COMPARATIVE SEQUENCE ANALYSIS OF THE INTERNAL TRANSCRIBED SPACER 2 REGION OF TURKISH RED PINE (*Pinus brutia* TEN.) AND NATURAL ALEPPO PINE (*Pinus halepensis* MILL.) POPULATIONS FROM TURKEY

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Approval of the Graduate School of Natural and Applied Sciences

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

COMPARATIVE SEQUENCE ANALYSIS OF THE INTERNAL TRANSCRIBED SPACER 2 REGION OF TURKISH RED PINE (*Pinus brutia* TEN.) AND NATURAL ALEPPO PINE (*Pinus halepensis* MILL.) POPULATIONS FROM TURKEY

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Turkish red pine (*Pinus brutia*) is wide-spread and an important forest tree species in Turkey, occurring mainly in southern, western and north-western Turkey and as small isolated populations in the Black Sea region. Aleppo pine (*Pinus halepensis)* has naturally found only in Adana and Muğla provinces as small population in mixture with Turkish red pine. Although Turkish red pine and Aleppo pine are morphologically different, Turkish red pine has been regarded as subspecies of Aleppo pine by some taxonomists due to occurrence of natural hybridization between these two species. However, the phylogenic relationship between these species needs to be explored further.

In the present study, by sampling overlapped populations of both species from Muğla and Adana provinces (4 populations of Turkish red pine and 3 populations of Aleppo pine), internal transcribed spacer (ITS) region of ribosomal DNA were comparatively studied with sequence analysis. Although ITS1, 5.8s and ITS-2 regions of ribosomal DNA were studied with ITS primers, only ITS-2 region was

successfully amplified with polymerase chain reaction (PCR). The complete data set for this region was analysed using MEGA3.1 and Arlequin softwares.

Analysis of molecular variance (AMOVA) demonstrated the highest genetic differentiation between Turkish red pine and Aleppo pine in Muğla with 100 percentage of variation. AMOVA analysis also indicated the possibility of low-level migration of genes between Turkish red pine and Aleppo pine populations in Adana with 50.65% of molecular variance. Haplotype comparison revealed that two major haplotypes were represented. One was detected in Aleppo pine samples, whereas the second was composed of samples that showed sequences and patterns of variability similar to those found in Turkish red pine. The significant genetic differentiation was detected between Aleppo pine and Turkish red pine populations in Muğla province. Also, the F_{st} values between Turkish red pine and Aleppo pine populations in Adana province were significant, but the lack of detectable differentiation between Turkish red pine population from Adana-pos-karsantı and Aleppo pine population from Adana kadirli-bahadırlı suggested the efficient amount of gene flow. Estimated sequence divergence values were low among closely related species Turkish red pine and Aleppo pine, revealing few fixed differences. In this study, phylogenetically informative characters were found in ITS-2, but the region was slightly variable. According to phylogenetic tree constructed in the analysis, the species were divided into two well-supported groups with a bootstrap value of 92%. Turkish red pine populations were grouped together in the same cluster, but apart from Aleppo pine group.

Since the beginning of botanical-genetical research within and among Mediterrenean pine species, there has been great interest in the relations between Turkish red pine and Aleppo pine. Based on the results of ITS-2 region sequence analysis, Turkish populations of Aleppo pine and Turkish red pine populations could not be fully differentiated. In Muğla province Turkish red pine and Aleppo pine revealed more differentiation due to reproductive isolation. But in Adana province, two species shared more common genetic background due to possible

hybridization. Since ITS-2 region of nuclear ribosomal DNA revealed a few variable and parsimony informative sites for both species, thus, only ITS-2 region of ribosomal DNA does not appear to be sufficient for fully resolving genetic relationships between Turkish red pine and Aleppo pine populations. Further studies including ITS-1 and 5.8s regions of ribosomal DNA and populations included from major Aleppo pine distribution areas will be useful to understand the evolutionary relationship between Aleppo pine and Turkish red pine populations in Turkey.

Key Words: Turkish red pine, Aleppo pine, ITS region, Phylogeny, DNA sequence analysis, AMOVA

ÖZ

TÜRKİYE KIZILÇAM (*Pinus brutia* TEN.) VE HALEP ÇAMI (*Pinus halepensis* MILL.) POPULASYONLARINDA ITS-2 BÖLGESİNİN KARŞILAŞTIRMALI DİZİ ANALİZİ

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Kızılçam, Türkiye'nin güney, batı ve kuzey-batı bölgelerinde geniş bir yayılış alanına sahip, önemli bir ağaç türüdür. Halep çamı ise sadece Adana ve Muğla yörelerinde küçük toplumlar halinde, kızılçam ile karışık olarak bulunmaktadır. Kızılçam ve halep çamı morfolojik açıdan farklı olmalarına rağmen, bu iki tür arasında ki doğal melezleme nedeniyle kızılçam, bazı taksonomistler tarafından halep çamının alttürü olarak kabul edilmiştir. Ancak bu iki tür arasındaki filogenetik ilişkinin daha kapsamlı olarak araştırılması gereklidir.

Bu çalışmada, Muğla ve Adana bölgelerinde bulunan kızılçam ve halep çamı toplumlarının (4 kızılçam populasyonu ve 3 halep çamı populasyonu) ribozomal DNA ITS-2 bölgesinin karşılaştırmalı olarak DNA sekans analizi yapılmıştır. ITS primerleri kullanılarak, ITS-1, 5.8S ve ITS-2 bölgeleri de çalışılmıştır. Ancak sadece ITS-2 bölgesi PCR ile başarılı bir şekilde çoğaltılabilmiştir. Tüm data seti MEGA3.1 ve Arlequin bilgisayar programları kullanılarak analiz edilmiştir.

Moleküler varyans (AMOVA) analizine göre; bu iki tür arasında en fazla genetik farklılık, Muğla bölgesine ait kızılçam ve halep çamı türleri arasında görülmüştür. Adana bölgesinde, bu iki tür arasında görülen %50.65 oranındaki moleküler varyasyon, bu bölgede bu iki tür arasında gen akışı olduğunu göstermektedir. Kızılçam ve halep çamı toplumları 2 haplotiple birbirlerinden ayrılmışlardır. İstatistiksel açıdan da anlamlı bulunan farklılıklar, Adana ve Muğla yörelerine ait kızılçam ve halep çamı toplumları arasında görülmüştür. Fakat Adana-pos-karsantı kızılçam toplumu ve Adana kadirli-bahadırlı halep çamı toplumu arasında farkın anlamlı bulunmaması, Adana bölgesinde bu iki türün melezleme yaptığına işarettir. Sekans farklılığını gösteren değerler düşük çıkmıştır ve bu iki türe ait toplumlar içindeki evrimsel ilişkiler çözülememiştir. Bu çalışmada, evrimsel açıdan informatif karakterler bulunmuştur ancak ITS-2 bölgesinde az miktarda varyasyon tesbit edilmiştir. Elde edilen filogenetik ağaçta, bu iki tür yüksek oranda güvenilirlik değeriyle (92%) 2 farklı grup olarak ayrılmışlardır. Ağaçta, Muğla ve Adana bölgelerine ait kızılçam toplumları bir dalı oluştururken; Muğla ve Adana bölgelerine ait halep çamı toplumları diğer dalı oluşturmuştur.

Botanik ve genetik alanlarında araştırmalar başladığından beri Akdenize özgü çam türleri arasında en çok ilgi çeken kızılçam ve halep çamı arasındaki ilişkidir. ITS-2 sekans analiz sonuçlarına göre Türkiye deki kızılçam ve halep çamı türleri tam olarak farklılık göstermemiştir. Kızılçam ve halep çamı türleri Muğla bölgesinde üreme bariyerleri nedeniyle daha fazla farklılık göstermiştir. Fakat bu iki tür Adana bölgesinde melezleme nedeniyle daha fazla genetik benzerlik taşımaktadır. Her iki tür için, ribozomal DNA ITS-2 bölgesinin az sayıda farklılık gösteren bilgi veren bölgeler taşıması, bu iki tür arasındaki genetik ilişki ve ayrımının daha iyi ortaya konulabilmesi için ITS-2 bölgesinin yeterli olmadığı görülmüştür.

ITS-1 ve 5.8S ribozomal DNA bölgelerini ve halep çamının ağırlıklı olarak yayılış gösterdiği bölgeleri de içine alan daha kapsamlı yeni çalışmalar, Türkiye deki kızılçam ve halep çamı türleri arasındaki evrimsel ilişkiyi çözmede yardımcı olacaktır.

Anahtar Kelimeler: Kızılçam, Halep çamı, ITS bölgesi, filogeni,DNA sekans analizi, AMOVA

to my mother and father…

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AMOVA	Analysis of Molecular variance
β-ME	Beta Mercaptoethanol
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribose triphosphate
EB	Elution buffer
EDTA	Ethylene Diamine Tetra Acetic Acid
ETS	External Transcribed Spacer
ETOH	Ethanol
ITS	Internal Transcribed Spacer Region
MEGA	Molecular Evolutionary Genetic Analysis
MST	Minimum Spanning Tree
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
nrDNA	Nuclear ribosomal DNA
NORs	Nuclear organizer regions
OTU	Operational Taxonomical Units
PCR	Polymerase Chain Reaction
SDS	Sodiumdodecylsulphate
TAE	Tris-acetate EDTA
TE	Tris EDTA Buffer

CHAPTER I

INTRODUCTION

1.1. Taxonomy of Turkish red pine and Aleppo pine

Pinus brutia and *Pinus halepensis* belong to Kingdom Plantae, Division Gymnospermae, Class Coniferae, Family Pinaceae, Genus Pinus L. *Pinus brutia* TEN. and *Pinus halepensis* MILL. are included in subsection *Sylvestres* of section *Pinus* (*Diploxylon*) (Critchfield and Little, 1966; Quezel, 2000). But Klaus (1989) placed them in sub-section *halepensis* of section *Pinus*. According to Quezel (2000), they belong to sub-genus *Pinus*, in the section *halepensis* and the sub-section group *halepensis* which is also accepted by (Boydak, 2006 and the reference there in). On the other hand, Frankis (1993) explained that *Pinus brutia* did not belong in section *Pinus* which differs markedly in cone, foliage and wood structure, and suggested a new classification.

Pinus brutia was first described by Tenore in Calabria-Italy and attributed to Brutium who thought that the occurence of the species in this region was native (Boydak, 2006 and the reference there in). But there are hesitations in regard of its distribution to be natural in Italy (Boydak, 2006 and the reference there in). According to Farjon (1984), it is also found in the Italian province of Calabria, but was probably imported there. *Pinus brutia* is also known by several other names, "Calabrian Pine" "Brutia pine" "East Mediterrenean Pine" and also "Turkish red pine". Aleppo pine is first described in Syria. It is closely related to Turkish red pine, Canary Island pine and Maritime pine, which all share many features with it.

Some authors include Turkish red pine as a subspecies of Aleppo pine, but it is usually regarded as distinct species. Aleppo pine is a relatively non-variable species, with constant morphology over the entire range.

