing of PCR Products

After the amplification of DNA fragment, PCR products *trnA* and *matK* regions were kept at 4°C. For the sequencing, the products were sent to Refgen Biotechnology, METU Teknokent, Ankara. Both the purification and sequencing processes were done by Refgen Biotech. A PCR purification process should be performed before the sequence analysis. In sequence analysis, ABI 310 Genetic Analyser User's Mannual was followed and sequencing was performed using the Big Dye Cycle Sequencing Kit (Applied Biosystems) with a ABI 310 Genetic Analyser (PE Applied Biosystem) automatic sequencer. For purification of PCR product Nucleospin Extract Kit (Clontech Laboratories,Inc.) was used. The purification processes were as follows:

- Mix 2 volumes of buffer NT (contains chaotropic salt) with 1 volume of sample.
- Place a NucleoSpin® Extract II column into a 2 ml collecting tube and load the sample.
- Centrifuge at 11000 g for 1minute.
- Discard flow-through and place NucleoSpin® Extract II column into the collecting tube.
- Add 600 µL ethanolic NT3 buffer and centrifugate at 11000 g for 1min.
- Discard flow-through and place the NucleoSpin® Extract II column back into the collecting tube.
- Centrifuge for 2minute at 11000 g to remove buffer NT3 quantitatively.
- Place the NucleoSpin® Extract II column into a clean 1.5 ml microcentrifuge tube.
- Add 15-50 µl elution buffer NE (5mM Tris-Cl pH: 8.5) and incubate at room temperature for 1 minute to increase he yield of eluted DNA.
- Centrifuge for 1 minute at 11000 g.

Table 3. 8 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. 9 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, the following precipitation procedure was applied

1. Add 2 µl of 125 mM EDTA.
2. Add 2 µl of 3 M sodium acetate.
3. Add 50 µl of 100% ethanol.
4. Invert 4 times.
5. Incubate for 15 min at room temperature.
6. Centrifuge at 2000-3000g for 30min.

7. Invert the plate and spin up to 185g.
8. Add 60 µl 70% ethanol.
9. Centrifuge at 4°C for 15 minutes at 1650 g.
10. Invert the plate and spin up to 185 g for 1 minute.

To prepare extension product-purification, the following procedure was applied:

1. Prepare 2.2% SDS in deionized water.
2. Add appropriate amount of SDS solution to sample to reach the volume of 0.2% SDS concentration.
3. Heat the tubes at 98C for 5min and cool at 25°C for 10 min.

For spin column purification, the following procedure was used:

1. Add 0.8 ml of deionized water.
2. Hydrate the gel at room temperature for at least 2 hours
3. Insert the column to wash tube.
4. Spin the column in a microcentrifuge at 730g for 2 minutes.
5. Remove the column from wash tube and insert into a sample collection tube.

The *trn*A and *matK* regions were amplified as three and two parts with the help of 6 and 4 primers, respectively. These parts were aligned visually before the analysis. For viewing the chromotogram data having ABI extension format, Finch TV Version 1.4.0 developed by the Geopiza Research Team, was utilized (Patterson *et al.*, 2004-2006) (Appendix B).

3.7. Analysis of Sequence Data of the *trn* and *mat*K Regions

For phylogenetic and molecular evolutionary analysis MEGA version 5.02 (Tamura *et al.*, 2011) and Arlequin (Schneider *et al.*, 2000) softwares were utilized. Before analysis, the sequences were processed in FASTA format manually by aligning the bases of sequences between the three regions *trn*cd, *trn*ef and *trn*VFVR of *trnA* and *MatK* regions. When the analyzed and unprocessed sequences were compared, it was found that the analyzed sequences were shorter than unprocessed sequence data due to the trimming of

unclear flanking regions.

The distances between *Picea orientalis* populations and other Pinaceae species were computed by using Maximum Composite Likelihood in MEGA version 5.02. A composite likelihood is defined as a sum of related log-likelihoods. Since all pairwise distances in a distance matrix have correlations due to the phylogenetic relationships among the sequences, the sum of their log-likelihoods is a composite likelihood. Tamura *et al*. (2004) showed that pairwise distances and the related substitution parameters are accurately estimated by maximizing the composite likelihood. They also found that, unlike the cases of ordinary independent estimation of each pairwise distance, a complicated model had virtually no disadvantage in the composite likelihood method for phylogenetic analyses.

Also when computing distances, pair wise deletion method was used. In the pairwise deletion option, sites containing missing data or alignment gaps are removed from the analysis as the need arises (e.g., pairwise distance computation). This is in contrast to the complete deletion option in which all such sites are removed prior to the analysis.

During visual alignment of the DNA sequences indels, insertion/deletion points in a sequence were not included. The data sets of DNA sequences were edited in mas extension file format and collected and organized in meg extension file format so that it could be analyzed with MEGA (Molecular Evolutionary Genetics Analysis) 5.02 software (Tamura *et al.,* 2011). Moreover, for the use of data in Arleqiun software (Version 2.000 for population genetics data analysis) (Schneider *et al.*, 2000) the input data was constructed in arp extension file format. The sequence statistics, containing nucleotide frequencies, transition/transversion (tr/tv) ratio and variability in different regions of the sequences were calculated by MEGA 5.02 (Tamura *et al.,* 2011).

3.7.1. Molecular Diversity and Phylogenetic Analysis with Other Pinaceae Species Based on Sequence Data of *trn* and *mat*K Regions

The differentiation between studied *Picea orientalis* populations with other *Picea* species were analyzed by obtaining relevant sequences from NCBI by using BLAST (Basic Local Alignment Search Tool) (Table 3.10, 3.11 and 3.12). Since the number of studied *trn*V region was considerably lower than other two *trn* regions, it was analyzed individually by using additional species from Pinaceae family (Table 3.10).

Table 3.10. The sequences used in molecular diversity analysis for *trn*V region

NCBI Accession No	Species Name	Location	Authors
AB019899.1	*Picea abies*	Japan	Wang *et al.,* 1999
DQ375447.1	*Picea sitchensis*	USA	Willyard *et al.,* 2007
EU998739.3	*Picea sitchensis*	USA	Cronn *et al.,* 2008
	Pinus nigra	Turkey	Gulsoy 2009
FJ899564	*Pinus torreyana* subsp. *torreyana*	USA	Parks *et al.,* 2010
AB19901.1	*Abies nuimidica*	Japan	Wang *et al.,* 1999
FJ899555.2	*Pinus ponderosa*	USA	Parks *et al.,* 2009
FJ899583.2	*Pinus pinaster*	France	Parks *et al.,* 2009
AB0198894.1	*Pinus brutia*	Japan	Wang *et al.,* 1999
AB019893	*Pinus halepensis*	Japan	Wang *et al.,* 1999
FJ899560.1	*Pinus strobus*	USA	Parks *et al.,* 2009
AB019891	*Pinus nigra*	Japan	Wang *et al.,* 1999
FJ899658.1	*Larix occidentalis*	USA	Parks *et al.,* 2009
AB480043.1	*Cedrus deodara*	Taiwan	Lin *et al.,* 2010
AB019900.1	*Larix decidua*	Japan	Wang *et al.,* 1999

Table 3.11 The sequences used in molecular diversity analysis for *trn*cd and *trn*ef regions

NCBI Accesion No	Species Name	Location	Authors
EF440557	*Picea orientalis*	Georgia	Bouille and Bousquet 2007
EF440563	*Picea rubens*	USA	
EF440561	*Picea purpurea*	China	
EF440550	*Picea maximowiczii*	Japan	
EF440549	*Picea martinezii*	Mexico	
EF440556	*Picea omorika*	Bosnia	
EF440551	*Picea mexicana*	Mexico	
EF440548	*Picea mariana*	USA	
EF440532	*Picea alcoquiana*	Japan	
EF440545	*Picea koraiensis*	China	
EF440569	*Picea wilsonii*	China	
EF440568	*Picea torano*	Japan	
EF440555	*Picea obovata*	Northern Eurasia	
EF440553	*Picea likiangensis* var. *montigena*	China	
EF440546	*Picea koyamae*	Japan	
EF440533	*Picea asperata*	China	
EF440531	*Picea Abies*	Poland	
EF440530	*Picea Abies*	Romania	
EF440567	*Picea spinulosa*	Bhutan	
EF440547	*Picea likiangensis*	China	
EF440535	*Picea breweriana*	USA	
EF440543	*Picea glehnii*	Japan	
EF440564	*Picea schrenkiana*	China	
EF440560	*Picea pungens*	USA	
EF440554	*Picea morrisonicola*	Taiwan	
DQ010626	*Picea orientalis*	Turkey	Ran *et al.,* 2006
DQ358151	*Picea chihuahuana*	United Kingdom	
DQ010612	*Picea jezoensis*	China	
DQ010598	*Picea alcoquiana*	Switzerland	
DQ010601	*Picea brachytyla*	China	
DQ010603	*Picea engelmanii*	Switzerland	
DQ010637	*Picea smithiana*	China	
DQ358156	*Picea neoveitchii*	China	

Table 3.12 The sequences used in Molecular diversity analysis for *mat*K region

Accesion No	Species Name	Location	Authors
EF440516	*Picea orientalis*	Georgia	Bouille and Bousquet 2007
EF440509	*Picea maximowiczii*	Japan	
EF440497	*Picea chihuahuana*	Mexico	
EF440529	*Picea wilsonii*	China	
EF440520	*Picea purpurea*	China	
EF440513	*Picea morrisonicola*	Taiwan	
EF440508	*Picea martinezii*	Mexico	
EF440495	*Picea brachtyla*	China	
EF440527	*Picea spinulosa*	Bhutan	
EF440519	*Picea pungens*	USA	
EF440523	*Picea schrenkiana*	China	
EF440522	*Picea rubens*	USA	
EF440510	*Picea mexicana*	Mexico	
EF440501	*Picea glauca*	Canada	
EU364788	*Picea engelmannii*	Canada	
AY035200	*Picea omorika*	Serbia-Bosnia	Germano *et al.*, 2002
AY035199	*Picea smithiana*	Nepal-Afganistan	
AY035194	*Picea mexicana*	Mexico	Germano *et al.*, 2001
AF133922	*Picea mariana*	USA	Germano and Klein 1999
AY035196	*Picea engelmannii*	USA	Germano *et al.*, 2002
EF440502	*Picea glehnii*	Japan	Bouille and Bousquet 2007
EF440503	*Picea jezoensis*	Japan	
EF440506	*Picea likiangensis*	China	
EF440500	*Picea farreri*	Myanmar	
AY289610	*Picea abies*	Europe	Germano *et al.*, 2002
AY035202	*Picea asperata*	China	Germano *et al.*, 2002
EF440528	*Picea torano*	Japan	Bouille and Bousquet 2007
EF440521	*Picea retroflexa*	China	
EF440514	*Picea obovata*	Northern Eurasia	
EF440511	*Picea meyeri*	China	
EF440505	*Picea koyamae*	Japan	
EF440498	*Picea crassifolia*	China	
EF440504	*Picea koraiensis*	China	
EF440525	*Picea sitchensis*	USA	

3.7.2. Models for Estimating Genetic Distance of *Picea orientalis*

Number of nucleotide substitution occuring between sequences are used for measuring the evolutionary distance between a pair of sequences. In order to study molecular evolution and phylogenetic reconstructions and for the estimation of divergence time, evolutionary distances are fundamental. There are several methods for distance estimation for nucleotide sequences further details of which and general guidelines for the use of these methods are given by Nei and Kumar (2000).