1.1.1 Subspecies of Turkish Red Pine

As recently proposed, *P.brutia* Ten. is a complex composed of four subspecies, i.e., subsp. *brutia*, subsp. *elderica*, subsp. *pithyusa* and subsp. *stankewiczii* (Boydak, 2006 and the reference there in). Of these four species, subsp. *brutia* has the largest natural range and it occurs in the eastern Mediterrenean area, i.e., mainly in the eastern part of the Aegean region, on the Crete and Cyprus, and also sparsely along the shores of the Black Sea in Turkey, and in Syria, Lebanon and Iraq. It grows from sea level up to 1500 m in the Taurus Mountains in Turkey (Selik, 1958; Critchfieldt and Little, 1966; Mırov, 1967), under several variations of the Mediterrenean climate (Emberger *et al*., 1963), and on various bedrock formations and soils (Arbez, 1974). Recently, attention has been given to this sub-species, which is the most important forest tree species in the region, providing both timber resources and amenity, especially in Turkey, Cyprus and Crete. This tree can be used for afforestation of degraded areas in the Mediterrenean region and elswhere, where there are homologues climates, because of its drought resistance (Oppenheimer, 1967). Subsp. *pithyusa* occurs in relict stands on the Black Sea coasts of Caucasus range while subsp. *stankewiczii* in Crimea and subsp. *elderica* occurs in a very small area in Caucasus and its land races probably extend to part of Iran and possibly Afghanistan (Schiller, 2000a).

1.1.2 Varieties of Turkish Red Pine

There are four known varieties of *Pinus brutia* Ten. (Yaltırık and Efe, 1994, Boydak, 2005)

1) *P. brutia* Ten. var. *agrophiotii* Papaj : It has a spherical appearance with dense and compact needles. The dimensions of the cones, seeds, needles and weight per 1000 seeds are small (Yaltırık and Boydak, 1989). It occurs frequently at higher elevations.

2) *P.brutia* Ten. var. *pyramidalis* Selik : It has a pyramidal compact crown. The needles are dense and compact (Boydak, 2006 and the reference there in). It occurs often at lower elevations with warmer and drier climates.

3) *P.brutia* Ten. var. *densifolia* Yalt. and Boydak : The variety has a spherical or subspherical, dense and compact crown with stiff, vigorous leaves which have dark green colors. It occurs in south-eastern Anatolia and seems to be more drought tolerant than other varieties.

4) *P.brutia* Ten. var. *pendufolia* Frankis : Frankis (1993) explained that this variety differs from var. *brutia* in longer and pendulous leaves (18-29 cm). This pendulous shape of leaves is a peculiar character of *Pinus brutia.* Depending on the site productivity these needles can be smaller or longer . As it may be a variation as a result of a good site condition, it is advised that this subspecies should be considered as a variety with reserve.

1.2. Phylogeny of Turkish Red Pine and Aleppo pine

Extensive studies (Boydak, 2006 and the reference there in; Panetsos, 1981, 1986b) suggested that Aleppo and Turkish red pines should be considered as two well-established and independently evolved pine species with common origin from a primitive pine population which existed in northern Europe during Tertiary era.

From fossil record, it appears that Turkish red pine in Tertiary era had a larger distribution than today while Aleppo pine occupied the same region with a considerable northern distribution in latitude (Boydak, 2006 and the reference there in).

In the Tertiary era, a species resembling Aleppo pine was growing in what is now the Baltic Sea area (Boydak, 2006 and the reference there in). Fossil findings indicate the existence of a species resembling Turkish red pine and Aleppo pine in the central Europe in the middle Miocene era (Schiller 2000a; after Klaus). Allozyme (Conkle, Schiller, and Grunwald, 1988) and morphology (Frankis, 1993) studies have suggested that *P.halepensis* is derived from a *P.brutia*-like ancestor and that *P.brutia* has retained greater ancestral variation, showing affinities not only to *P.halepensis* but also to other Mediterrenean pines e.g., *P.pinaster* and *P.canariensis* (Frankis, 1993).

Fossils of Aleppo pine were found at some mine reservoirs in the Aegean Region-Turkey (Gemici *et al*., 1990; 1991). Surprisingly fossils of Turkish red pine were not found in the same places. But Turkish red pine fossils were discovered in Ağaçlı-İstanbul mine reservoir (Boydak, 2006 and the reference there in). Aleppo pine has natural distribution in Sarıçam Ormanı-Adana (Kayacık, 1954) and assumed Aleppo pine-Turkish red pine mixed forest in Yumurtalık-Dalyan-Adana (Yaltırık and Boydak, 1989) in the Mediterrenean Region of Turkey. In addition, some scattered small natural populations of *P.halepensis* occur in Çeşme, Urla, Güvercinlik and Gökova localities in the Aegean Region of Turkey (Boydak, 2006 and the reference there in). These natural distributions in the Aegean Region of Turkey and fossil findings in the same region mentioned above could be attributed to much wider occurence of *P.halepensis* in the past in the Aegean Region of Turkey (Boydak, 2006).

1.3. Natural and Artificial Hybridization of Turkish Red Pine and Aleppo pine

Turkish red pine has been regarded by some taxonomists as a variety of *Pinus halepensis* Mill. (*P. halepensis* var. *brutia*) (Duffield, 1952) because these two species hybridize naturally with each other. Several researchers have reported the existence of natural hybrids between the two species (Papajoannou, 1954; Panetsos, 1975, Yaltırık and Boydak, 1993). The species have developed several kinds of barriers such as spatial, elogical, seasonal, partial embryo and F1 sterility (Panetsos, 1981, 1986b). Despite these isolation mechanisms, they form natural hybrids when they come in contact (Panetsos, 1975). Hybrid identification and description can be possible using morphological and anatomical characteristics (Papajoannou, 1936; Panetsos, 1981, 1986). Cortical terpene characters can also be used successfully to identify hybrids between Turkish red pine and Aleppo pine (Gallis and Panetsos, 1997). Artificial hybridization has revealed that the two species can be successfully crossed and even that natural hybrids are formed in areas where the two species come in contact, due to human interference (Panetsos, 1975; Moulalis *et al*., 1976). According to the results obtained from the artificial crossings, there exists an overlapping of pollen shedding from the two species (Papajoannou, 1954) and the production of natural hybrids is to be expected whenever two species come in contact. Artificial crosses between the two species were performed in 1948 and in 1961. It was reported that crossings were successful when Turkish red pine was used as the female parent, but not reciprocally. Evaluation of F1 and advanced generation hybrids showed that F1 hybrids possess impressive hybrid vigor over parent tree species in growth and adaptation (Moulopoulos and Bassiotis, 1961).

Their superiority in growth varied from 5 % to 190 %. They can also resist freezing temperatures much better then parental species.

Advanced generation hybrids were always inferior in growth compared to F1 hybrids (Panetsos *et al*., 1983; Panetsos, 1986b; 1989; 1990).

1.4. Research results on relationship between Turkish red pine and Aleppo pine

Turkish red pine which was treated as a variety of Aleppo pine is at present considered to be a well-established species (Mırov, 1955). The same conclusion was made by (Boydak, 2006 and the reference there in) after morphological, geographical, biochemical, and ecological studies of the two species. These species systematically differ from each other according to the data obtained from biochemical markers (Mirov, 1953; 1961, Acar,1993), serotaxonomical methods (Prus-Glowacki *et al*., 1985; Schirone *et al*., 1991), analysis of chloroplast genome simple sequence repeats (cpSSR) (Bucci *et al*., 1998; Morgante *et al*., 1998) and from some anatomical and morphological characteristics (Boydak, 2006 and the reference there in; Vidakovic, 1991; Panetsos, 1981). Turkish red pine and Aleppo pine are considered as two distinct species based on palinological research (Boydak, 2006 and the reference there in), physical and chemical analysis of the gum terpentine as genetic markers (Mirov and lloff, 1955; Mirov 1953, 1955; 1961; Mirov *et al.,* 1966; Gallis and Panetsos, 1997) and isoenzyme analysis (Korol *et al*.,2002 b). Quantitative differences in the terpene composition can also be used to seperate the two species (Mırov *et al*., 1966; Schiller and Grunwald, 1987a; Gallis and Panetsos, 1997). Bud terpene analysis by headspace chromatography seems to be a valuable tool to seperate the taxa and to help to identify even hybrids of Aleppo pine and Turkish red pine (Gallis, Lang and Panetsos, 1998).

Electrophoretic analysis of diversity and phylogeny supported the hypothesis that Aleppo pine and Turkish red pine complex are different taxa, which probably evolved from a common progenitor species (Schiller, 1994; 2000a).

Morover, allozyme analysis (Schiller, 1994; 2000a after Conkle *et al*.), HPLC chromatography of needle flavanoids (Schiller, 2000), chemical analysis (Vildrich *et al*., 1993) and karyotype analysis (Schiller 2000a) indicate a highly significant divergence between Turkish red pine and Aleppo pine. In accordance with other genetic markers (Boydak, 2006 and the reference there in; Mirov *et al*., 1966, Conkle *et al*., 1988), the flavonoid patterns support the seperation of Aleppo pine and Turkish red pine as two independent species. Goncharenko *et al*.(1998) included *P. stankewiczii, P.pithyusa* and *P.elderica* as subspecies of Turkish red pine by using isozyme analysis of the megagametophytes.

1.5. Natural Distribution of Turkish red pine and Aleppo pine

Aleppo pine and Turkish red pine form a distinct group within the Eurasian hard pines and their combined geographic distribution reflects their prominence among low elevation Mediterrenean forest species (Panetsos, 1981). Aleppo pine forests cover extensive areas in the Western Mediterrenean: Spain, France, Italy, Croatia, Albania, Greece, Morocco, Algeria, Tunisia, Libya and Malta. A few natural and artificial populations can be found in the eastern Mediterrenean in Turkey, Syria, Israel, Jordan and Lebanon (Figure 1.2). Total forest cover is estimated to be approximately 3.5 million hectares (EUFORGEN Conifer Network). According to Panetsos (1975); Aleppo pine is widely distributed in the Mediterrenean region ranging from Morocco to the main land of Greece. It grows also on some small islands of the west Aegean Sea, while in Asia Minor, it is rarely found, only in two localities in Muğla and Adana (Figure 1.1). It is native in coastal region of Syria and is also found in Israel and Jordan.

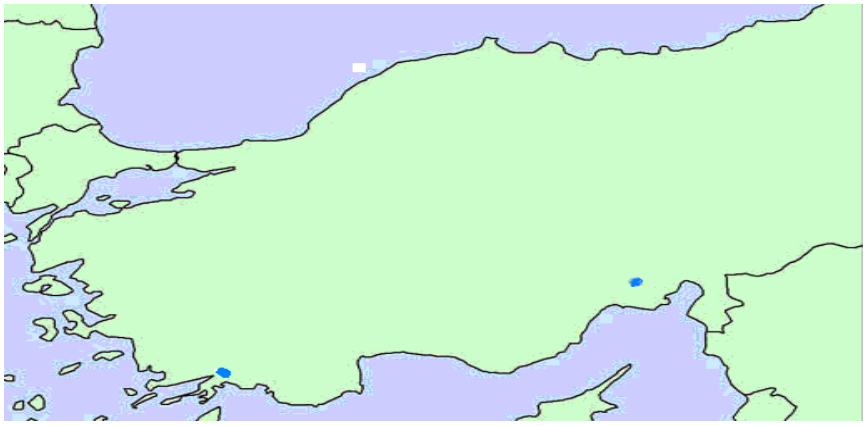


Figure 1.1. Natural Distribution of Aleppo pine in Turkey

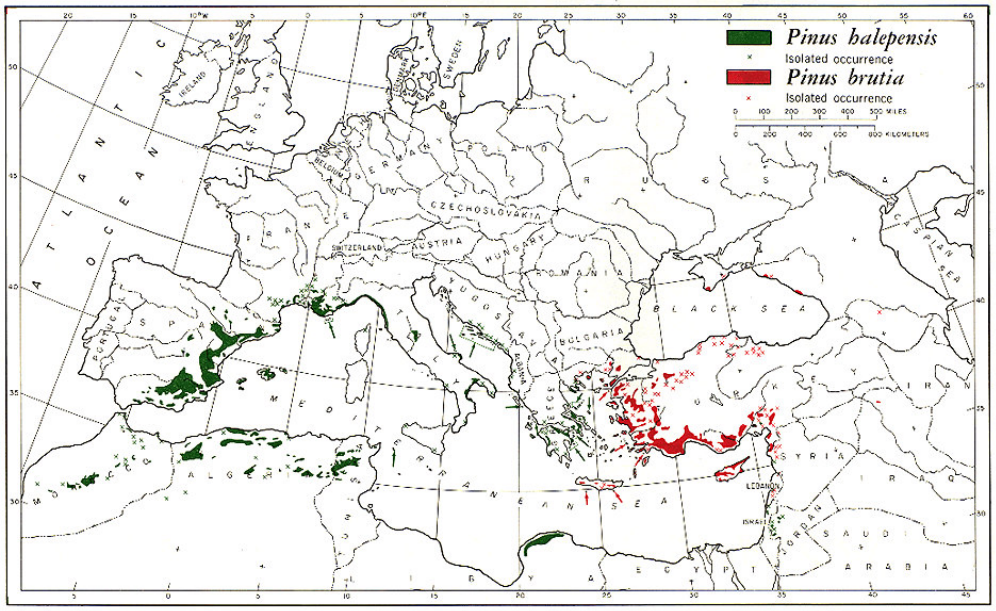


Figure 1.2. Distribution of Turkish red pine and Aleppo pine throughout the world. (Obtained from dendrome.ucdavis.edu)

The natural distribution of Turkish red pine generally occurs at eastern Mediterrenean region, but it is a typical tree species of Mediterrenean climate like Aleppo pine and olive tree. From these species, Turkish red pine distributes on eastern Mediterrenean while Aleppo pine on western Mediterrenean region. But there are some isolated stands or some mixed stands of Aleppo pine with Turkish red pine at the Aegean and Mediterrenean regions of Turkey (Kayacık, 1954; Yaltırık, 1993; Yaltırık and Boydak, 1989).

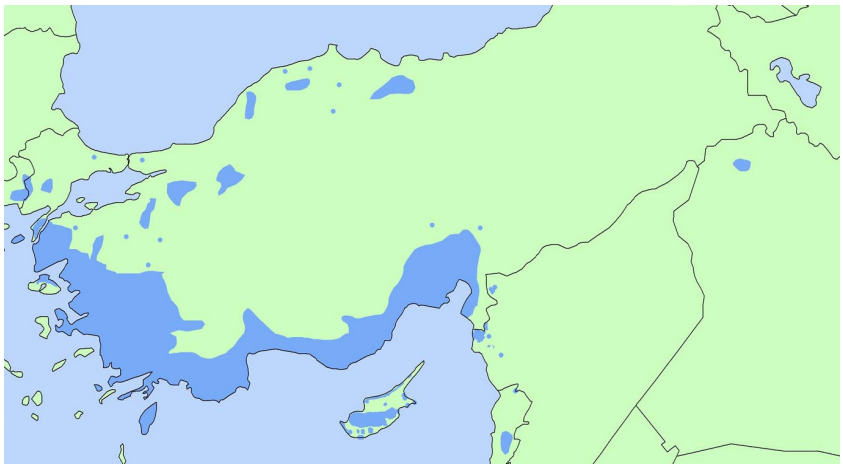


Figure 1.3. Natural Distribution of Turkish red pine in Turkey (Obtained from EUFORGEN Conifer Network)

In the world, Turkish red pine forests cover extensive areas in the Eastern Mediterrenean: Greece, Turkey, Crete, eastern Aegean islands, Crimea, Caucasus coast, Georgia, Azerbaijan, Lebanon and western Syria. A few small populations can be found in Iran and northern Iraq (Figure 1.2).

In Turkey, it is a natural found forest tree species which has the largest natural distribution area and covers about 4.2 million ha. (Anonymous, 2001a).

Turkish red pine is naturally distributed mainly in Mediterrenean, western and north-western parts of Turkey and in small isolated populations within the areas in the Black Sea Region with a micro climate similar to Mediterrenean climate (Figure 1.3) (Kayacık, 1954; Davis, 1965; Arbez, 1974; Atalay, 1982) There are four main distribution areas of Turkish red pine in Turkey;

1. Black Sea Region : Turkish red pine shows a scattered distribution beginning from Samsun-Çamgölü at Black Sea region, diffusing from coastal to interior along the valleys (for example; Kelkit Valley-Erbaa-Niksar-Koyunhisar; Kızılırmak-Gökırmak-Devrez; Yenice Brook; Sakarya Valley) and reaching up to 800-1000 m (Akıncı, 1963; Saatçioğlu, 1971, Atalay *et al*. 1998). In the Black Sea region, Turkish red pine forests are only seen within the Dikmen valley located in eastern part of Sinop Province.

2. Marmara Region : Turkish red pine stands, which are associated with rich maquis elements, are wide-spread at the low elevations of the southern Marmara Region. Mainly, it is found on the coasts of Marmara, being distributed on coasted Marmara, Princess island, Keşan, and Gallipdi at Thrace. About 10 % of Turkish red pine forests in Turkey grow in Marmara Region extensively in Gelibolu and Biga peninsula (Boydak, 2006)

3. Aegean Region : 40 % of Turkish red pine forests in Turkey are distributed in this region. The forests in Aegean Region generally occur at the low elevations, but most of which have been degraded. The areas where natural Turkish red pine stands were destroyed, were replaced by the garique and maquis vegetation. The distribution ranges from coast to 800-1000 m at western Anatolia (Çanakkale, İzmir, Aydın, Denizli, Balıkesir, Bursa, Uşak, Bilecik).

Approximately 200 km inland from the Aegean Coast, along the valley, (Gediz, Büyük Menderes, Küçük Menderes) and at the Lakes Region, it is also found naturally (Boydak, 2006).

4. Mediterrenean Region : The Mediterrenean region provides optimum growing areas for the Turkish red pine. About 47 % of Turkish red pine forests in Turkey are found in the Mediterrenean Region, mainly along the coastal zone. The Turkish red pine forests are distributed from the coast to 1500 m. Specially the slopes facing south of the Taurus Mountains and thus, receiving sea breezes forms a suitable habitat for the species. The species are also located in the vicinity of Lake Burdur, at an elevation of 900 m, along the valleys of Aksu, Seyhan and Ceyhan rivers, which are located in the interior parts of the Mediterrenean region. In south eastern Anatolia, the species has scattered distributions at Gaziantep, Adıyaman, and Siirt Provinces (Atalay *et al*., 1998; Kaya and Raynal, 2001).

1.6. Biology and ecology of Aleppo Pine

It is a a small to medium size tree, reaching 15-25 m in height and with a trunk diameter of up to 60 cm, exceptionally 1 m (Figure 1.4). The form of the trees is generally poor due to overexploitation of the species for a long time in the past. (Matziris, 1997). The crown of Aleppo pine changes its geometrical shape when the tree matures; from conical to cylindirical and to hemispherical. The bark is light gray and smooth when young, turning reddish brown and furrowed, with rounded scaly ridges. It is thick and deeply fissured at the base of the trunk, and thin and flaky in the upper crown. Evergreen needles are 5 to 10 cm long in fascicles of 2, thin, straight and have yellowish green persistent fascicle sheath.The cones are narrow conic, stalked, unarmed, 5-10 cm long and 2-3 cm broad at the base when closed, green at first, ripening glossy red-brown when 24 months old (Figure 1.5).

They open slowly over the next few years, or after being heated by a forest fire, to release the seeds, opening to 5-8 cm broad. Aleppo pine produces female cone and male strobili. Male strobili are produced in clusters of 4 to 40 strobili and are located on the apex of lateral shoots. Female cones are contained from up to 100 scales, each containing two ovules. They can occupy different positions on the main shoot, either apical or more basal (Mirov, 1967). Female cones appear early in the spring (February) and are pollinated between March and April. Female cone production starts at the age of 3-4 years, while male strobili production occurs later on (Thanos, 2000). Small trees can therefore be either female or bisexual, whereas mature trees are all bisexual. Pure male trees are usually rare and are often related to poor growth conditions (Schmida *et al*., 2000).

Figure 1.4. A view of Aleppo pine tree in Italy (Photo is obtained from www.meditflora.com/flora/pini.htm)

Altough pollination starts between March and April, fertilization takes place much later in February of the following year. Cone growth and maturation set in after fertilization and continue till June of the subsequent year, more than 2 years after cone emergence (Panetsos, 1981). The twig is modaretely stout, ash-gray to gray-brown. The flowers are monoecious; males are cylindrical, in tight cluster at branch tips; females are small, reddish purple with loose scales at branch tips. The seeds are 5-6 mm long, with a 20 mm wing, and are wind-dispersed (Figure 1.6). The species is extremely prolific seed disperser and can colonize open and disturbed areas easily.

Aleppo pine can grow on all substrates and almost all climates of the Mediterrenean region. The range of Aleppo pine include arid and semi-arid climates, whereas Turkish red pine prefers the more humid climate (Quezel, 2000). Aleppo pine can be found at altitudes of 0-600 m in the northern Mediterrenean and 0-1400 m in the southern Mediterrenean. However, it can reach higher altitudes, e.g. 2600 m in the Atlas Mountains of Morocco. Optimal development of Aleppo pine forests occur at annual rainfalls of 350-700 mm and absolute mean minimum temperatures between -2 and +10˚C (semi-arid and sub-humid bioclimates). Aleppo pine grows on a variety of forest sites and tolerates different substratums. However, it prefers mostly limestone, both marl and chalk (Schiller *et al.*,1981). A distict relation between the nature of the bed rock and growth of the species exists. The growth is superior on marl and chalk compared to that on dolomite or limestone. This behaviour was attributed to differences in moisture retention capacity by the different substratums. It tolarates high content of free carbonate in the soil and avoids heavy clay soils with poor drainage (Panetsos, 1981). It prefers light (sandy) and medium (loamy) soils, requires well-drained, nutritionally poor, as well as acid, neutral and basic (alkaline) soils. It requires dry or moist soil and can tolerate drought and strong winds. Aleppo pine demonstrates a higher resistance to environmental stresses, such as drought and high temperature than Turkish red pine (Schiller, 2000).