With the help of analytical formulas and bootstrap method, standard errors of estimates were also computed. Considering nucleotides, the sequences were compared nucleotide-by-nucleotide. Composite maximum likelihood method was chosen in this study so that multiple hits were corrected, taking into account the differences in substitution rate between nucleotides and the inequality of nucleotide frequencies. It also distinguishes between transitional substitution rates between purines and transversional substitution rates between purines and pyrimidines. The maximum composite likelihood is used for describing the sum of log-likelihoods for all pairwise distances in a distance matrix (Tamura *et al*. 2004) estimated by using the Tamura-Nei (1993) model.

3.7.3. Molecular Clock Estimation

It is expected that the number of differences between two taxa would have a straightforward time if the evoluiton had only a divergent property and if all lineages evolved at a constant rate because they diverged from a common ancestor. In this manner it is possible to find the phylogeny by the rate of difference between pairs of taxa since DNA sequences evolve and diverge at a constant rate (Futuyma, 2005). This concept was named as molecular clock in which a phylogeny is easily estimated in a simple way (Zuckerkandl and Pauling, 1965). Moreover, it is also possible to estimate the absolute time

since divergence. In order to estimate the rate of molecular evolution, the parsimony informative total variable sites in the base pairs among compared taxa is used. In order to calculate molecular clock the following equations are used:

$$\text{Molecular Clock} = \frac{k}{\text{mutation rate}}$$

$$k = -\left(\frac{3}{4}\right)\ln\left(1 - \frac{4}{3}d\right)$$

$$d = \frac{Variable\ Site}{Total\ Number\ of\ Base\ Pairs\ Sequenced}$$

where *d* is the number of substitutions per base pair and k is the substitutions since divergence. According to these equations, the molecular clock estimations were performed with respect to *trn*A and *mat*K regions among Pinacea species. In this study, the mutation rate was considered constant as $2x10^{-9}$ (Pevsner, 2009).

3.7.4. Estimation of Pairwise Genetic Distances (F_{st}) among Populations

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 is obtained by permuting haplotypes between the populations. For the construction of F_{st} values Tajima and Nei (1984) method was used in order to output a corrected percentage of nucleotides for which two haplotypes are different. The correction is an extension of Jukes and Cantor (1969) method, allowing for unequal nucleotide frequencies. The overall nucleotide frequencies are computed from the data.

3.7.5. Construction of Phylogenetic Trees for *Picea orientalis* Populations and Other Pinaceae Species

Phylogenetic trees are important to show the evolutionary interrelationships among various species or other entities which probably have a common ancestor. These phylogenetic relationships of genes or organisms are presented in a treelike form with either a root (rooted tree) or without a root (unrooted tree). An unrooted trees only illustrate the relatedness of the leaf nodes.

In this study, 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 single species. 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 (i.e., permutations with replacement). 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 using MEGA 5.02.

CHAPTER 4

RESULTS

4.1. Amplification of the t-RNA and *mat*K Regions of the Chloroplast DNA

For all three regions of non coding *trn* (*trn*cd, *trn*ef and *trn*V5V3) and *mat*K regions, highly qualified single bands were observed. PCR conditions for *trn*cd and *trn*ef regions were optimized by including 2 µL of template DNA (7.5 ng/µL); 1X of 10X buffer; 0.5 µL of *Taq* DNA polymerase (Fermentas, Ontorio, Canada); 0.2 mM of dNTP mix (Fermentas, Ontorio, Canada); 2.5 mM $MgCl_2$ and 50 pmol of each primer (200 pmole for *trn*ef) in the reaction mixture. For *trn*V5V3 primer the mixture contained 3mM $MgCl_2$, 0.4mM of dNTP mix and 100 pmole of each primer. The concentrations of other components of the reaction mixture were same with that for *trn*cd primer. For the amplification of *mat*K region, the reaction mixture with 1.2X Buffer, 3mM MgCl2, 0.8mM dNTP and 0.4 µM each primer as well as 15ng/µL template DNA was found to be optimum (Table 3.6). After obtaining good quality bands for all three *trn* (Figure 4.1) and *mat*K regions, amplifications were performed in order to obtain their sequences.

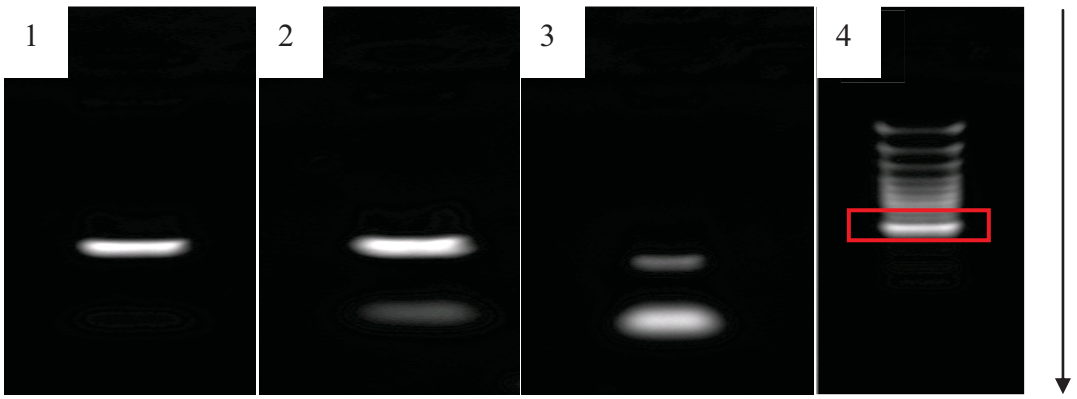


Figure 4.1 The amplified DNA of three regions of *trn*A of cpDNA (lane 1: *trn*V region, Lane 2: *trn*cd region, lane 3:*trn*ef region, and lane 4: Fermentas GeneRuler^{TM} 100bp marker. The thicker band indicated with red rectangle shows the 500bp region and the black arrow shows the direction of DNA fragments running on electrophoresis)

4.2. Molecular Diversity of Studied cpDNA Regions

4.2.1. Molecular Diversity of the Three Non-coding *trn* Regions

Although the studied three non-coding *t-RNA* regions were located at different loci on cpDNA, they are combined and analyzed in together. The order of the sequences was the same with the locations of the regions starting with *trn*V5V3 along with *trn*cd and finally *trn*ef regions. The region of *trn*V5V3 was 520 bp with 40.2% GC content. The *trn*cd and *trn*ef regions were 568 bp with 39.8 % GC content and 399 bp with 34.1% GC content, respectively (Table 4.1). The *trn*cd and *trn*V5V3 regions were highly conserved that there were no variable sites in order to use them in phlyogenetic analysis of studied *P. orientalis* populations. However, the *trn*ef region had a variable site that was parsimony informative (Table 4.1 and Table 4.2).

4.2.2. Amplification of *mat*K Region

The *mat*K region starts with start codon and ends with one of the stop codons (TGA) which could be amplified by specifically designed primers as well as by partial amplification of the region through *trn*K sequence at 3' region. Due to the length of the *mat*K region, it was divided in two sectors and amplified with two pairs of primers (*mat*MAF1-*mat*MAR1 for the first region and *mat*MAF2-*mat*MAR2 for the second region) which possess some overlapping sequences between 900-1000th bases. The entire region was obtained by first trimming unclear flanking regions and then assembling the overlapping region.The first region and second regions were 999 bp and 905 bp in length, respectively. According to analysis performed with MEGA 5, the first region of *mat*K possess 35% GC content; while second region had 39.1% GC content. On average, the whole *mat*K region contained 37.1 % GC content (Table 4.1).

Table 4.1 Estimated molecular diversity parameters for *trn*V, *trn*cd, *trn*ef and MatK gene regions for *Picea orientalis* populations

Molecular Diversity Parameters	**Total *trn***	***trn*-Val (v region)**	***trn*-Leu (cd region)**	***trn*L3'-F (ef region)**	**Entire MatK+*trn*K partial sequence**	***mat*K1**	***mat*K2 + *trn*K partial sequence**
Total sample size	53	53	53	53	14	14	14
Total Length (bp)	1480	520	568	385	1804	999	905
GC content (%)	38.3	40.2	39.8	34.1	37.1	35	39.1
Conserved sites	1479	520	528	384	1804	999	905
Variable sites	1	0	0	1	0	0	0
Parsimony informative sites	1	0	0	1	0	0	0

Table 4.2 Information on variable sites among *Picea orientalis* populations (Numbers show the each individual samples)

The *trn* sequence sources	**The base position**
	1460
Giresun Ordu Cambasi 146	A
Giresun Ordu Cambasi 148	
Giresun Ordu Cambasi 153	
Giresun Ordu Cambasi 154	
Giresun Ordu Golkoy 164	
Giresun Ordu Golkoy 171	
Giresun Ordu Golkoy 172	
Giresun Tirebolu Akilbaba 131	T
Giresun Tirebolu Akilbaba 133	A
Giresun Tirebolu Akilbaba 135	T
Giresun Tirebolu Akilbaba 141	
Trabzon Macka Hamsikoy 191	
Trabzon Macka Hamsikoy 201	
Trabzon Macka Hamsikoy 206	
Trabzon Macka Hamsikoy 211	
Trabzon Torul Orumcek 178	A
Trabzon Torul Orumcek 183	T
Trabzon Torul Orumcek 185	
Trabzon Pazar Camlihemsin 267	A
Trabzon Pazar Camlihemsin 272	
Artvin Merkez Tutunculer Taslica Hatila 116	
Artvin Merkez Tutunculer Taslica Hatila 120	
Artvin Merkez Tutunculer Taslica Hatila 126	T
Artvin Merkez Tutunculer Taslica Hatila 127	
Artvin Sacinka Artvin 248	
Artvin Sacinka Artvin 250	
Artvin Sacinka Artvin 252	
Artvin Sacinka Artvin 254	
Artvin Sacinka Artvin 259	
Artvin Borcka Balci 281	
Artvin Borcka Balci 283	
Artvin Borcka Balci 288	
Artvin Borcka Balci 291	
Artvin Borcka Balci 293	
Artvin Yusufeli Altiparmak 56	A
Artvin Yusufeli Altiparmak 64	
Artvin Yusufeli Altiparmak 67	
Artvin Yusufeli Altiparmak 73	T
Artvin Ardanuc Tepeduzu Ovacik 83	
Artvin Ardanuc Tepeduzu Ovacik 88	
Artvin Ardanuc Tepeduzu Ovacik 103	A
Artvin Savsat Yayla 1	
Artvin Savsat Yayla 3	
Artvin Savsat Yayla 5	
Artvin Savsat Meydancik 16	A
Artvin Savsat Meydancik 21	
Artvin Savsat Meydancik 28	
Artvin Savsat Velikoy 35	
Artvin Savsat Meydancik 36	
Artvin Savsat Meydancik 38	
Artvin Savsat Meydancik 45	T
Erzurum Ardahan Posof 51	
Artvin Savsat Yayla 10	

4.3. Molecular Diversity between *Picea orientalis* and Other Pinaceae Species

4.3.1. Molecular Diversity with respect to *trn* Regions

The studied species that were obtained from NCBI were shown in table 3.10 and 3.11. Although there were sequences of *trn*cd and *trn*ef were available from the NCBI database (Table 3.11), there was no adequate number of useful sequences within *Picea* genus for *trn*V5V3 region. Thus, other species of Pinacea were also considered to establish molecular relationship of *Picea orientalis* with other conifers (Table 3.10).