The species is very well adapted to dry mild Mediterrenean ecosystem and it is better adapted to drought but less adapted to cold than Turkish red pine. Aleppo pine does not have fire adaptive traits such as thick bark and resprouting ability (Keely and Zedler, 1998). Aleppo pine is a post-fire obligate seeder and is partially serotinous. This pine is a successful post-fire regenerator (Saracino *et al*., 1997; Arianoutsou and Ne'eman, 2000) as well as a successful colonizer of disturbed areas (Lepart and Debussche, 1991; Trabaud, 1987,1993). Partially serotinous species, like Aleppo pine, divide the available seed pool in two parts: a seed pool which disperses after maturation and a seed pool which is stored in the canopy and disperses after fire (serotiny). The degree of the serotiny is positively related to fire frequency (Gauthier *et al*., 1996; Enright *et al*, 1998). In absence of fire, seeds from non-serotineous cones disperse over summer during hot dry winds (Nathan *et al*., 1999). Some of the serotineous cones open eventually in absence of fire, under the dry weather conditions (Nathan *et al*, 1999). If a tree flowers at an early age (3rd year), it produces a large number of serotineous cones which persist for many years on the crown of the trees. After fires, the cones open, releasing viable seeds which germinate promptly in the favourable environment, making natural regeneration succesfull. (Matziris, 1997). Reproductive succes is dependent on several inherent characteristics which includes cone production, pollination, abortion, seed dispersal as well as the level of serotiny.

Figure 1.5. Photo showing the cones of Turkish red pine (left) and Aleppo pine (right) (Photo is obtained from www.agaclar.net)

Figure 1.6. Photo showing the seeds of Turkish red pine (left) and Aleppo pine (right)

1.7. Biology and ecology of Turkish red pine

The Turkish red pine is a tree to 27-35 m in height, with a usually open crown of irregular branches and with a trunk diameter of up to 1 m (Figure 1.7).

The bark on the lower trunk is thick, scaly, fissured, patterened red-brown and buff, and thin, flaky, and orange-red higher in the crown.

The shoots are slender, 3-7 mm thick, grey-buff, and rough with persistent small decurrent scale-leaf bases. The winter buds are ovoid-acute, with red-brown scales with long free tips revolute and frined with white hairs.

Figure 1.7. A view of Turkish red pine tree and stand in Turkey (Photograph is the coustesy of Michael Frankis)

The adult leaves are retained for 1.5-2.5 years, with a persistent 1-1.5 cm sheath; on most trees they are in fascicles of two, and 10-18 cm long. They are bright green to yellow-green, slender, about 1 mm thick, with serrulate margins, fine lines of stomata on both faces, and several marginal resin canals. The juvenile leaves are glaucous, 1.5-4 cm long, and continue to be grown for 2-4 years, mixed with the first adult foliage produced from 9 months from seed. The cones are erect to forward pointing on short stout stalks, symmetrical, broad conic, (4-)6-10(-12) cm long (Figure 1.5). 4-5 cm broad when closed, green, ripening shiny red-brown in April two years after pollination.

The cones open the same summer or 1-2 years later, to 5-8 cm broad, though the seeds are often not shed till winter rain softens the scales. The scales are short, broad, thick, woody and very stiff; the apophysis is 10-15 ×15-20 mm, smoothly rounded, with a slight to moderate transverse ridge; the umbo is dorsal, flat to slightly raised, 5-7mm wide, and grey-buff. The seeds are grey-brown, 7-8×5 mm with a broad, auricled 15-20 × 10 mm wing, yellow-buff streaked darker brown (Figure 1.6) (Frankis, 1993).

Turkish red pine is wind-pollinated and allagamous. Male and female flowers are located on different parts of the tree. Like Aleppo pine, it is a prolific seed disperser and can colonize open and disturbed areas easily. According to Zohary (1973), Turkish red pine 'possesses a rather wide range of ecological requirements' and is a highly invasive species. The species grows on much different geological formations and in areas with quite different rain fall and climatic variations (Arbez, 1971; Panetsos, 1981). Within its area of distribution, Turkish red pine grows under various ecological conditions, resulting from topography and climatic conditions (Emberger *et al*., 1963), bedrock formations and a wide variety of soil types, which are the weathering

products of schist, serpentine, sandstone, dolomite, chalk and limestone, marl, loess (Zohary, 1962; 1973; Laban, 1972; Lise, 2000 and the reference there in). It is confined to various silicious and calcareous soils in Anatolia, igneous rocks in the Trodos mountains in Cyprus, while in south Lebanon it occurs on Jurassic and Aptial strata (Feinbrun, 1959), and on very calcareous sites (Lise, 2000 and the reference there in). Turkish red pine can be found at altitudes of 0-600 m in the Aegean Region and 0-1400 m in the Mediterrenean Region. Its occurence can reach at higher altitudes, e.g 1650 m in the Taurus Mountains of Turkey. Optimal development of Turkish red pine forests require higher rainfalls, but they accept a wider range of temperatures (absolute mean minimum temperatures between -5 and +10˚C, sub-humid and humid climates) (EUFORGEN Conifer Network).

1.8. Importance and Use of Turkish Red Pine and Aleppo pine

The pine and mixed pine forests of the Mediterrenean basin are extremely important ecosystems. They provide the last refuge for many Mediterrenean plants and animals, which are areas of cultural wealth and scenic beauty, and are important environmental buffers for the densely populated lowlands. Aleppo and Turkish red pines represent the only or main source of wood and forest cover in many Mediterrenean countries. Mediterrenean pines are used for many purposes: construction, pulp, paper and furniture industry, and also they are used as package and fuel. They are planted in sand dunes for stabilization and used in shelterbelt planting. Economically, Turkish red pine is the most important conifer species in Turkey; Aleppo pine is the most important forest species of North Africa, and has high ecological importance in southern France and Italy. For both species, resin and terpentine that are obtained from the bark are very important. Resin is a hydrocarbon secretion that is obtained by making incisions in bark or wood or extracted by leaching

the tissues with alcohol. Resin seals the plant's wounds, kills insects and fungi, allows plant to eliminate excess metabolites and used as varnish and adhesive. There are information (Hillis, 1987) that resin was used in the Mediterrenean region as far back as 3000 years ago, mainly for water proofing and various pharmaceutical purposes. Resin yield is a highly inherited characteristic (Moulalis, 1991). Range of yield among Aleppo trees from 1 kg to 6 kg per year with an average of 2.7 kg have been reported (Geogoulis, 1964). Terpentine is obtained by distillation from resin. It is used medically and also used as solvent in industry.

Turkish red pine is considered a fast growing conifer when compared to other native forest tree species in Turkey (Işık *et al*., 1987), therefore it is an important timber source for the country. This tree can be used for afforestation of degraded areas in the Mediterrenean region and elsewhere, where there are homologues climates, because of its drought resistance (Oppenheimer, 1967). Thus, there is an urgent need to understand the nature and scale of diversity exhibited by the species, in order to aid the selection of suitable seed sources. The species has taken a considerable interest and introduced to several countries in the Mediterrenean region and to several overseas countries such as Australia and Mexico (Palmberg, 1976; Fischer *et al*., 1986; Weinstein 1989a; Weinstein 1989b). The distribution of Turkish red pine in the eastern Mediterrenean basin and its ability to grow in adverse climatic and soil conditions make this species be very important for multiple purpose forestry. Aleppo pine is widely planted for timber in its native area, being one of the most important trees in forestry in Algeria and Morocco. It is also a popular ornamental tree, extensively planted in parks and gardens in hot dry areas such as southern California in the United States, where heat and drought tolerance of plants is highly valued.

1.9. Genetic Diversity of Turkish red pine and Aleppo pine

The maintenance of genetic diversity throughout a tree-breeding operation is of vital importance and it is essential for the effective management of a species. Genetic variation in natural populations is a resource for the survival and future evolution of a species, as well as potential resource for improving its productivity (Frankel *et al*., 1995). For the evaluation of the genetic diversity among populations of Aleppo pine, some studies used biochemical traits as genetic markers, such as resin monoterpene composition analyzed with the gas chromatography technique (e.g., Schiller and Grunwald, 1986, 1987; Baradat *et al*., 1989, 1995). High levels of genetic diversity among circum-Mediterrenean populations of Aleppo pine was revealed by means of the chloroplast microsatellites technique (Bucci *et al*., 1998; Morgante *et al*., 1998; Vendramin *et al*., 1998). Many studies, based on the relatively sparse seed collections which covered the geographically wide range of Turkish red pine (Anonymous, 1973; Arbez, 1974), used morphological, anatomical and biochemical traits to determine the extant of intra and interpopulation genetic diversity. These studies established the existence of altitudinal zonation within the wide geographic range of this species, in allele frequencies (Conkle *et al*., 1988), in cortex and needle resin composition (Schiller and Grunwald, 1987; Schiller and Genizi, 1993), and in morphological and anatomical needle characters, resistance of seeds to water stress, and shoot morphology (Calamassi *et al*., 1980a and b, 1988; Calamassi, 1982). Later studies by Işık (1993), Işık and Kaya (1993), and Yahyaoğlu *et al*. (1993) yielded further evidence of higher intra than interpopulation genetic variability. Also high genetic variation between and within Turkish red pine populations has been observed previously by Işık (1986), and Kaya and Işık (1997) on various seedling traits, biomass traits; and by Yıldırım (1992) on shoot growth patterns. Genetic diversity in Turkish red pine populations from Mediterrenean region of Turkey was studied by using RAPD (Lise, 2000) and SSR (Ersöz, 2001). Estimation of heterozygosity in both studies indicated that Turkish red pine

exhibits high levels of genetic differentiation and highest proportion of genetic diversity was found within population. Understanding the nature and scale of diversity exhibited by Turkish red pine is important in order to aid selection of suitable seed sources and for the success of tree improvement programs at the early stages (Anonymous, 1989).

IUFRO-FAO project on Mediterrenean pine species was the basis of genetic diversity analysis, done by means of the isoenzyme starch gel electrophoresis technique (Conkle *et al*., 1982), among 19 circum-Mediterrenean populations of Aleppo pine and 10 Turkish red pine populations.(Schiller *et al*., 1986; Conkle *et al*., 1988). Subsequent studies that utilized the same technique concerned themselves only with the regional distribution of the genetic diversity in Aleppo and Turkish red pines (e.g., Grunwald *et al*., 1986; Teisseire *et al*., 1995; Agundez *et al*, 1999; Korol and Schiller 1996; Puglisi *et al*., 1999; Kara *et al*., 1997).

In situ gene conservation networks specifically designed for the target species, forest reserves or national parks including the target species, and *ex situ* measures including clonal archieves, cold storage seed banks, DNA banks; are the most commonly used conservation measures at national level. In Turkey, in recently prepared the *National Plan for in situ Gene Conservation of Plant Genetic Diversity in Turkey* (Kaya *et al*., 1997), Turkish red pine has been determined as one of the priority species in which *in situ* gene conservation areas needed to be set aside soon in addition to the existing ones. A concerted management effort should be carried out range wide to increase the efficiency of *in situ* genetic resource conservation. Transfer of seed material should be avoided across zones and countries with different ecological requirements, notably because of cold, drought and insect damage risks. Locally, some populations require specific attention and appropriate forestry practise.

Marginal populations as populations at high altitudes, in desert margins and mixed forests may contain valuable genes (resistance to drought, cold, pests) for adaptation under global warming. Efforts such as gene reserves should be made to conserve them.