Among 520 bp sequence of *trn*V5V3, there were 32 variable sites. Twenty of these are parsimony informative and 12 of them were singleton sites (Table 4.4). There were 502 identical pairs (ii) as well as 5 transition (si) and transversion (sv) pairs exist with the ratio of 0.89 (R=si/sv) (Table 4.3).

Considering *trn*cd and *trn*ef region together 33 different sequence data for *Picea* were used in analysis, including sequence data of *Picea orientalis* from this study (Table 3.11).

There were 26 variable sites. Nine of these were parsimony informative and 17 of them were singleton sites (Table 4.5). Among 972 bp, 37 % GC content, 951 identical pairs (ii), 1 transitional pair, 2 transversional pairs and 0.49 si/sv ratio were observed (Table 4.3).

Table 4.3 Estimated molecular diversity parameters for *trn*V5V3 and *trn*cd-*trn*ef regions among Pinaceae and *Picea* species, respectively

Molecular Diversity Parameters	*trn*V region	*trn*cd-*trn*ef region
Total sample size	16	34
Total Length (bp)	585	972
Average GC content (%)	39.2	37
Conserved sites	488	946
Variable sites	32	26
Singleton sites	12	17
Parsimony informative sites	20	9
Identical pairs	502	951
Transitional pairs	5	1
Transversional pairs	5	2
Transition/ Transversion Ration	0.89	0.49
Usable Site	520	972
Polymorphic Site	12	38
Substutitions	5	9
Indels	7	36
Nucleotide Diversity ± S.D. (average over total site)	0.013462 ±0.009569	0.028464 ± 0.021671

Table 4.4 The position of variable sites in *trn*V region among Pinaceae species

Sequence sources of *trn*V	Position of Base Changes
Base Numbers	38 52 53 56 90 100 101 115 144 170 190 201 238 239 240 245 251 255 256 300 307 308 322 346 349 358 374 386 405 449 482 484 501
Picea orientalis	A C A G G C C A C A G C T C T G C T G G G C T G A C T G T A T C
Picea abies AB019899.1	A C A G G C C A C A G C T C T G C T G G G C T G A C T G T A T C
Picea sitchensis DQ375447.1	A C A G G C C A C A G C T A T G C C G G G C T G A C T G T A T C
Picea sitchensis EU998739.3	A C A G G C C A C A G C T A C G A T A G G C T G A C T G T A T C
*Pinus nigra*_Gulsoy(2009)	T A A T T C T G T A G C T A C G A T A A A A T G A A C G G A T C
Pinus torreyana subsp. *torreyana* FJ899564	T C A T G C T G C A G C T A C G A T A A G A T G A A C G G A T C
Pinus ponderosa FJ899555.2	T A A T G C T G C A G C T A C G A T A A G A T G A A C G G A T C
Pinus pinaster FJ899583.2	T A A T G C T G T A T C T A C G A T A A G A T G A A C G T A T C
Pinus brutia AB0198894.1	T A A T G C T G T A G C T A A G A T A A G A T G A A C G G A T C
Pinus halepensis AB019893.1	T A A T G C T G T A G C T A C G A T A A G A T G A A C G G A T C
Pinus strobus FJ899560.1	T C C T G C T G C A G C T A C G A T A A T A T T A C G G T T T C
Pinus nigra AB019891.1	T A A T T C T G T A G C T A C G A T A A A A T G A A C G G A T C
Larix occidentalis FJ899658.1	T C A T G C C A C A G C C A C T A T G G G A T T A C T G G A C C
Larix decidua AB019900.1	A C A G G C C A C A G C T C T G C T G G G C T G A C T G T A T C
Abies numidica AB19901.1	T C A T G T C A C A G C T A C G A T G G G A T T G C T A T A T T
Cedrus deodara AB480043.1	T C A T G C C A C T T T T A C G A T G G G A A A A C T G T A C C

Table 4.5. The positions of variable sites in *trn*cd-*trn*ef region among *Picea* species

Base Numbers	1 3 5	3 1 0	3 5 5	3 6 4	3 7 9	3 8 9	4 0 8	4 7 3	5 6 4	5 9 7	6 3 8	6 5 7	7 6 4	7 7 4	8 0 3	8 4 7	8 8 6	8 9 3	9 3 7	9 3 8	9 3 9	9 4 0	9 4 1	9 4 2	9 4 3	9 4 4	9 4 5	9 4 6	9 4 7	9 4 8	9 4 9	9 5 2	9 5 3	9 5 5
Picea orientalis	A	T	T	T	C	T	A	A	C	C	G	G	G	G	C	C	C	G	G	T	T	C	G	T	T	-	-	-	-	-	C	G	A	C
Picea orientalis EF440557.1	A	T	T	T	C	T	A	A	A	N	N	N	G	G	N	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea orientalis DQ010626.1	A	T	T	T	C	T	A	A	A	C	G	G	G	G	C	C	A	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea purpurea EF440561.1	A	T	T	T	C	T	A	A	C	C	G	G	G	G	C	C	T	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea maximowiczi EF440550	A	T	T	T	C	T	A	A	A	C	G	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea martinezii EF440549	A	T	T	T	C	T	A	A	A	C	G	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea omorika EF440556	A	T	T	T	C	T	A	A	A	C	T	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea mariana EF440548	A	T	T	T	C	T	A	A	A	C	T	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea alcoquiana EF440532	A	T	T	T	C	T	A	A	A	T	G	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea neoveitchii DQ358156	A	T	T	T	C	T	A	A	A	T	G	G	G	G	C	C	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea chihuahuana DQ358151	A	T	T	T	C	T	A	A	A	C	G	G	G	G	C	C	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea jezoensis DQ010612	A	T	T	T	C	T	A	A	A	C	T	G	G	G	C	C	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.5. (Cont.) The positions of variable sites in *trn*cd-*trn*ef region among *Picea* species

Base Numbers	1 3 5	3 1 0	3 5 5	3 6 4	3 7 9	3 8 9	4 0 8	4 7 3	5 6 4	5 9 7	6 3 8	6 5 7	7 6 4	7 7 4	8 0 3	8 4 7	8 8 6	8 9 3	9 3 7	9 3 8	9 3 9	9 4 0	9 4 1	9 4 2	9 4 3	9 4 4	9 4 5	9 4 6	9 4 7	9 4 8	9 4 9	9 5 2	9 5 3	9 5 5
Picea alcoquiana DQ010598	A	T	T	T	C	T	A	A	A	C	T	G	T	G	C	C	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea pungens EF440560	A	T	T	T	C	T	A	A	A	C	T	G	T	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea pungens EF440560	A	T	T	T	C	T	A	A	A	C	T	G	T	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea morrisonicola EF440554	A	T	T	T	C	T	A	A	A	T	T	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea brachytyla DQ010601	A	T	T	T	C	T	A	A	A	T	G	G	G	G	C	C	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea mexicana EF440551	A	T	T	T	C	T	A	T	A	C	T	G	G	C	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea engelmanii DQ010603	A	T	T	T	C	T	C	T	A	C	T	G	G	G	C	C	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea koraiensis EF440545	A	T	T	T	T	T	A	A	A	C	T	N	G	G	N	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea wilsonii EF440569	A	T	T	T	C	T	A	A	C	C	G	G	G	G	C	C	T	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea torano EF440568	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G

Table 4.5. (Cont.) The positions of variable sites in *trn*cd-*trn*ef region among *Picea* species

Base Numbers	1 3 5	3 1 0	3 5 5	3 6 4	3 7 9	3 8 9	4 0 8	4 7 3	5 6 4	5 9 7	6 3 8	6 5 7	7 6 4	7 7 4	8 0 3	8 4 7	8 8 6	8 9 3	9 3 7	9 3 8	9 3 9	9 4 0	9 4 1	9 4 2	9 4 3	9 4 4	9 4 5	9 4 6	9 4 7	9 4 8	9 4 9	9 5 2	9 5 3	9 5 5
Picea obovata EF440555	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea likiangensis EF440553	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea koyamae EF440546	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea asperata EF440533	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea abies EF440531	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea spinulosa EF440567	A	T	T	T	C	T	A	A	A	C	-	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea abies EF440530	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea breweriana EF440535	A	T	T	T	C	T	A	A	A	C	-	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea breweriana EF440535	C	G	G	C	C	C	A	A	A	C	G	G	G	G	C	A	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea smithiana DQ010637	A	T	T	T	C	T	A	A	A	C	-	T	G	G	C	C	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea glehnii EF440543	A	T	T	T	T	T	A	A	A	C	T	T	G	G	A	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G
Picea schrenkiana EF440564	A	T	T	T	C	T	A	A	A	C	-	G	G	G	C	C	C	G	C	T	C	A	G	T	T	G	G	T	A	G	A	A	G	G

4.3.2. Molecular Diversity with respect to *mat*K Region

After the analysis of the *mat*K sequences of *Picea* species from the NCBI databases and sequences obtained in the present study, it was found that there were 28 variable sites. The 13 of these are parsimony informative while the remaining 15 of them were singleton sites (Table 4.7). There were, on the average, 1776 identical pairs, 2 transitional and 3 transversional pairs with 0.59 si/sv ratio (Table 4.6).

Table 4.6. Estimated molecular diversity parameters for *mat*K regions among *Picea* species

Molecular Diversity Parameters	MatK region
Total sample size	35
Total Length (bp)	1827
Average GC content (%)	37
Conserved sites	1776
Variable sites	28
Singleton sites	15
Parsimony informative sites	13
Identical pairs	1796
Transitional pairs	2
Transversional pairs	3
Transition/ Transversion Ratio	0.59

Table 4.7. Variation sites with respect to *mat*K region among *Picea* species

Base Numbers	73	219	346	376	444	450	473	494	508	545	582	666	736	789	790	994	1173	1225	1231	1449	1467	1468	1474	1475	1476	1477	1478	1479	1480	1508	1521	1522	1523	1524	1525	1572	1573	1574	1575	1576	1585	1652	1662	1697	1786
Picea orientalis	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea orientalis EF440516	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
*Picea maximomiczii*EF440509	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea chihuahuana EF440497	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea wilsonii EF440529	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	C	A	G
Picea pupurea EF440520	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	C	A	G
Picea morrisonicola EF440513	A	G	C	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea martinezii EF440508	A	G	T	A	C	G	A	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea brachytyla EF440495	A	G	T	A	A	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea spinulosa EF440527	A	G	T	A	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	A	A	G	A	G
Picea pungens EF440519	A	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea schrenkiana EF440523	C	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	A	G	A	G
Picea rubens EF440522	A	G	T	G	C	G	C	A	A	A	G	A	C	T	G	T	G	G	C	T	T	A	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea mexicana EF440510	A	G	T	G	A	G	C	G	T	A	T	A	C	T	G	T	G	A	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea mexicana AY035194	A	G	T	G	A	G	C	G	T	A	T	A	C	T	G	T	G	A	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea glauca EF440501	A	G	T	G	A	G	C	G	T	A	T	A	C	T	G	T	G	A	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea engelmannii EU364788	A	G	T	G	A	G	C	G	T	A	T	A	C	T	G	T	G	A	C	T	T	C	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea omorika AY035200	A	G	T	G	C	G	C	A	A	A	G	A	C	T	G	T	G	G	C	T	T	A	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea mariana AF133922	A	T	T	G	C	G	C	A	A	A	G	A	C	T	G	T	G	G	C	T	T	A	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea engelmannii AY035196	A	G	T	G	A	G	C	G	T	A	T	A	C	T	G	T	G	A	C	T	T	C	T	C	A	G	T	T	A	C	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea glehnii EF440502	A	T	T	G	C	G	C	G	A	A	G	A	C	A	T	T	G	G	C	T	T	A	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea jezoensis EF440503	A	N	T	G	C	G	C	G	A	A	G	A	C	A	T	T	G	G	C	T	N	A	T	C	A	G	T	T	A	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea likiangensis EF440506	A	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	-	-	-	-	-	-	-	-	-	-	-	-	G	A	G	A	G
Picea farreri EF440500	A	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	G	C	T	T	C	T	C	A	G	T	T	A	T	-	-	-	-	-	-	-	-	-	-	-	-	G	A	G	A	G
Picea abies AY289610	A	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea asperata AY035202	A	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G

Table 4.7. (Cont.)Variation sites with respect to *mat*K region among *Picea* species

Base Numbers	73	219	346	376	444	450	473	494	508	545	582	666	736	789	790	994	1173	1225	1231	1449	1467	1468	1474	1475	1476	1477	1478	1479	1480	1508	1521	1522	1523	1524	1525	1572	1573	1574	1575	1576	1585	1652	1662	1697	1786
Picea torano EF440528	A	G	T	G	C	G	C	G	T	A	G	A	G	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea retroflexa EF440521	A	G	T	G	C	G	C	G	T	A	G	A	G	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea obovata EF440514	A	G	T	G	C	G	C	G	T	T	G	A	C	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea meyeri EF440511	A	G	T	G	C	G	C	G	T	A	G	A	G	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea koyamae EF440505	A	G	T	G	C	G	C	G	T	A	G	A	C	T	G	T	G	A	C	G	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea crassifolia EF440498	A	G	T	G	C	G	C	G	T	A	G	A	G	T	G	T	G	A	C	T	T	C	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea koraiensis EF440504	A	G	T	G	C	G	C	G	T	A	G	A	G	T	G	T	G	A	C	T	T	A	-	-	-	-	-	-	-	T	A	A	T	A	C	-	-	-	-	-	G	C	G	A	G
Picea sitchensis EF440525	A	G	T	G	C	T	C	G	T	A	G	G	C	T	G	C	A	G	C	T	T	A	T	C	A	G	T	T	A	T	A	A	T	A	C	A	T	C	C	G	G	A	G	C	A

4.4. Genetic Distance Variation (Evolutionary Divergence) of the Studied *Picea orientalis* Populations

4.4.1. Genetic Distances within Studied *Picea orientalis* Populations

Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*.,2011) in MEGA 5.02. The analysis involved 53 *trn*A (including *trn*V, *trn*cd and *trn*ef) nucleotide sequences. There were a total of 1473 positions in the final dataset. According to the results with respect to polymorphic region on *trn*ef region, although the variations ranged from 0 to 0.001, in most populations, individuals had the same sequences within population level. Only 3 populations which are Trabzon-Torul-Örümcek, Artvin-Merkez-Tütüncüler Taşlica-Atila and Artvin-Ardanuç-Tepedüzü-Ovacık had the highest variation (0.00045) (Table 4.8) among the 15 studied populations. Since the population from Erzurum Posof had only one sample, genetic distance for this population could not be calculated.

Table 4.8. Average genetic distances within populations of *Picea orientalis*

Population Code	Genetic distance within *Picea orientalis* Populations (±standard error)
OG-OC	0.00000 (±0.00000)
OG-OG	0.00000 (±0.00000)
OG-TA	**0.00034 (±0.00033)**
TR-MH	0.00000(±0.00000)
TR-TO	**0.00045 (±0.00044)**
TR-PC	0.00000 (±0.00000)
AB-MT	**0.00045 (±0.00044)**
AB-SA	0.00000 (±0.00000)
AB-BB	0.00000(±0.00000)
AY-YA	**0.00034 (±0.00033)**
AY-AA	**0.00045 (±0.00044)**
AS-SY	**0.00034 (±0.00033)**
AS-SM	0.00000 (±0.00000)
AS-SV	**0.00034 (±0.00033)**
AS-AP	N/A

4.4.2. Genetic Distances among *Picea orientalis* Populations Grouped with respect to Geographical Regions

Genetic distances were computed among geographical regions (groups) of *Picea orientalis* populations. Average genetic distance within geographic regions ranged from 0.00018 to 0.00039. The *Picea orientalis* populations from Artvin-Borçka were the least diverged while Artvin-Yusufeli populations were the most diversed oned. Among 5 geographical regions, the populations from Artvin-Borçka (AB) and Artvin-Şavşat (AŞ) were genetically the most distant (0.00046) while the populations of Artvin-Şavşat and Ordu-Giresun as

well as Artvin-Borçka and Trabzon-Rize region were the most simlar ones (0.00026). (Table 4.9).

Table 4.9. Average genetic distances computed among populations of regional groups of *Picea orientalis*

		Average Genetic Distance Between Geographic regions Groups			
	Average Genetic distance within Gegraphic Region ± SE	OG	TR	AB	AY
Ordu-Giresun (OG)	0.00029 ±0.00029				
Trab-zon-Rize (TR)	0.00034 ±0.00033	0.00039 ±0.00037			
Artvin-Borçka (AB)	0.00018 ±0.00017	0.00045 ±0.00043	0.00026 ±0.00025		
Artvin-Yusufeli (AY)	0.00039 ±0.00038	0.00032 ±0.00030	0.00036 ±0.00034	0.00037 ±0.00036	
Artvin-Şavşat (AŞ)	0.00028 **±**0.00027	0.00026 ±0.00025	0.00040 ±0.00038	0.00046 ±0.00044	0.00032 ±0.00030

4.5. Genetic Distance among Genera of Pinaceae Family with respect to *trn*V Region

The samples were grouped depending on the type of genera which are *Picea, Pinus, Abies, Larix* and *Cedrus*. Bootstrap method was used as variance estimation method with 500 numbers of bootstrap replication with maximum composite likelihood model. The included substititons were both transitions

and transversions with complete deletion data treatment. According to the genetic distance analysis, the genus *Picea* is genetically closer to *Larix* than it is to other genera. There were high variations between groups such that the divergence of selected species from *Picea* is considerably high (0.005).

Table 4.10 shows the the genetic distances between species with standard errors below the diagonal.

Table 4.10. The molecular distance estimation within species and between group average with respect to *trn*V region

Average diversity within Pinaceae genera ± SE		**Average diversity between taxa**			
		Picea	*Pinus*	*Abies*	*Larix*
Picea	0.005±0.002				
Pinus	0.008±0.002	0.028±0.007			
Abies	NA	0.021±0.006	0.028±0.007		
Larix	0.02±0.006	0.012±0.003	0.028±0.006	0.019±0.005	
Cedrus	NA	0.023±0.006	0.030±0.007	0.020±0.006	0.020±0.005

4.6. Genetic Distance between *Picea* Species with respect to *trn*cd-*trn*ef Regions

In order to determine the genetic distance between *Picea* species, they were grouped depending upon their sections as *Picea*, *Omorika* and *Casicta* Moreover, *Picea orientalis* individuals were grouped separately as a fourth group including *P. orientalis* contig sequence obtained from this study and two *P. orientalis* sequences obtained from NCBI database (Table 3.11). One of these was from Georgia (Bouille and Bousquet, 2007) and the other was from Samsun, Turkey (Ran *et al*., 2006). According to complete deletion method with bootstrap variance estimation method *Picea orientalis* had a high divergence within sequences. Moreover, this taxon has a close relationship with both species from *Picea* and *Casicta* sections. In fact *Picea orientalis* belongs to section *Picea*. The spruce species from section *Omorika* were the most diverged one from the other spruce species of *Picea* and *Casicta* (Table

4.11).

Table 4.11. The Molecular Distance Estimation within *Picea* species with respect to *trn*c-*trn*ef regions combined

Average diversity within *Picea* species from geographic regions ± SE		***Picea orientalis***	**Section *Picea***	**Section *Omorika***
P.orientalis	0.004±0.001			
Section *Picea*	0.003±0.001	0.004±0.001		
Seciton *Omorikae*	0.005±0.001	0.005±0.001	0.004±0.001	
Section *Casicta*	0.003±0.001	0.004±0.001	0.003±0.001	0.004±0.001

4.7. Genetic Distance between *Picea* Species with respect to *mat*K Region

The genetic distance between selected *Picea* species were analyzed considering *mat*K region considering the sections (*Picea*, *Omorika* and *Casicta*) as well as *Picea orientalis* as a fourth group. The group containing *P. orientalis* species included only the contig sequence of the current study and the sequence from Georgia (Bouille and Bousquet, 2007) (Table 3.12). According to this study, there is no variation among two *P. orientalis* samples and much of the variation was seen in the species from section *Casicta* (Table 4.12). Considering the average diversity between *P. orientalis* and other species, the result was the fact that of the 4 groups with respect to *trn*cd-ef region, *P. orientalis* is close to section *Picea*. The species of section *Casicta* were more divergent than spruce species from all other groups including *P. orientalis* (Table 4.12).

Table 4.12. The molecular distance estimation within *Picea* species with respect to *mat*K region

Average diversity within *Picea* species from geographic regions ± SE		**Average diversity between *P. orientalis* and geographic regions**		
		Picea orientalis	**Section *Picea***	**Section *Casicta***
Picea orientalis	*0.000±0.0000*			
Section *Picea*	0.0020±0.0006	0.0010±0.0005		
Section *Omorikae*	0.0020±0.0008	0.0030±0.0007	0.0020±0.0006	
Section *Casicta*	0.0030±0.0009	0.0020±0.0007	0.0020±0.0006	0.0030±0.0007

4.8. Molecular Clock Estimation

According to this study the molecular clock estimation were performed with respect to *Picea orientalis* samples that were studied as well as the species within Pinaceae species with respect to *trn*V and the species between *Picea* species with respect to *trn*cd-ef and *mat*K regions. Table 4.13 shows the molecular clock times for *trn*A and *mat*K regions. According to results, the groups of studied *Picea orientalis* have diverged about 300,000 mya. Considering trnV region, the Pinaceae species have diverged about 20 mya. Within Picea genus, the divergence of the samples were between 4.5-3.5 mya with respect to *trn*cd-ef and *mat*K regions.

Table 4.13. Molecular clock estimations

Calculated Regions	Number of Parsimony informative sites	Total number of site sequenced	d	k	MCE (mya)*
***Picea orientalis trn*A region**	1	1480	$6.75x10^{-4}$	$6.75x10^{-4}$	0.3375
Pinaceae species with respect to *trn*V region	20	520	$3.85x10^{-2}$	$3.95x10^{-2}$	19.750
***Picea* species with respect to *trn*cd-ef regions**	9	972	$9.26x10^{-3}$	$9.3x10^{-3}$	4.650
***Picea* species with respect to *mat*K (complete) and *trn*K (partial) region**	13	1827	$7.12x10^{-3}$	$7.15x10^{-3}$	3.575

*Molecular Clock Estimation (million years ago)

4.9. Estimation of Pairwise Genetic Distances (F_{st}) among Populations and Taxa

Pairwise genetic distances were calculated to represent the average number of pairwise differences within populations sampled and between populations. Moreover, the fixation index for Pinaceae species with respect to *trn*V region and *Picea* species for both *trn*cd-ef and *mat*K regions.