Populations under recurrent forest fires also require specific attention. Aleppo pine and Turkish red pine usually regenerate well after fire, using the seed bank released from serotinous cones. If the seed production happens to be poor in the 2 years after fire, and if only a few isolated seed trees remain in the burnt area, artificial regeneration should be used to counteract the risk of genetic erosion in regenerated threats. In this case, lots collected from large gene pools should be used. Also, populations where hybridization may occur should be under consideration. Planting Aleppo pine where Turkish red pine is present should be avoided in areas where frost and potential pest damage are limiting factors, or strictly monitored in areas where drought is the limiting factor. Owing to the anisotropy of between-species gene flow, the impact should be reduced when planting Turkish red pine in the vicinity of Aleppo pine forests (Euforgene Conifer Network).

1.10. Threats to Genetic Diversity of Turkish Red Pine and Aleppo pine

Insects such as *Thaumatopea pityocampa* can induce severe defoliation throughout the distribution area of both pines altough it does not often lead to mortality. Aleppo pine has an increased sensitivity to the fungus *Sphaeopsis sapinea* under severe water-stress conditions. Aleppo pine is also sensitive to the pine best scale *Matsococcus josephii*, but Turkish red pine is resistant to it. Recently, the canker *Crumenulopsis sororia* has started to cause severe defoliation and dieback on Aleppo pine in France (EUFORGEN Conifer Network).

The effect of forest fires is ambivalent. The fire frequency has increased significantly in the 20th century in the whole Mediterrenean basin. In some areas, the fire return interval may be less than 5 years (Trabaud *et al*., 1993). These fires cause major damage to forest stands in the region, but result in expansion of pine forests (Quezel, 2000). Although the Mediterrenean basin inhabits about ten pine species, the most common species are Aleppo pine and Turkish red pine (Barbero *et al*., 1998). Stands of these pines have major ecological and economical values as they cover about 7.5 million hectares (Quezel, 1985) and are main landscape features in forested regions (Quezel, 2000). Although fires actually promote regeneration, they could be responsible for rare allele changes over generations, explained by the very low diversity found in Aleppo pine and promote the spreading of Aleppo pine genes into Turkish red pine forests. Among–region seed transfers have led to significant frost or water-stress damage occurences after planting when ill-adapted material was used. One of the risks is reducing local population adaptability through gene flow from plantations. Both species are often the last forest species to be found at desert or steppe margins. Global warming and its collateral modification of rainfall regimes may dramatically modify the distribution ranges of these species (EUFORGEN Conifer Network).

Human influences also must be considered, especially in Turkey and the near East, where ancient civilizations have had considerable impact on the landscape. There are many factors, which have caused the loss or decline of forest genetic diversity as well as resulted in habitat alteration or loss in Turkey. Some of these factors, such as agricultural activities, industrialization and urbanization, touristic developments, unregulated use of plant materials, forest fires and forestry activities and environmental pollution are still a threat to forest genetic resources.

Frequent forest fires, changes in land use and grazing occured in the past have narrowed the original distribution of the Turkish red pine in Turkey (Kaya and Işık, 1997; Özer, 1997; Muthoo, 1997; M'Hirit, 1999, Kaya and Raynal, 2001).

These anthropogenic effects are also most likely impacted the genetic diversity of Turkish red pine populations, however, the magnitude of the impact is unknown (Kaya and Işık, 1997). Today, there are large areas of degraded forest lands present in Turkey that could be reforested with Turkish red pine.

1.11. Internal transcribed spacers (ITS) and Use in Taxonomic Studies

Nuclear ribosomal DNA (nrDNA) has two internal transcribed spacers. The first (ITS-1) is located between the small subunit (16s-18s) and 5.8S rRNA cistronic regions, and the second (ITS-2) is located between the 5.8S and large subunit (23S-28S) rRNA cistronic regions. The two spacers and the 5.8S subunit are collectively known as the internal transcribed spacer (ITS) region and have become an important nuclear locus for molecular systematic investigations (Baldwin *et al*., 1995) of closely related taxa because the ITS regions evolve much more rapidly than other conserved regions of the rDNA. Thus, the sequence of the ITS regions may vary among species within a genus or even among populations (Bridge and Arora, 1998).

Since ITS region is highly conserved intraspecifically, but variable between different species, it is often used in taxonomy (Bruns *et al*., 1991; Hillis and Dixon, 1991). Phylogenetic studies based on nrDNA ITS sequences have provide novel insights into plant evolution and hybridization (Baldwin *et al*., 1995; Sang, Crawford, and Stuessy, 1995; Wendel, Schnabel, and Seelanan, 1995; Buckler and Holtsford, 1996a).

nrDNA is phylogenetically useful in part because of sequence homogeneity among repeats within the same species (Hillis and Dixon, 1991; Baldwin *et al*., 1995). This homogeneity is attributed to concerted evolution, a process that leads to greater similarity among members of a repeated family within a species than among species (Dover, 1982; Arnheim, 1983). The popularity of the ITS region can be attributed to the relatively high rate of nucleotide substitution in the transcribed spacers, permitting the systematic comparison of relatively recently diverged taxa. In addition, the ITS region can be readily PCR-amplified and sequenced with conserved primers positioned in the cistronic regions.

There are thousands of copies of rDNA cistrons in the plant nuclear genome, these are arranged in tandem repeats distributed at one to several chromosomal loci, the nuclear organizer regions (NORs) (Hamby and Zimmer, 1992). This repeated gene family undergoes rapid concerted evolution via unequal crossing over and gene conversion (Arnheim *et al*., 1980; Hillis *et al*., 1991; Wendel *et al.*, 1995). As a consequence, the ITS region is generally homogeneous, or nearly so, within a genome (Karvonen and Savolainen, 1993; Suh *et al*., 1993), and a single ITS sequence can characterize an individual. Furthermore, concerted evolution and sexual recombination tend to promote ITS region uniformity within interbreeding populations (Soltis and Kuzoff, 1993). The effectiveness of concerted evolution on the ITS region in most plants makes it a primary choice for phylogeny reconstruction at lower taxonomic levels (Baldwin *et al*., 1995). In particular levels of variation in the ITS-1 and ITS-2 generally allow resolution of interspecific and intergeneric relationships (Figure 1.8).

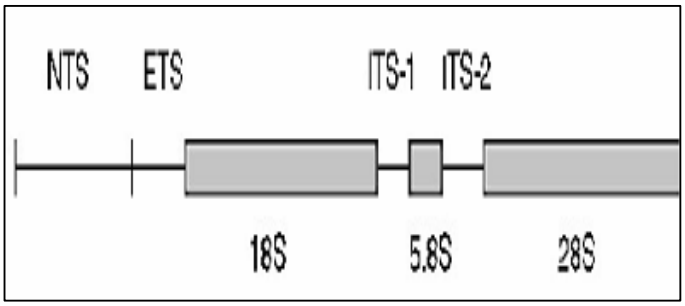


Figure.1.8. The map of the rDNA (NTS: non-transcribed spacer, ETS: external transcribed spacer, ITS: ınternal transcribed spacer region, 18S: small subunit, 5.8S: rRNA cistronic regions, 28S: large subunit)

A limitation of the ITS region in most plants is that it provides a relatively small amount of data, as the 5.8S rDNA is highly coserved and the two spacers total only ca. 400-535 bp in length. In angiosperms, the ITS region varies from ca. 565-700 bp in length (Baldwin *et al*., 1995). The ITS region of some non-flowering seed plants has been shown previously to be longer than the typical angiosperm ITS. The ITS region is exceptionally long in some species of Pinaceae. For example, it ranges from approximately 1550 bp in *Pseudotsuga* to between 3150 and 3660 bp in *Picea* (Liston *et al*., 1996; Germano and Klein, 1999; Maggini, 2000).

Pinus is exceptional among land plants in possessing a ca. 3000-bp ITS region (ITS-1+5.8S+ITS-2) (Liston *et al*., 1996; Marrocco *et al*., 1996). Karvonen *et al.* (1993) determined the ITS region to be ca. 3000 bp. in length in *Pinus sylvestris* L. A 3037 bp ITS region of *Pinus pinea* from a genomic library was reported (Qu *et al*., 1993; Marrocco *et al*., 1996). Thus, the sequence of a longer ITS region can provide more information for comparative systematic studies.

CHAPTER II

JUSTIFICATION OF THE STUDY

In Turkey, Turkish red pine (*P.brutia)* is a naturally occuring forest tree species, which has the largest natural distribution area and covers about 4.2 million ha; (Anonymous, 2001a). Turkish red pine is naturally distributed mainly in Mediterrenean, western and north-western parts of Turkey and in small isolated populations within the areas in the Black Sea Region with a micro climate similar to Mediterrenean climate

Aleppo pine in Turkey has very limited distribution**.** A few natural and artificial Aleppo pine populations can be found in the Mediterrenean region in Turkey. The natural distribution of Turkish red pine generally occurs in the eastern Mediterrenean region, but it is a typical tree species of Mediterrenean climate like Aleppo pine and olive tree. Off these species, Turkish red pine is found in eastern Mediterrenean, mainly in Turkey while Aleppo pine is naturaly found in western Mediterrenean region. However, there are some isolated pine stands or mixed stands of Aleppo pine with Turkish red pine in the Aegean and Mediterrenean regions of Turkey.

At large scale, the relationship between two pine species has been extensively studied by using morphological, anatomical, ecological traits, biochemical, chemical and molecular markers, karyotype analysis and sexual hybridization. Most of these studies indicated the divergence between Turkish red pine and Aleppo pine. But genetic and evolutionary relationships between naturally

occuring Aleppo pine population and natural Turkish red pine populations in the same localities have not been investigated yet. In the light of this study, taxonomic and evolutionary relationship existing between these species in Turkey could be further elaborated. This kind of information could be also a reference for future studies dealing with evolutionary relationship between these two Mediterrenean pine species.

CHAPTER III

OBJECTIVES OF THE STUDY

The general objective of this study was to reveal genetic and evolutionary relationship between natural Aleppo pine (*Pinus halepensis*) and Turkish red pine (*Pinus brutia)* populations by limiting the sampling to Adana and Muğla provinces where Aleppo pine is naturaly found, with a comparative study on nrDNA ITS region of the species with DNA sequencing analysis.

Specifically the following objectives were also set for the study:

1. To compare sequence divergence of nrDNA ITS-2 region in both pine species.

2. To determine the levels of genetic differentiation and estimate the amount of gene flow occuring among Turkish red pine and Aleppo pine populations with respect to ITS-2 sequence data.

3. To reevaluate the molecular systematics of Turkish red pine and Aleppo pine in the light of nrDNA ITS-2 sequence analysis.

CHAPTER IV

MATERIAL AND METHODS

4.1. Plant Material

Seeds from 4 populations of Turkish red pine and 3 populations of Aleppo pine were obtained from Muğla and Adana Provinces with collaboration of Turkish Forestry Tree Seeds and Tree Breeding Research Directorate, Ankara, detailed description of Turkish red pine and Aleppo pine populations used in the study was provided in Table 4.1.

Table 4.1 Detailed description of Turkish red pine and Aleppo pine populations used in the study.