4.9.1. Estimation of Pairwise Genetic Distances (Fst) among Studied *Picea orientalis* Populations

According to the fixation index calculation among *Picea orientalis* groups showed that most of the divergence was found between Artvin-Şavşat and Artvin-Borçka (0.050) and between Ordu-Giresun and Artvin Borçka (0.048). In fact, according to F_{st} values the populations from Artvin-Borçka is the most divergent group among other populations of other regional groups (Table

4.14).

Table 4.14 Pairwise genetic distances among *Picea* orientalis geographic regions

	OG	TR	AB	AY
Ordu-Giresun (OG)				
Trab-zon-Rize (TR)	**0.019**			
Artvin-Borçka (AB)	**0.048**	**0.001**		
Artvin-Yusufeli (AY)	0.000	0.000	**0.030**	
Artvin-Şavşat (AŞ)	0.000	**0.020**	**0.050**	0.000

4.9.2. Estimation of Pairwise Genetic Distances (Fst) among Pinaceae Species

The fixation index values were calculated by considering Pinaceae species with respect to *trn*V region as well as *Picea* species with respect to *trn*cd-ef and *mat*K reigons (Tables 4.15, 4.16 and 4.17). The results showed that *Picea* species were close to *Larix* species for *trn*V region. Moreover, *Cedrus* species were the most isolated species (Table 4.15). Furthermore, the fixation index for *Picea* species first with *trn*cd-ef region and then *mat*K region regarding *Picea orientalis* sequences as well as the geographic regions (Asia, North America and Europe). According to *trn*cd-ef region, *Picea orientalis* was close to species from section *Picea.* Species from section *Casicta* constituted the most divergence (Table 4.16). Finally, the F_{st} values among *Picea* species considering *mat*K region were calculated. The result showed similarity with *trn*cd-ef region such that *P.orientalis* species possessed the least divergence

with the species from section *Picea*. The species from section *Omorika* are the divergent taxa (Table 4.17).

Table 4.15. Pairwise genetic distances among Pinaceae genus with respect to *trn*V region

	Picea	***Pinus***	***Abies***	***Larix***
Pinus	0.748			
Abies	0.751	0.708		
Larix	0.092	0.618	0.002	
Cedrus	0.763	0.720	0.091	0.002

Table 4.16 Pairwise genetic distances among *Picea* section with respect to *trn*cd-ef region

	P. orientalis	**Section *Picea***	**Section *Omorakae***
Section *Picea*	0.0002		
Section *Omorakae*	0.0008	0.0003	
Section *Casicta*	0.0004	0.0008	0.0004

Table 4.17 Pairwise genetic distances among *Picea* section with respect to *mat*K region

	P. orientalis	**Section *Picea***	**Section *Omorakae***
Section *Picea*	0.0004		
Section *Omorakae*	0.0009	0.0004	
Section *Casicta*	0.0005	0.0001	0.0006

4.10. Phylogenetic Tree Construction

In this study, five different phylogenetic trees were constructed including the relationship between *Picea orientalis* populations with respect to the one parsimony informative site on *trn*A and *mat*K regions with outgroups (Figure 4.2 and 4.3). Moreover, the relationship between *Picea orientalis* populations with other Pinaceae species with respect to *trn*V region was also shown by a phylogenetic tree (Figure 4.4). Finally, the phylogenetic trees for *Picea orientalis* with other *Picea* species with respect to *trn*cd-ef and *mat*K regions were constructed (Figure 4.5 and 4.6). During phylogenetic tree construction maximum parsimony method with bootstrap analysis (with 500 replications) were used. According to the results, there is no considerable variation between *Picea orientalis* populations. Moreover, the population from different groups showed highly dispersed allocations with recpect to *trn*A region. However, this one parsimony informative site separated groups from each other with a high bootstrap value. (Figure 4.2). Considering *mat*K region, there was no variation among studied *Picea orientalis* populations. Moreover, they were closer to *P. purpurea* from China and *Picea orientalis* from Georgia than other included outgroups (Figure 4.3). When other Pinacea species were considered with respect to *trn*V region, *Picea orientalis,* as expected*,* is highly divergent from other Pinaceae especially from *Pinus* species such that *Pinus* species and other included taxa constructed separate clusters. *Picea* species were closer to *Larix* species with respect to *trn*V region (Figure 4.4). Considering other *Picea* species with respect to *trn*cd-*trn*ef and *mat*K regions, although the result were not phylogenetically informative there seems some variations between *Picea orientalis* and other *Picea* species such that the studied *Picea orientalis* taxon was closer to species from section *Picea* although there were also some exceptions with the presence of close relationship of *Picea* species from section Casicta or Omorakae (Figure 4.4).

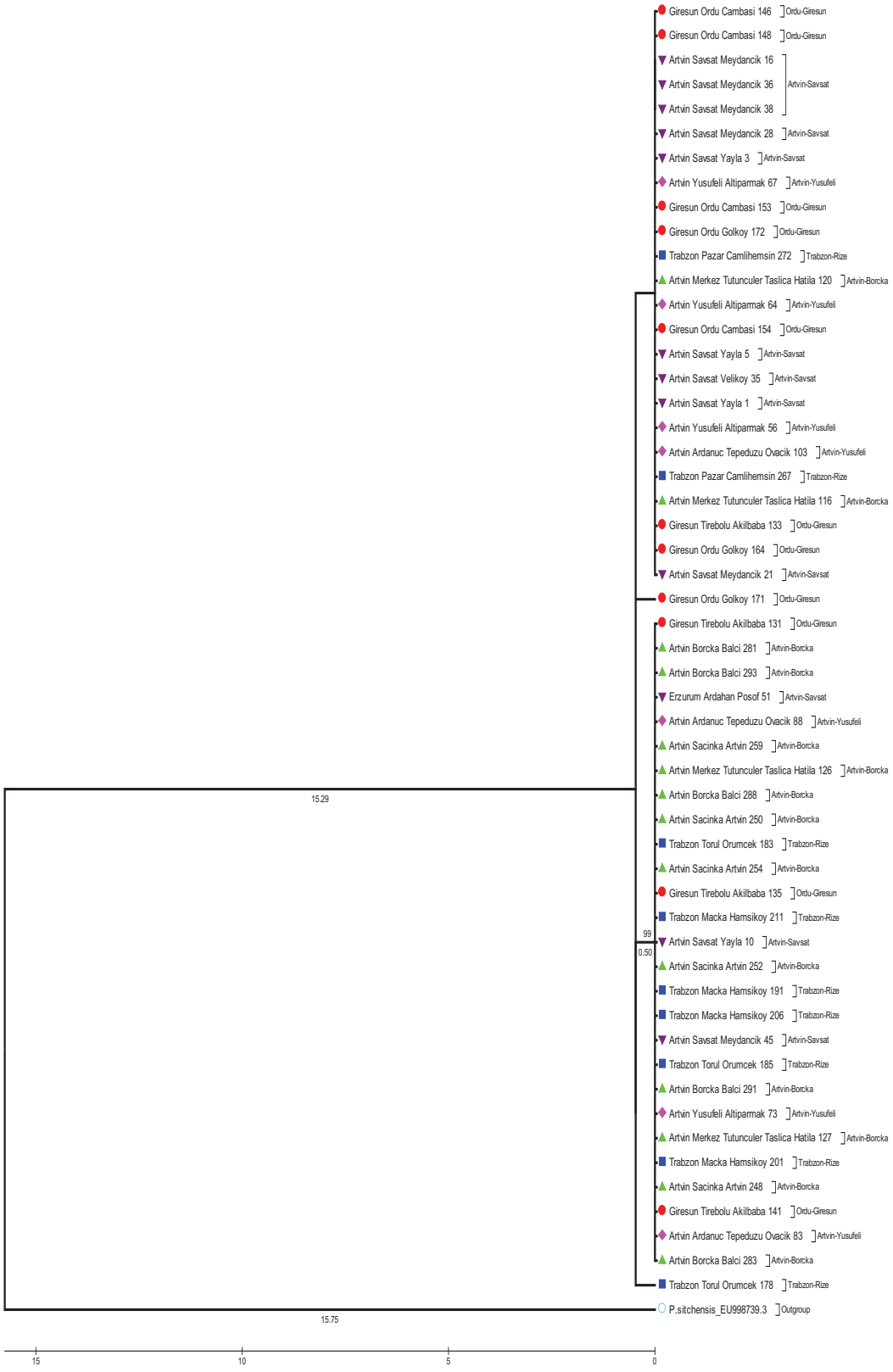


Figure 4.2. The phylogenetic tree constructed by *Picea orientalis* populations and *P. sitchensis* as outgroup (the values above and below branches are the bootstrap values and branch lengths, respectively)

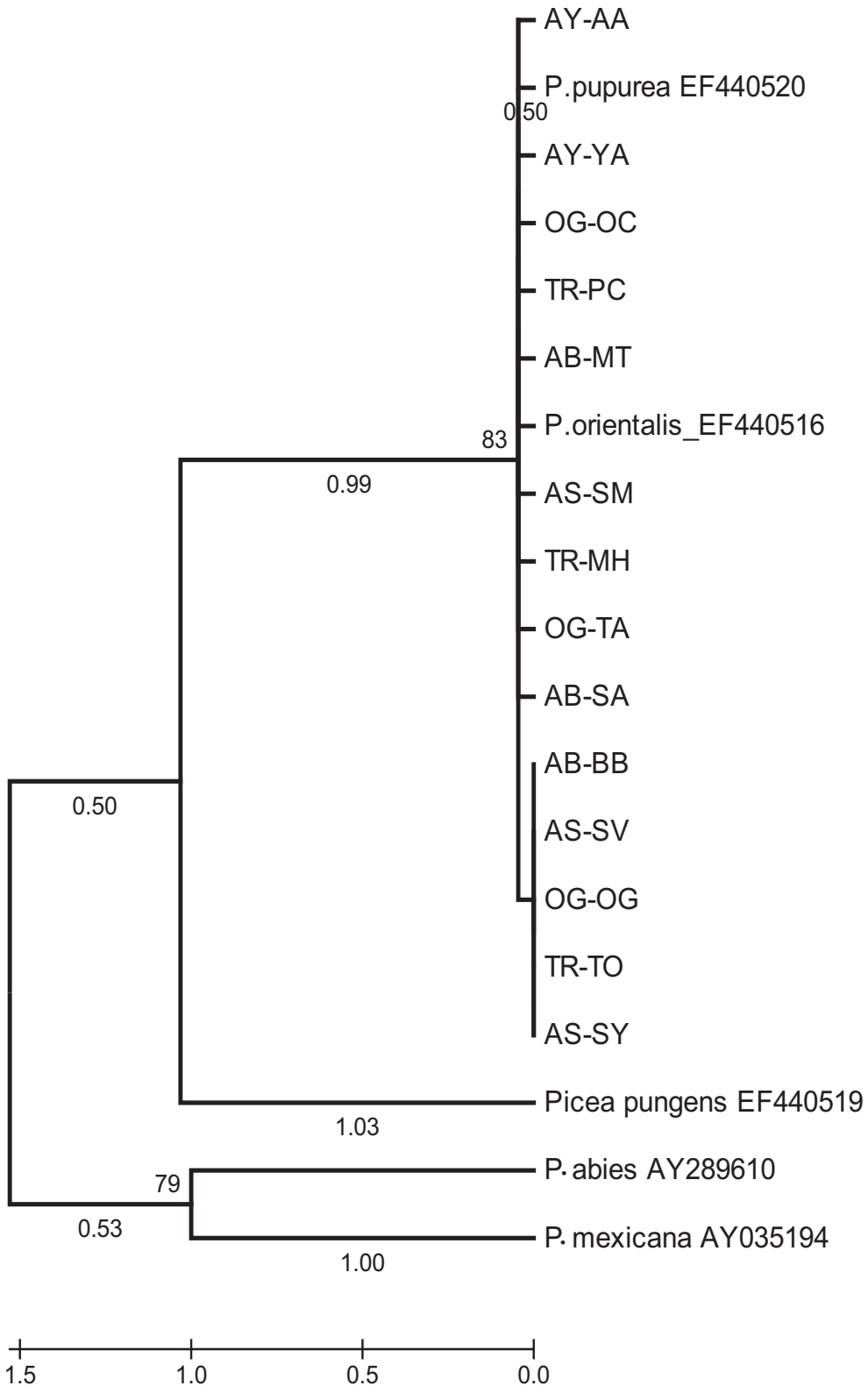


Figure 4.3 The phylogenetic tree constructed by *Picea orientalis* populations and outgroups (the values above and below branches are the bootstrap values and branch lengths, respectively)