Species	Regional Directorate of Forest	Forest Management Directorate	Forestry Unit	Lattitude	Longitude	Altitude (m)
Aleppo pine	Adana	Kadirli	Bahadırlı	37˚ 32' 30	35˚ 23' 00	745
Turkish Red pine	Adana	Pos	Karsantı	37˚ 34' 30	35˚ 24' 00	735
Turkish Red pine	Adana	Pos	Soğukoluk	37 ˚35 '30	35 ˚21 10	735
Aleppo pine	Muğla	Ula	Kızılyaka	37˚ 01' 45	28˚ 06' 25	50
Turkish Red pine	Muğla	Ula	Kızılyaka	37˚ 05' 33	2˚8 32' 22	680

4.2. Storage of Seeds

Seeds were kept in cold (+4˚C) storage until they were used for DNA extraction.

4.3. Chemicals

The chemicals used in this study and their suppliers were listed in Appendix A.

4.4. Methods

4.4.1. DNA isolation

For each of 7 populations of Turkish red pine and Aleppo pine, megagametophyte tissues from at least 36 seeds were used for DNA extraction. A combination of methods from Dellaporta *et al*. (1983) and Kreike (1990) were adopted (Lise, 2000) to our laboratory conditions and used as the DNA extraction method.

Seeds were soaked in distilled water at 4 ˚C for 24 hrs before excising and removing the seed embryo. The excised megagametophyte was homogenized in 400 µl of extraction buffer (1 M Tris (Base) pH:8.0, 0.5 M EDTA, 5 M NaCl, 10 mM β-ME) in 1.5 ml Eppendorf tubes and the mixture was ground with a closely fitting and rotating pestle. The buffer contained mercaptoethanol to prevent protein contamination and NaCl to aid in disasociating proteins from DNA. 400 µl of additional extraction buffer containing 2% SDS (Sodiumdodecylsulfate) was added and the mixture was incubated at 65 ˚C for at least 30 minutes. The duration of incubation is very important, as the duration gets longer, the isolated DNA becomes purer, because heat increases solibilization of lipids and aid protein dissociation from DNA. 2% SDS was used to solubilize plant membranes.

Then, 250 µl of 5 M potassium acetate was added to settle the DNA down, mixed and incubated on ice for at least 30 minutes. Then the mixture was centrifuged for 15 minutes at 0 ˚C at 13000 rpm. The supernatant was transferred to a new tube and mixed with 500 µl chloroform:octanol (24:1). Chloroform was used instead of phenol to denature proteins and inactivate DNase, since phenol has inhibitory effect on polymerase chain reaction (PCR) reaction. The mixture was spinned at 0 ˚C for 10 minutes at 13000 rpm and the top aqueous layer was transferred to a new tube. Afterwards 800 µl of absolute ethanol/0.3 M sodium acetate mixture was added to precipitate DNA and the mixture was incubated at -20 ˚C for at least 40 minutes. However, overnight incubations were found to yield higher DNA concentrations. The mixture was spinned at 0 ˚C for 15 minutes at 13000 rpm. Supernatant was poured off and the pellet was washed twice with 400 µl of 70% EtOH. The pellet was dried in laminar flow hood and then resuspended in 50 µl Tris EDTA (Ethylenediaminetetraaceticacid disodium salt) (1 M Tris pH,8.0, 0.5 M EDTA), the DNA samples were stored at -20 ˚C until they were used for PCR.

4.4.2 DNA Quantification

DNA quantification of all samples was performed with Hoefer DyNA QuantTM 200 Fluorometer which is a filter fluorescence photometer with a fixed excitation band pass source (365 nm) and an emission bandpass filter (460 nm). Bisbenzimide, commonly known as Hoechst 33258 (H 33258) dye, exhibits changes in fluorescence characteristics in the presence of DNA that allow accurate DNA quantification. The determination of DNA concentration of all isolated DNA samples was done by using the fluorometric assay of Cesarone *et al*. (1979). Fluorometer was zeroed by using 2 ml of assay solution. Then, the instrument was calibrated to 100 ng/ µl by using DNA standart solution. DNA concentration of samples was determined by mixing 2 µl of DNA sample with 2 ml of assay solution.

DNA yields per megagametophyte varied from 1 ng/ µl to 106 ng/ µl (Table 4.2). The highest DNA yield was selected and diluted to 3 ng/ µl for PCR applications and data colection. Diluted samples were stored at -4 ˚C throughout the course of the study.

Table 4.2 Average with standard deviation, maximum and minimum DNA concentrations of Aleppo pine and Turkish red pine populations

Species	Population	Average ± SD (ng/µl)	Minimum (ng/µl)	Maximum (ng/µl)
Aleppo pine	Kadirli-Bahadırlı	9.44±4.04	1	18
Aleppo pine	Ula-Kızılyaka	10.83±5.49	1	27
Aleppo pine Average		10.14±0.98	1	27
T. red pine	Pos-Karsantı	15.38±6.43	4	30
T. red pine	Pos-Soğukoluk	24.80±23.74	2	106
T. red pine	Ula-Kızılyaka	20.73±10.51	6	57
T. red pine Average		20.30±4.72	2	106
Overall Mean		**16.236±6.51**	**1**	**106**

4.4.3 Polymerase Chain Reaction (PCR)

The specifically designed primers for PCR were used to amplify internal transcribed spacers (ITS-1 and ITS-2) of Turkish red pine and Aleppo pine.

For the ITS-1 region, primers ITS Plant1 (forward primer) and ITS-Gym2 (reverse primer) were used. For the ITS-2 region, the primers ITS-Plant4B (forward primer) and ITS-Gym3 (reverse primer) were utilized. These primers were designed by Rogers and Kaya (2006). Also, for the ITS-1 region forward primer ITS105 (GAAGTTGTGTCATCCTTTGC) and reverse primers ITS594 (CAGGAGACCCTTCTTTGTAG) and ITS1467 (CTTCAATGCTCCGATGGCC) (White *et al*., 1990) were tested for PCR amplifications and these primers were previously screened for *Picea* and amplifies successfuly (Campbell *et al*., 2005). The list of the primers and their sequences were provided in Table 4.3.

Table 4.3 The list of ITS Primers used in the study (Roger and Kaya, 2006).

Region	Primer	5` -3` sequence
ITS-1 (Forward)	ITS-Plant1	TCCGTATGTGAACCTGCGG
ITS-1 (Reverse)	ITS-Gym2	GCTACATTCTTCATCGGTGC
ITS-2 (Forward)	ITS-Plant4B	GGGGAATCCTGGTTAGTTTC
ITS-2 (Forward)	ITS-Gym3	GCACCGATGAAGAATGTAGC

In this study, optimized PCR reactions contained 4 µl of DNA sample (3 ng/µl); 2.5 µl of buffer (10X, $MgCl_2$ free; Bioron); 0.25 µl (1 unit) of *Tag* DNA polymerase (5 u/ µl, Bioron); 0.5 µl of dNTPs (5 mM, Larova); 2.5 µl of $MgCl_2$ (25 mM, Bioron); 1 µl of 20 mM primer pairs (Elips Health Products, Turkey); 0.13 µl of Tween 20 (Sigma,USA); 1 µl of BSA (Sigma, USA) and 12.12 µl of doubled distilled sterile water. Optimum reaction conditions were provided in Table 4.4.

The reaction mixtures were prepared in thin-walled 0.2 ml Eppendorf tubes and run on a thermocycler (Eppendorf-Mastercycler, Eppendorf, Canada and Techne-genius Thermocycler, Techne, USA) .

Table 4.4 Optimized PCR conditions for ITS-2 region

Compenent	Quantity used (µl)	Final concentration
10x buffer	2.5	1x
dNTPs (5mM)	0.5	0.1 mM
MgCl2 (25mM)	2.5	2.5 mM
Primer (1 picomole)	1	1 picomole
Tag DNA polymerase (5u/ µl)	0.25	1 unit
DNA (3ng/ µl)	4	12 ng
BSA (1.8 µg/µ)	1	1.8 µg
Tween 20	0.13	
H_2O	12.12	
Total reaction mixture	25	

The steps of PCR amplification cycles used during optimization of primer-template concentrations were presented in Table 4.5. For all of the primer-pairs, after 5 minutes at 94 ˚C, the PCR involved 30 cycles of amplification, including 30 seconds at 94 ˚C, 1.5 minutes at 55 ˚C and 1.5 minutes at 72 ˚C, and a final extension step of 5 minutes at 72 ˚C was applied.

For ITS-Plant1/ITSGym2, ITS105/ITS594 as well as ITS105/ITS1467 primer pairs (to amplify ITS-1 region); additionally three different PCR conditions were tested. For the first PCR test, the steps and the temperature regime were used as follows; 1 minute at 94 ˚C , then 35 cycles of 1 minutes at 94 ˚C, 4 minutes at 55 ˚C, a ramp of 1 ˚C per 8 seconds to 72 ˚C and 4 minutes at 72 ˚C. This was followed by 10 minutes at 72 ˚C .For the second PCR test, the PCR steps were as follows; (1) 94 ˚C for 3 minutes, (2) 80 ˚C with a pause for the addition of 1.9 of $MgCl_2$, (3) 58 ˚C for 1 minutes, (4) 72 ˚C for 4 minutes (5) 94 ˚C for 3 minutes,(6) 58 ˚C for 1 minutes, (7) 72 ˚C for 4 minutes, (8) repetition of steps 5-7 34 times, (9) 72 ˚C for 10 minutes (Campbell *et al*, 2005). The third PCR cycling steps were presented in Table 4.6.

Table 4.5. PCR Cycling steps and conditions tested for ITS1 and ITS2 regions

Step	Temperature	Time	Cycle #	Description
1	94 ˚C	5 minutes	1	Initial denaturation
2	94 ˚C	30 seconds	30	Denaturation
	55 ˚C	1.5 minutes		Annealing
	72 ˚C	1.5 minutes		Extension
3	72 ˚C	5 minutes	1	Final extension
4	4 ˚C	_	_	Hold

Table 4.6. PCR Cycling steps and conditions tested for ITS-1 region primers (Gernandt and Liston, 1999)

Step	Temperature	Time	Cycle #	Description
1	94 ˚C	3 minutes	1	Initial denaturation
2	94 ˚C	60 seconds	35	Denaturation
	55 ˚C	60 seconds		Annealing
	72 ˚C	3 minutes		Extension
3	72 ˚C	7 minutes	1	Final extension
4	4 ˚C	_	_	Hold

4.4.4. Agorose Gel Electrophoresis

Agorose gels were prepared by dissolving and boiling the agarose in 1XTAE (Tris-acetate EDTA) buffer in a microwave oven. The solution was poured into horizontal gel tray in which combs were inserted and the gel was left to be polymerized. After polymerization, the combs were gently removed from the gel. 1XTAE buffer was poured into electrophoresis apparatus.

The samples were mixed with formamide loading dye and loaded into wells of the gel by using a micropipette. A θX174 DNA/BsuRI (HaeIII) Marker (MBI Fermentas) was used to determine the size of ITS-2 band. The range of the ladder was between 72-1353 base pairs with intervals of 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 base pairs. Although primers designed for both ITS1 and ITS2 regions were tested, primers for ITS-1 did not yield any PCR amplification. Thus, here on, information only for ITS-2 region will be provided.