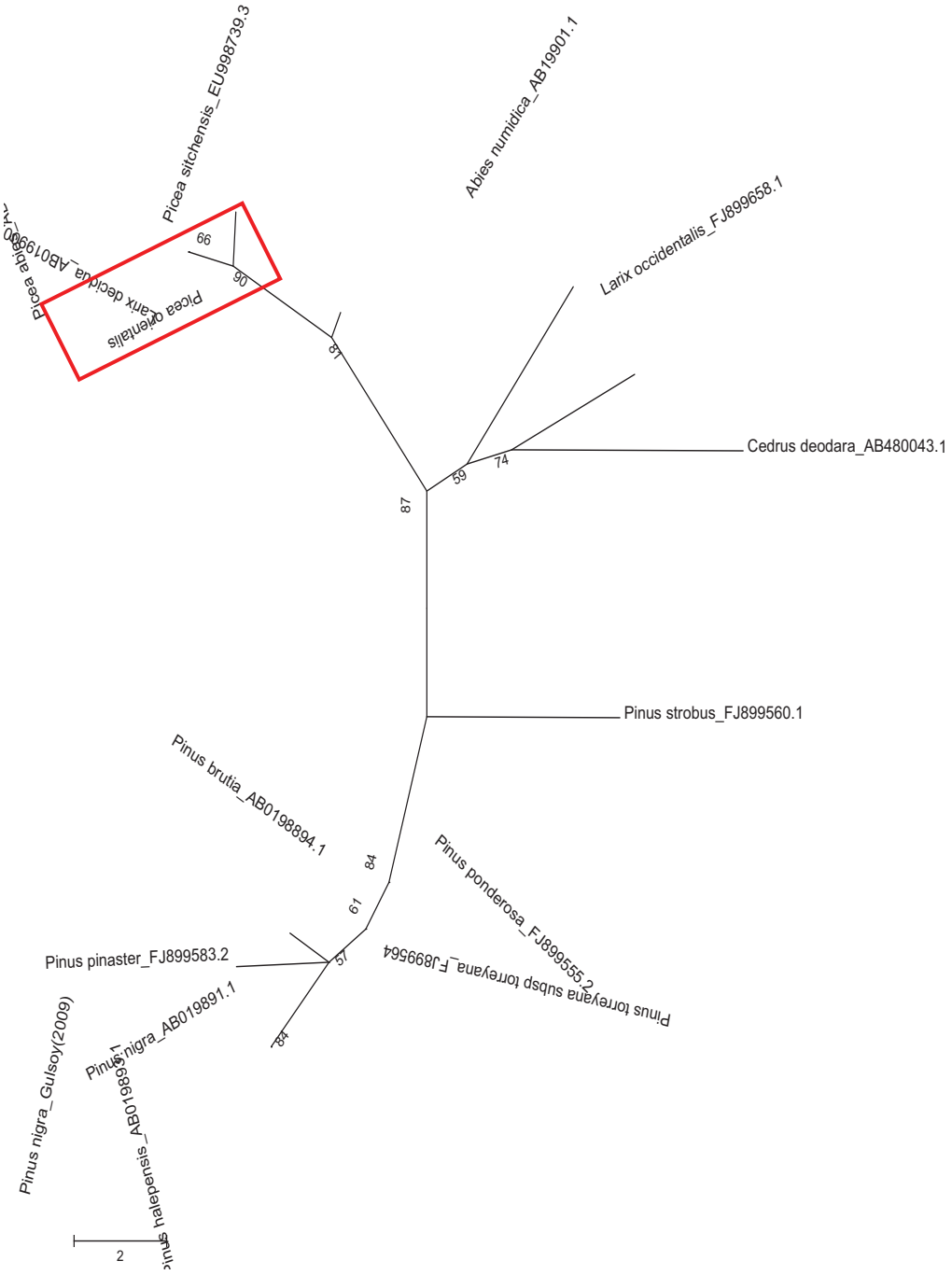


Figure 4.4 The phylogenetic tree constructed by Pinaceae species with respect to *trn*V region (The values next to the branches are the bootstrap values. The contig taxon of the studied *Picea orientalis* populations were shown in red rectangular)

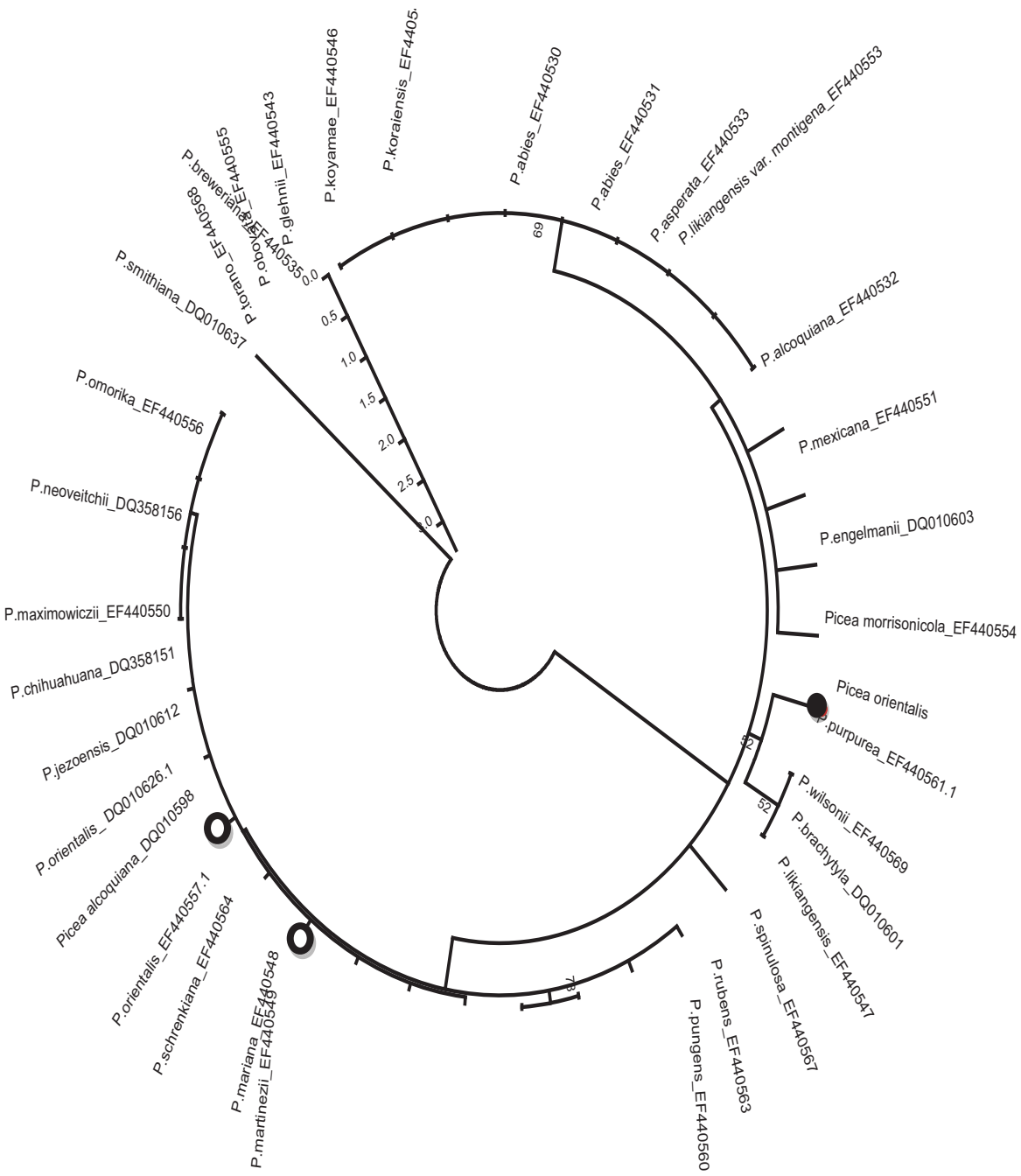


Figure 4.5 The phylogenetic tree constructed by *Picea* species with respect to *trn*cd-ef region (the values above and below the branches are the bootstrap values and scale lengths, respectively) (The contig taxon of the studied *Picea orientalis* populations were shown in filled circle and other *Picea orientalis* species from database shown with empty circles)

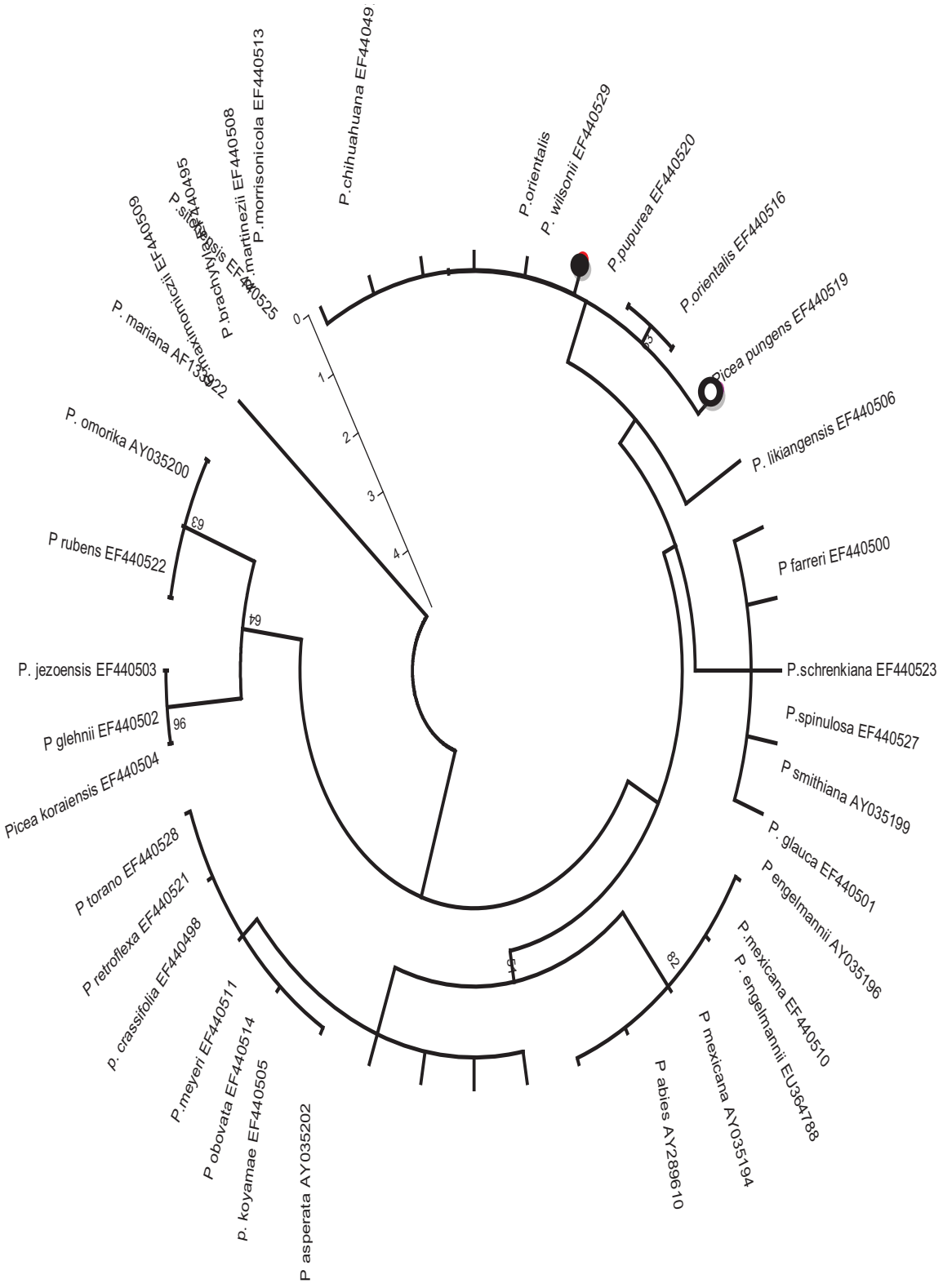


Figure 4.6 The phylogenetic tree constructed by *Picea* species with respect to *matK* region (the values above and below the branches are the bootstrap values and scale lengths, respectively) (The contig taxon of the studied *Picea orientalis* populations were shown with filled circle and other *Picea orientalis* species from database shown with empty circle)

CHAPTER 5

DISCUSSION

5.1. Molecular Diversity in *Picea orientalis*

5.1.1. Molecular Diversity with respect to *trn*A Region

In this study, because of trimming of unclear flanking ends, *trn* region was about 1480 bp. The length of *trn*V region was 520 bp in length which was close to the reported lengths (Wang *et al*.,1999; Willyard *et al*., 2007; Cronn *et al*., 2008). The previous studies reported that this region ranges from 500 bp to 540 bp among *Picea* species. Moreover, according to other studies this region is 577 bp in *Larix* species (Parks *et al*., 2009). For *Cedrus* species the studies showed that *trn*V region was 467 bp (Lin *et al*., 2010). The region were also between 460 bp to 630 bp for other Pinaceae species including *Abies*, *Keteleeria*, *Pseudolarix*, *Cathaya* and *Pseudotsuga* (Wang *et al*., 1999; Wu *et al*., 2009; Lin *et al*, 2010; Eckert and Hall, 2006).

The *trn*cd-ef region was found to be 953 bp in length. In the study performed by Bouille and Bousquet (2007), it was about 1000 bp among 37 *Picea* species and according to Ran *et al*. (2006), it was about 970 bp in length. Since the *trn* region yielded high variation among Pinaceae species, it could be possible that *trn*A region is different in length because of possible indels.

In the sequence analysis, the entire *trn* region in *Picea orientalis* had 38.3 % GC content, one variable site which was also parsimony informative and 1479 conserved sites. The variable site, which was located in *trn*ef region, were transversion between A and T. The studies dealing with *trn*V regions revealed that there were 32 variable sites (20 parsimony informative and 12 singleton sites) (Wang *et al*., 1999; Willyard *et al*., 2007; Cronn *et al*.,2008; Parks *et al*., 2009; Gulsoy, 2009; Lin *et al*., 2010). For *trn*cd-ef region there were 26

variable sites 7 of them were singleton with respect to the studied *Picea orientalis* populations. This may indicate the effect of habitat isolation through evolutionary time and hence the variation were considerebly high although there were only 1 variable site within studied *Picea orientalis* populations.

With respect to *trn*V region, the total nucleotide diversity was 0.013462 (±0.009569) and for *trn*cd-ef region, it was 0.028464 (± 0.021671) among Pinaceae and *Picea* species, respectively. The results suggest that different portions of the *trn*cd-ef region had different evolutionary patterns and might not share the same evolutionary history. However, the reason of the such small change in nucleotide diversity over many different taxa might be due to (i) low variation in cpDNA among plant species (Clegg, 1990; Powell,1994; Provan *et al*., 2001), (ii). the ascertainment bias which is the selection of loci from an unrepresentative sample of individuals yielding loci that are not representative of allele diversities in a population (Allendorf and Luikart, 2007) and (iii) the young evolutionary history of *trn*A region (Gülsoy, 2009).

5.1.2. Molecular Diversity with respect to *mat*K Region

In this study, the primers designed to amplify *mat*K region included *mat*K and partial amplification of the *trn*K region at 3'. For this reason, the *mat*K region had 1906 bp in length within which 1545 bp was composed of *mat*K region. According to Bouille and Bousquet (2007), the reported *mat*K length among *Picea* species including *Picea orientalis* from Georgia Caucasus Mountains were between 1537-1547 bp. Moreover, other reported studies have also indicated similar range among *Picea* species (Germano and Klein 1999; Germano *et al.,* 2001; Germano *et al*., 2002).

The entire *mat*K and partial *trn*K regions had 37.1% GC content within studied *Picea orientalis* populations. However, there was no variation. According to several studies, the high rate of substitution in this gene has resulted in an increased number of parsimony informative sites and strong phylogenetic signals, making it useful to determine evolutionary histories at

various taxonomic levels (Müller *et al*. 2006; Hao *et al*. 2008). Moreover, the abundant phylogenetic information derived from *matK* has made it an extremely valuable gene for DNA barcoding, systematic and evolutionary studies (Hao *et al*., 2008). The reason for not detecting variable sites in *mat*K region in *Picea orientalis* is probably due to high conserved rate within matK region in *Picea orientalis* populations. According to the analysis of *P. orientalis* population with *Picea* species retrieved from GenBank, there were 28 variable sites with 10 indels, 2 of which were unique in the studied *Picea orientalis* populations. There were also 0.004248 (±0.002255) nucleotide diversity. This also indicates the much lower variablity of *mat*K region that of *trn*A region. According to Hilu and Liang (1997) the relativley low nucleotide divergence (1-8%) even at the intrafamiliar level and consistent monophyly indicates representation of the study by a small number of taxa. The sample adjustment by increasing the order level provides more comprehensive analysis. Moreover, *mat*K region might be useful at higher taxonomic levels.

5.2. Genetic Distance Variation (Evolutionary Divergence)

5.2.1. Genetic Distance Variation within *Picea orientalis* Populations

According to the results conducted based on the five geographic location differences within *Picea orientalis*, the populations from Artvin was the most variable ones (0.00034-0.00045). This result is meaningful if there were a probable pollen based gene flow between populations because cpDNA is caried paternally in conifers (Allendorf and Luikard, 1997) and pollen dispersal makes remarkably high contribution to gene flow (Silvertown and Doust, 1993). Since in some species, especially in coniferous tree species, individuals are distributed continously across large landscape (Artvin in this case) and are not subdivided by distinct boundries, there may not be any barrier to inhibit gene flow. However, still gene flow may be finite around short distances due to isolation by distance (Wright, 1943). For that purpose

the variation within population from Artvin might be due to the gene flow but the low proportion in this variation was probably the isolation by distance.
The reason why SE's are higher than expected is related with sample size. The standard error becomes smaller as the sample size increases. The sample mean becomes a more accurate estimate of the parametric mean, so the standard error becomes smaller (Sokal and Rohlf, 2001).

5.2.2. Genetic Distance Variation with respect to Geographic Regions

The genetic variations within the groups constructed by the criteria of Alptekin (1986) showed that the most variable group was Artvin-Yusufeli including the sub-demes Altıparmak, Ardanuç, Tepedüzü and Ovacık. This indicates that there is a proportion of differentitation due to differences between sub-demes and these differences are probably potentially adaptive differences (Silvertown and Doust, 1993). Among 5 geographical regions, the populations from Artvin-Borçka (AB) and Artvin-Şavşat (AŞ) were genetically the most distant while the populations of Artvin-Şavşat and Ordu-Giresun as well as Artvin-Borçka and Trabzon-Rize region were the most similar ones. The distinctness between very close geographic regions (Artvin-Borçka and Artvin-Şavşat in this case) were also probably due to the adaptive differences. However, the close relationship between geographically distinct groups may probably due to the fact that when variation within populations is higher than expected, the proprotion of total variation between populations could not be very high since the measure of genetic distance is often bias towards downwards (Hedrick, 1999).

5.2.3. Genetic Distance Variation within Pinaceae Species

According to the study with respect to *trn*V region, the most variable genus was *Pinus* followed by *Picea.* This result is meaningful since more than half of the Pinaceae species (over 100 species) are in the genus *Pinus* (Richardson, 1998). The genus *Picea has* 35 to 40 species naturally found in the regions from North America to Eurasia (Hardin *et al*., 2001). Considering differences among genera, *Picea* is the closest taxon to *Larix*. However, this result were unexpected since according to several studies *Larix* which is a decidious tree, is distinct from evergreen conifers and resemble other decidious trees due to their resemblance in seed proteins (Prager *et al.,* 1976; Price *et al.,* 1989). Moreover, according to Lin *et al.* (2010), in chloroplast phylogenomics of Pinaceae showed the distinctness of *Picea* from *Larix*. Furthermore, according to several studies *Picea* is much closer to *Pinus* than other taxa (Hart, 1987; Frankis, 1988; Price, 1989; Farjon, 1990; Wang *et al*., 2000; Gernandt *et al*., 2005). Hence, the reason for this result was probably due to inadequate sampling. Regretably, there were limited number of studies for *trn*V region among Pinaceae species so the sampling from genebank became unqualified to make meaningfull comparison among conifer species.

According to the analysis for *trn*cd-ef region, *Picea* species were more or less possessed equal diversity within taxa due to the usage of the different types of *Picea* species during analysis. Moreover, *Picea orientalis* had closer relationship from species of the section *Picea* indicating the effect of monophyly of Picea species and is congruent with previous studies (Ran *et al*.,2006, Sigurgeirsson and Szmidt, 1993; Farjon, 1990). According to Sigurgeirsson and Szmidt (1993), RFLP analysis of cpDNA on *Picea* species showed that level of differentiation among Eurasian species were lower than that among American species.

5.3. Molecular Clock Estimation and Origin of *Picea*

5.3.1. Origin of *Picea orientalis*

According to the molecular clock estimation with respect to 1 parsimony informative site on *trn*ef region, *Picea orientalis* diverged about 338.000 years ago from its recent ancestor. This time falls into late quarternary period indicates the very recent divergence of the taxa. According to Comes and Kadereit (1998), climatic oscillations in the Quaternary have played a major role in changing the geographical distribution of plant species. A comparative analysis shows that phylogeographic patterns in Europe appear to be less concordant than in North America. The change of geographic distribution provided speciation through isolation. This hypothesis were also supported by several other studies (Qian and Ricklefs, 2000; Liu *et al*., 2002; Petit *et al*., 2003; Hewitt, 2004).