ITS amplicons from PCR amplification with ITS-2 primers were about 420 bp long (Figure 4.1). Gels were run at 75 volts for 1.30 hours. When electrophoresis was completed, DNA fragments were stained with 5 µg/ml ethidium bromide. After staining, the bands were visualized by direct examination of the gel under UV light.

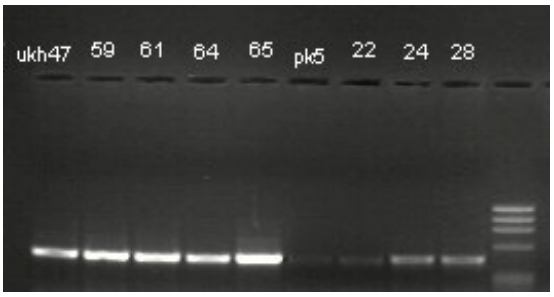


Figure 4.1. Banding pattern of Turkish red pine from Muğla-Ula (lanes labeled as 47, 59, 61, 64 and 65) and Aleppo pine from Adana-Pos (lanes labeled as 5, 22, 24 and 28).

4.4.5. PCR Purification

All PCR products were purified with QIAquick PCR purification kit (Qiagen) before sequencing. Purification system combines the convenience of spin-column technology with selective binding properties of a uniquely-designed silica-gel membrane. Silica-gel membrane is uniquely adapted to isolate DNA from both aqueous solutions and agarose gels, and up to 10 µg DNA can bind to each column.

Efficient recovery of DNA and removal of contaminants provided by special buffers in each application. The binding buffers provide the correct salt concentration and pH for adsorption of DNA to the membrane. QIAquick Gel Extraction Kit Protocol was used in the study. That protocol was designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. According to the protocol, firstly DNA fargments were excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in an Eppendorf tube and 3 volumes of Buffer QG (250 ml solubilization and binding buffer with pH indicator) was added to 1 volume of gel. Buffer QG solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica-membrane. The solution was incubated until the gel slice had completely dissolved. During the incubation, the solution was mixed by vortexing the tube every 2-3 minutes to help dissolve the gel. Then 1 gel volume of isopropanol was added to the sample and mixed. This step increases the yield of DNA fragments <500 bp and >4 kb. The spin column was placed in a provided 2 ml collection tube and the sample was applied to the column and centrifuged for 1 minute to bind DNA. The flow-through was discarded and the column was placed back in the same collection tube. During the DNA adsorption step, unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, and detergents do not bind to the silica membrane, but flow through the column. Then 0.5 ml of QG Buffer was added to the column and centrifuged for 1 min. to remove all traces of agarose. To wash, 0.75 ml of Buffer PE (100 ml wash buffer for use in DNA clean-up) was added to the column and centrifuged for 1 minutes and the flow- through was discarded. Salts are quantitatively washed away by the ethanol-containing Buffer PE. Residual ethanol from Buffer PE which may interfere with subsequent enzymatic reactions, was completely removed by an additional centrifugation step at 13,000 rpm for 1 min. Then the column was placed into a clean 1.5 ml microcentrifuge tube and 23 µl Buffer EB (Elution buffer; 10 mM Tris-Cl, pH:8.5) was added to the center of the column membrane.

After additional 1 minute incubation (for optimal DNA yield), the solution was centrifuged for 1 minute and the flow-through was the purified DNA and stored at -20˚C before sequencing.

4.4.6.DNA Sequencing

Purified PCR products were sequenced with the amplification primers using the primer strategy of ABI 310 Genetic Analyser User's Manuel by a biotechnology company (Refgen Biotechnology, METU Teknokent, Ankara). Sequencing was performed using the Big Dye Cycle Sequencing Kit (applied biosystems) and carried out with a ABI 310 Genetic Analyser (PE applied Biosystem) automatic sequencer. For each sample, forward and reverse sequencing reactions were compared for sequence confirmation. Resulting ITS-2 sequences were checked by eye with the software CHROMAS Lite (version 2.01). The comparison of each sequencing data was done by using Sequencher Software (Demo version, Gene Codes Corp; Ann Arbor, MI, USA) and the consensus sequence for each sample was formed.

4.4.7. Analysis of Data

Sequence alignment was performed using Clustal W (Thampson and Higgins, 1994). The 5'and 3' ends of the alignment were trimmed to remove missing data or unreliable readings from the analysis with the help of Bioedit (Version 5.0.6). The data of ITS-2 region DNA sequences were collected and organized in Fasta format so that it could be analyzed with MEGA 3.1 software (Kumar *et al*, 2005) and it could be used for construction of input data for the analysis by Arlequin software (version 2.000 for population genetics data analysis) (Schneider *et al*., 2000)

4.5. BLAST searches and Phylogenetic Analysis

Outgroup and the most closely related species were chosen on the basis of previous phylogenetic studies within *Pinus* (Liston *et.al*., 1999). From the NCBI (National Center for Biotechnology Information) site, BLASTN search (http://www.ncbi.nlm.nih.gov/) was used to determine the sequences of outgroup (*Picea rubens*) and the most closely related species to Turkish red pine and Aleppo pine sequences. Sequences were compared with 7 conifer rDNA ITS-2 Sequences from NCBI (*Pinus resinosa*-Genbank accession number AF37002, *Pinus sylvestris*-AF37003, *Pinus pinea*-PPITS12RN,*Pinus pinaster*-AF037024, *Pinus halepensis*-AF037007, *Pinus strobus*-AY430069.1, *Picea rubens*-AF136611).

The analysis were done to determine the ITS-2 polymorphism within and between species (Aleppo pine and Turkish red pine). For this purpose, the Arlequin software (Schneider *et al*., 2000) and MEGA 3.1 software (Kumar *et.al*, 2005) used, and the following parameters were estimated: The component of molecular variance by Analysis of Molecular Variance Approach Analysis (AMOVA), minimum spanning tree, matrix of significant Fst values between populations, pairwise comparison of Fst between populations, haplotype distribution between populations (Schneider *et al*., 2000) and molecular diversity indices, pairwise differences according to p-distance method, the average distances between populations, bootstrap test of phylogeny, interior branch test of phylogeny (Nei and Kumar, 2000). Finally, construction of phylogenetic trees was caried out by using neighbour-joining method (Saitou and Nei; 1987)

4.5.1. Population Gentic Structure Inferred By Analysis of Molecular Variance (AMOVA)

The differentiation between groups and the genetic structure of the population is investigated by an analysis of variance framework, as initially defined by

Cockerham (1969, 1973), and extended by others (e.g. Weir and Cockerham, 1984; Long, 1986). The Analysis of Molecular Variance Approach (AMOVA) used in Arlequin software (Schneider *et al.*, 2000). is essentially similar to other approaches based on analysis of variance of the gene frequencies, but it takes into account the number of mutations between molecular haplotypes (which first needed to be evaluated). The covariance components are used to compute fixation indices, as originally defined by Wright (1951, 1965), in terms of inbreeding coefficients, or later in terms of coalescent times by Slatkin (1991).

Formally, in the haploid case, it is assumed that the *i*-th haplotype frequency vector from the *j*-th population in the *k*-th group is linear equation of the form.

$$x_{ijk} = x + a_k + b_{jk} + c_{ijk}$$

The vector **x** is the unknown expectation of x_{ijk} , averaged over the whole study. The effects are **a** for group, **b** for the population within a group, assumed to be additive , random, independent, and to have the associated covariance components, σ_a^2, and σ_b^2, σ_c^2 respectively. The total molecular variance (σ^2) is the sum of the covariance component due to differences among haplotypes within a population

(σ_c^2), the covariance components due to the differences among haplotypes in different populations within a group, (σ_b^2), and the covariance components due to the differences among the **G** groups (σ_a^2). The same framework could be extended to additional hierarchical levels, such as to accomodate, for instance, the covariance component due to differences between haplotypes within diploid individuals.

In terms of inbreeding coefficients and coalescent times, this F*st* can be expressed as

$$F_{ST} = \frac{f_0 - f_1}{1 - f_1} = \frac{\bar{t}_1 - \bar{t}_0}{\bar{t}_1}$$

Where f_0 is the probability of identify by descent of two different genes drawn from the same population, f_1 is the probability of identity by descent of two genes drawn from two different populations, $\bar{t}_1$ is the mean coalescence time of two genes drawn from the same population. The significance of the fixation indices is tested using a non-parametric permutation approach described in Excoeffier *et al*.(1992), consisting in permuting haplotypes, individuals or populations, among individuals, populations or groups of populations. After each permutation round, all statistics were recomputed to get their null distribution. Depending on the tested statistic and the given hierarchical design, different types of permutations are formed. Under this procedure, the normality assumption usual in analysis of variance tests is no longer necessary, nor is it necessary to assume equality of variance among populations or groups of populations. A large number of permutations (1000 or more) is necessary to obtain some accuracy on the final probability.

All estimations were performed using Arlequin, version 2000 (Schneider *et al*., 2000). The AMOVA design and expected mean squares are given in Table 4.7.

Table 4.7 AMOVA table for the grouped data of 7 populations of Turkish red pine and Aleppo pine

Source of variation	d.f	Sum of Squares	Expected Mean Squares
Among groups	1 (G-1)	SSD(AG)	$n''\sigma_a^2 + n'\sigma_b^2 + \sigma_c^2$
Among populations within groups	5 (P-G)	SSD(AP/WG)	$n\sigma_b^2 + \sigma_c^2$
Within populations	21 (N-P)	SSD(WP)	σ_c^2
Total	27 (N-1)	SSD(T)	σ_T^2

SSD(T) :Total sum of squared deviations

SSD(AG) :Sum of squared deviations Among Groups of populations

SSD(WP) :Sum of squared deviations Within Populations

SSD(AP/WG) :Sum of squared deviations Among Populations, Within Groups

G :Number of groups in the structure

P :Total number of populations

N :Total number of individuals for genotypic data or total number of gene copies for haplotpic data

4.5.2. Population Pairwise Genetic Distances (Fst)

The pairwise Fst s can be used as short term genetic distances between populations, with the application of a slight transformation to linearize the

distances with the population divergence time (Reynolds *et al*, 1983; Slatkin, 1995). The pairwise Fst values are given in the form of a matrix. The null distribution of pairwise Fst values under the hypothesis of no difference between the populations is obtained by permuting haplotypes between populations.

The P-value of the test is the proportion of permutations leading to a Fst value larger or equal to the observed one. The P-values are also given in matrix form. Pairwise Fst values were also computed using Arlequin software version 2.000.

4.5.3 Minumum Spanning Network among haplotypes

Computation of Minimum Spanning Tree (MST) (Kruskal, 1956; Prim, 1957) between Operational Taxonomic Units (OTUs). The MST is computed from the matrix of pairwise distances calculated between all pairs of haplotypes using a modification of the algorithm described in Rohlf (1973). The Minimum Spanning Network embedding all MSTs (see Excoeffier and Smouse, 1994) were also computed.

4.5.4. Models for estimating distances

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

In addition to the distance estimates, also the standard errors of the estimates were computed using the analytical formulas and the bootstrap method. In nucleotide method, sequences were compared nucleotide-by-nucleotide.