5.3.2. Divergence of Species within Pinaceae

The study with *trn*V region showed that the divergence of species from their common ancestor was about 20 million years ago which falls into late Tertiary period. However, according to Wang *et al* (2000), based on their molecular clock estimations, the divergence of Pinaceae family were assumed to be initiated about 140 mya during Cretaceous period. This assumption was supported by the fossil records of *Pinus* dating back to the early Cretaceous (Miller 1977; Florin 1963). They also found that the widely distirbuted taxa like *Picea* were also started to diverge at Cretaceous period. Based on these results, analysis with *trn*V region was not so reliable due to the inadequate sampling.

However, analysis with *trn*cd-ef and *mat*K region showed consistent results by getting divergence times about 4.5 and 3.5 mya, respectively. These times overlapped with late tertiary, pliocene epoch. The result were also supported with several studies that based on fossil records (Miller, 1989; LePage, 2001)

and molecular clock estimation of the *mat*K gene (Wang *et al*., 2000), the origin of *Picea* could date back to the early Tertiary or late Cretaceous, when there were exchange between North America, Asia and Europe. Moreover, considering the fossil records Ledig *et al*. (2004) inferred that *Picea* probably originated in North America, then spred to Asia, and from Asia to Europe. Moreover, when low genetic variation results were combined with molceular clock estimation, the result become more consistent because a high species diversity resulted from recent and rapid diversification would be characterized by low genetic diferentiation among taxa and a poorly resolved phylogeny (Richardson *et al*., 2001).

5.4. Estimation of Pairwise Genetic Distances (F_{st})

5.4.1. Pairwise Genetic Distance among *Picea orientalis* Populations

The analysed F_{st} values showed that the most genetically divergent groups were Artvin-Şavşat and Artvin-Borçka. This result is consistent with the results of genetic diversity analysis obtained by using composite maximum likelihood method. The high F_{st} differences between these spatially close population is probably due to the adaptive differentiation. Furthermore, the expected amount of frequency differentiation with given amont of gene flow is different for cpDNA and nuclear genes due to haploidy and uniparental inheritance. Since individuals hemizygous more differentiaiton at cpDNA is seen because of smaller effective population size (Allendorf and Luikard, 1997). F_{st} values showed with zero were actually possessed minus F_{st} values indicates that F_{st} was biased downwards since there were comparatively high variation within populations.

5.4.2. Pairwise Genetic Distances (F_{st}) among Pinaceae species

Considering *trn*V region, the similar problem of genetic distance variation was also seen in Fst analysis which means that the genus *Larix* was seen more

closer to *Picea* than other taxa. Probably this problem was overcome by increasing the sampling of *Larix*. However, as indicated before there were no so many study with the *trn*V region.

According to *trn*cd-ef region, species from section *Casicta* had the highest Fst values and the most divergent groups from other constructed groups. This result may become meaningful when the phylogenetic classifications are considered. Many phylogenetic studies were highly based on morphology (Corrigan *et al*., 1978; Schmidt, 1989). However, according to Sigurgeirsson and Szmidt (1993), species positions in taxon were different than expected such that there are several species that were considered in the same section but posessed different possessed different positions with respect to several markers. This kind of difference is generally due to geographic distribution and different evolutionary rates of taxa. Considering *mat*K region, similar result were seen. However, species from section Omorakae are the most distinct groups. *Picea orientalis* is closer to *Picea* species for both *trn*cd-ef and *mat*K regions. This result is also consistent with the genetic divergence analysis and indicates the Quaternary period dispersal among *Picea* species and congruency of the species with its previously defined position.

5.5. The Constructed Phylogenetic Trees

The constructed phylogenic tree with studied *Picea orientalis* populations with respect to *trn*A region showed that the Artvin population was especially distinct from other populations with 99 % bootstrap value. This result is also in accord with genetic divergence analysis including maximum likelihood and Tamura-Nei methods. The result is probably due to gene flow between subpopulations of Artvin together with local adaptation of these groups. Moreover, the populations from Trabzon, Giresun and Ardahan are diverged from other populations that also indicates the adaptive evolutionary divergence.

Picea orientalis populations were also used to construct phylogenetic tree with other *Picea* species for *mat*K region. The results indicated that *Picea orientalis*

has close relationship with the species from Asia (*P. orientalis* and *P. purpurea*); however, it was highly distinct form the species from Europe (*P. abies*) and North America (*P. pungens* and *P. mexicana*). The result is meaningful when it is combined the results obtained from genetic divergence and fixation index analysis.

According to the analysis with *trn*V region, the species within Pinaceae showed 2 cluster formation. The first cluster was composed of *Pinus* species and the second was included other Pinaceae species. The close relationship of *Picea* with *Larix* were also seen here. For the verification of these findings should be done with further studies including better sampling of spruce species.

The analysis of *trn*cd-ef with *Picea* species showed a dispersed allocation in the tree although the studied *Picea orientalis* was close to section *Picea* species. Similarly, the constructed phylogenetic tree with matK reigon showed very close results with the tree from *trn*cd-ef region although many branches were not strongly supported by the high bootstrap values.

According to phylogenetic tree based on trncd-ef region, *P. breweriana*, a phenetically most distinct species as well as *P.rubens* and *P.pungens* with a high bootstrap value. These all three species are naturally located in North America. The remaining species showed a dispersed allocation with respect to division by clade but they showed consistent results according to their geographical distribution. For example the studied *Picea orientalis* contig sequence showed high resemblence with *P.purpurea* and *P.wilsonii* which are both derived from China but belong to clade Casicta and Picea, respectively. Depending on previous classifications based on morphology and chemical composition, *Picea orientalis* belong to clade Picea (Corrigan *et al*., 1978; Schmidt, 1989). However, several other phylogenetical analysis showed incogruent results with this classification and showed similar results with this study (Mikkola, 1969; Sigurgeirsson and Szmidt,1993; Ran *et al.,* 2006). Likewise, with respect to matK region, *Picea orientalis* showed dispersed divergence with all three groups but still closer to species from Asia. These

results also supported another hypothesis which is the fact that based on the cpDNA RFLP analysis, *Picea* originated in North America and colonized to Eurasia. Moreover, the North American origin hypothesis of *Picea* was supported by Ran *et al*. (2006).

CHAPTER VI

CONCLUSION

- The main purpose of this study was to explore the taxonomic status of *Picea orientalis* by means of studying *trn*A and matK regions of cpDNA.
- *Picea orientalis trn*A region was found to be 1480 bp in length. Since *Picea orientalis* was studied at population level, the region showed very little (*trn*ef) or even no variation (*trn*V and *trn*cd). Nevertheless, the presence of this one parsimony informative site on *trn*ef region might become useful for the diversification analysis of populations.
- The Artvin population among the sudied populations showed higher divergence and more distinction. This results were probably due to the fact that there may be high gene flow between subpopulations within Artvin so they possessed higher genetic variation. Moreover, the populations from Artvin-Borcka, Artvin-Sacinka, Ordu-Giresun and Trabzon-Rize showed highest distinction which indicates the local adaptations of demes within populations.
- The analysis with other Pinaceae species with respect to *trn*V region were used to provide better resolution for the relationship of *Picea orientalis* with other Pinaceae species. However, due to the lack of adequate amount of sample data for *trn*V region on database lead to biased result such that the genus *Picea* was showed closed relationship with *Larix* which could not be so meaningful when *Larix* and *Picea* were compared both morphologically and genetically. Hence, this part of the study should be improved further by trying to include more genera of Pinaceae and providing *Larix* as an outlier taxa.

- The analysis of the *Picea* speices considering *trn*cd-ef and matK region implemented very consistent results in both genetic distance calculations and phylogenetic tree construction. *Picea orientalis* was found to be closer to section *Picea* species than other sections. This result were also supported by molecular clock estimation and origin of divergence in *Picea* species such that the current hypothesis suggest that dispersal of *Picea* species form North America to Asia and then Europe occurred during glacial times of Quaternary period . Moreover, *Picea orientalis* was shown in the section of Picea which is consistent with several other studies.

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APPENDIX A

BUFFERS, CHEMICALS and EQUIPMENTS

Buffers and solutions for DNA extraction and qualification

DNA Extraction

2X CTAB: 10 g CTAB (Cetyl Trimethyl Ammonium Bromide), (SIGMA)

50 mL (pH: 8.0) Tris HCl, (SIGMA)

40 mL (pH: 8.0) 0.5M EDTA, (Ethylenediaminetetraaceticacid disodium salt) (SIGMA)

41 g 5M NaCl is completed with 500 mL with dH2O

Chloroform-Octanol, (FLUKA): (24:1)

β-Mercaptoethanol, (SIGMA): 17.5 ml β-Mercaptoethanol is completed with 250 mL dH2O

Isopropanol, (FLUKA): Pure isopropanol, ice cold

Ethanol: 70% in dH2O

TE buffer: 10 mM Tris HCl (pH: 7)

DNA Qualification

Agarose Gel Electrophosesis (%0.8)

Running Buffers: 1XTBE prepared in dH2O

Agarose, (SIGMA): 1 or 1.7 percent (w/v) Agarose gel

Ethyidium Bromide, (SIGMA): 5mg/mL

Loading Buffer: 9.5mL Formamide, (SIGMA)

500μL EDTA (0.5 M)

15mg Bromophenolblue, (SIGMA)

15mg Xylene cyanol, (SIGMA)

Buffers and solutions for Polymerase Chain Reaction (PCR)

10X PCR Buffer ($MgCl_2$ free) (Fermentas)

$MgCl_2$ Stock Solution (Fermentas): 25mM $MgCl_2$

dNTPs (Fermentas): 5mM

Taq DNA polymerase (Fermentas): 5U/µL

Sterile Water: dH2O

Primer-pairs: 100µM

Electrophoresis buffers and gel systems

Agarose Gel Electrophoresis (0.8%)

Running Buffers: 1XTBE prepared in dH2O

Agarose, (SIGMA): 1 or 1.7 percent (w/v) Agarose gel

Ethyidium Bromide, (SIGMA): 5mg/mL

Loading Buffer: 9.5mL Formamide, (SIGMA)

500µL EDTA (0.5 M)

15mg Bromophenolblue, (SIGMA)

15mg Xylene cyanol, (SIGMA)

Equipments

Centrifuge: Sigma 3-18 K

Deepfreezer: Bosch – Freezer

Horizontal Electrophoresis System: Maxicell EC360M Elect. Unit

Thermocyclers: Eppendorf- Mastercycler, Techne-genius

Magnetic Stirrer: Labor Brand/Hotplate L-81

Power Supplies: EC135-90 E-C

Refrigerator: Arçelik

UV Transilluminator : Vilbor Lourmant

Vortex: Nuve NM110

Water Bath: Memme

APPENDIX B

AN EXAMPLE OF CHROMOTOGRAM DATA

Figure A.1. An example of chromotogram data of *trn*cd region

APPENDIX C

AN EXAMPLE OF MEGA DATA FILE

>Giresun Ordu Cambasi 146
CGATTCGTCCGATCCACGAAATAGATTCTATGTGAAAAAGTCTTACT
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TCGATCCGATTCGAATTACGAATCTAATTGATATGGTCAATCCCAGCT
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APPENDIX D

AN EXAMPLE OF ARLEQUIN SEQUENCE DATA

[Profile]

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

[Data]

[[Samples]]

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SampleSize=11
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ACTTCCAGATTGAAAATAAGAAAGATCATTCTCAAAACTGGAAAAATAGT
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ACAGGGAAGAGCCGGGATAGGTTCGTTCGCGAACGAC
}

[[Structure]]

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"POP02_Trabzon_Rize"
"POP03_Artvin_Borcka"
"POP04_Artvin_Yusufeli"
"POP15_Artvin_Savsat"
}