4.5.4.1 Nucleotide /p-distance Model

This distance is the proportion (p) of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. It does not make any correction for multiple substitutions at the same site, substitution rate biases (for example, differences in the transitional (Transition: A transition occurs when a purine is substituted by a purine, or a pyrimidine by a pyrimidine.) and transversional rates (Transversion: A change from a purine to a pyrimidine, or vice versa, is a transversion.), or differences in evolutionary rates among sites.

Following p-distances and related quantities were also computed:

d: Transitions+Transversions : Proportion of nucleotide sites that were different

p, n_d / L, p (1-p) /L

s : Transitions only : Proportion of nucleotide sites with transitional difference

s, P, s(1-s) /L

v :Transversions only : Proportion of nucleotide sites with transversional difference

v, Q, v(1-v) /L ,

R= s/v : Transitions/Transversions ratio

L :No of valid common sites : Number of sites compared

R, P/Q, $(c_1^2 p + c_2^2 Q - (c_1 p + c_2 Q))^2 / L$

Where $c_1 = 1/s$ and $c_2 = -s/v^2$

P and Q were the proportion of sites showing transitional and transversional differences, respectively (Nei and Kumar, 2000).

4.5.4.2. Nucleotide/Number of Differences Model

This distance is the number of sites at which the two compared sequences differ. For this distance, the following quantities were computed:

d: Transitions+Transversions : Number of different nucleotide sites

$$\text{Var}(d) = n_d (L-n_d) / L$$

s : Transitions only : Number of nucleotide sites with transitional differences

$$\text{Var}(s) = s (L-s)/ L$$

v :Transversions only : Number of nucleotide sites with transversional differences

$$\text{Var}(v) = v (L-v) / L$$

R= s/v : Transitions/Transversions ratio

L :No of valid common sites : Number of compared sites

Formulas for computing these quantities and their variances are as follows.

$$R = s/v$$

$$\text{Var}(R) = (c_1^2 p + c_2^2 Q - (c_1 p + c_2 Q)^2) / L$$

where $c_1 = 1/s$ and $c_2 = -s/ v^2$

P and Q were the proportion of sites showing transitional and transversional differences, respectively (Nei and Kumar, 2000).

4.5.5. Phylogenetic Trees

Phylogenetic relationships of genes or organisms usually are presented in a treelike form with a root, which is called a rooted tree. It also is possible to draw a tree without a root, which is called an unrooted tree. The branching pattern of a tree is called a topology.

In the case of the neighbour-joining (NJ) method (Saitou and Nei; 1987), the S (smallest value of the sum of all branches) value is not computed for all or many topologies, but the examination of different topologies is embedded in the algorithm, so that only one final tree is produced. The algorithm of the NJ method is somewhat complicated and is explained in detail in Nei and Kumar (2000). The NJ method produces an unrooted tree because it does not require the assumption of a constant rate of evolution. Finding the root requires an outgroup taxon. In the absence of outgroup taxa, the root is sometimes given at the midpoint of the longest distance connecting two taxa in the tree, which is referred to as mid-point rooting. Thus, in this study, NJ method was used to construct the phylogenetic tree.

4.5.6. Phylogeny Tests

There are two different types of methods for testing the reliability of an obtained tree; the Bootstrap test and the Interior branch test. Interior branch test of phylogeny is a t-test, which is computed using the bootstrap procedure, is constructed based on the interior branch length and its standard error. It tests the topological difference between the tree and its closely related tree by using a certain quantity (For example, the sum of all branch lengths in the neighbour-joining method). This type of test examines the reliability of every interior branch of the tree, and is generally a conservative test as compared to other tests. If the confidence probability value is greater than 95% for a given branch, then the

inferred length for that branch is considered significantly positive. If it is greater than %50, it is informative (Nei and Kumar; 2000).

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

One of the most commonly used tests of the reliability of an inferred tree (Inferred Tree is a tree reconstructed from the observed sequence or other appropriate data using any tree-making method is known as an inferred or reconstructed tree.) is Felsenstein's (1985) bootstrap test, which is evaluated using Efron's (1982) bootstrap resampling technique. If there are *m* sequences, each with *n* nucleotides (or codons or amino acids), a phylogenetic tree can be reconstructed using some tree building method. From each sequence, *n* nucleotides are randomly chosen with replacements, giving rise to *m* rows of *n* columns each. These now constitute a new set of sequences. A tree is then reconstructed with these new sequences using the same tree building method as before. Next the topology of this tree is compared to that of the original tree. Each interior branch of the original tree that is different from the bootstrap tree partitions is given a score of 0; all other interior branches are given the value 1. This procedure of resampling the sites and the subsequent tree reconstruction is repeated several hundred times, and the percentage of times each interior branch is given a value of 1 is noted. This is known as the bootstrap value. As a general rule, if the bootstrap value for a given interior branch is 95% or higher, then the topology at that branch is considered "correct" if it is greater than 50%, it is informative. Details of these procedures are given in Nei and Kumar (2000).

CHAPTER V

RESULTS

5.1. Genetic and Evolutionary Differences between Aleppo pine and Turkish red pine populations in Turkey

AMOVA analysis according to geographic regions between two groups was carried out. One of the groups was composed of Turkish red pine populations from Adana and Muğla; the second group was formed with Aleppo pine populations from Adana and Muğla. The results indicated that great amount of total variation (81.23%) exist between these species. Percentage of variation among populations within groups produced negative values. These values which were not interpretable and were considered to be zero. Thus, there was not any differentiation among Turkish red pine populations from Adana and Turkish red pine populations from Muğla. Also there was no variation between Aleppo pine populations from Adana and Aleppo pine populations from Muğla (Table 5.1.).

Table 5.1 : Results of AMOVA for taxonomic groups * sampled from two geographic regions

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among species	1	7.122	0.51536 Va	81.23
Among populations within species	5	0.271	0.00 Vb	0.00
Within populations	21	2.500	0.11905 Vc	19.26
Total	27	9.893	0.61819	

*Group-1: Turkish red pine (populations: Muğla-Gökova, Muğla-Ula/kızılyaka, Adana-Pos/Karsantı, Adana-Pos/Sogukoluk), Group-2: Aleppo pine (populations: Adana-Kadirli/Bahadırlı, Muğla-Gökova, Muğla-Ula/Kızılyaka)

The second AMOVA analysis was carried out between Turkish red pine and Aleppo pine populations originating from Muğla Province. The percentage of variation attributed to species was 100%. There was no variation among populations within species (Table 5.2).

Table 5.2 : Results of AMOVA between Aleppo and Turkish red pine species within Muğla Province

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among species	1	4.00	0.5 Va	100
Among populations within species	2	0.00	0.0 Vb	0.00
Within populations	12	0.00	0.0 Vc	0.00
Total	15	4.00	0.5	

Group-1: Turkish red pine (populations: Muğla-Gökova, Muğla-Ula/Kızılyaka)
Group-2 : Aleppo pine (populations: Muğla-Gökova, Muğla-Ula/Kızılyaka)

The third AMOVA analysis was performed between Turkish red pine and Aleppo pine populations originating from Adana. The half of variation (50.65%) was between Turkish red pine and Aleppo pine in Adana, while among populations within species was 14.72% of the total variation. The percentage of variation among populations within species was made up substantially when it was compared to the results of first AMOVA analysis (Table 5.3).

Table 5.3 : Results of AMOVA between Aleppo and Turkish red pine species within Adana Province

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among species	1	2.917	0.40625 Va	50.65
Among populations within species	1	0.750	0.11806 Vb	14.72
Within populations	9	2.500	0.27778 Vc	34.63
Total	11	6.167	0.80208	

Group-1: Turkish red pine (populations: Adana-Pos/Karsantı, Adana-Pos/Soğukoluk),
Group-2 : Aleppo pine (populations: Adana-Kadirli/Bahadırlı)

5.2. Haplotype comparison between Turkish red pine and Aleppo pine

The populations that had the haplotype-1 were Aleppo pine populations of Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka and Muğla-Gökova. The populations that had the haplotype-2 were Turkish red pine populations of Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Soğukoluk, Adana-Pos-Karsantı, Aleppo pine (AF037007) and the outgrup *Picea rubens* had the haplotype close to haplotype-2. The haplotype distribution between 12 populations from two species, Aleppo pine (AF037007) and *Picea rubens* was given in Table 5.4.

Table 5.4. Haplotype distribution among populations of Aleppo pine, Turkish red pine and outgroups

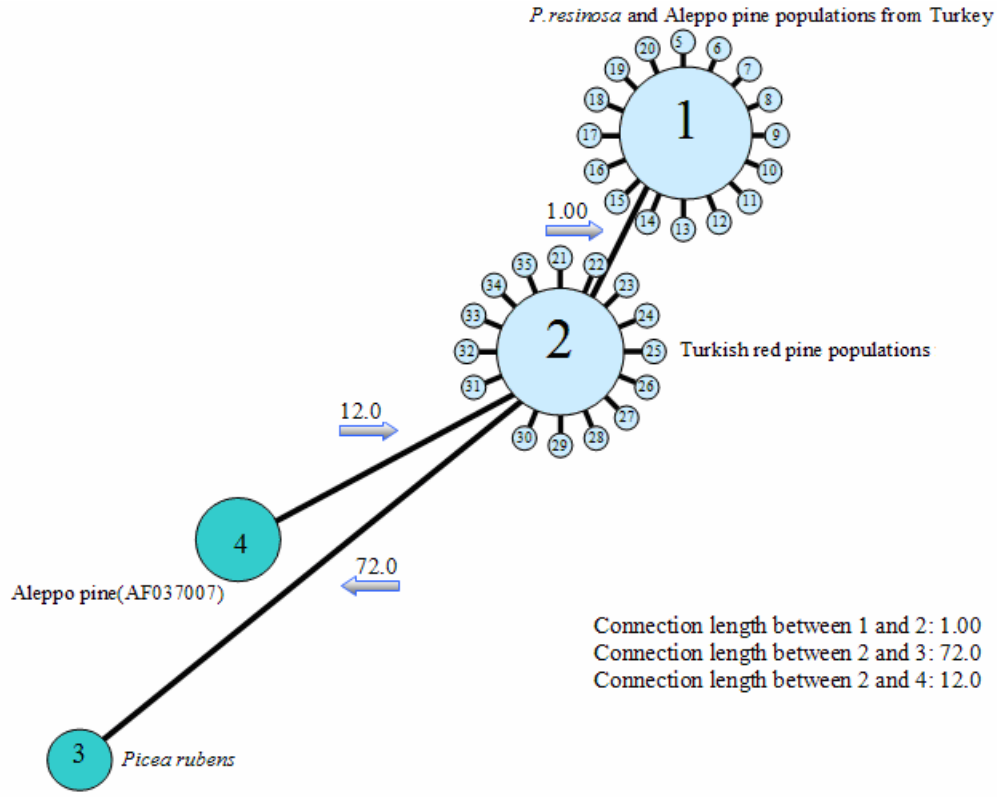
Species	Turkish red pine				Aleppo pine			*Picea rubens*	Aleppo pine (AF037007)
Populations	Muğla UK*	Muğla G*	Adana PK*	Adana PS*	Muğla G*	Muğla UK*	Adana KB*		
Haplotype-1					+	+	+		
Haplotype-2	+	+	+	+				+	+

* Codes are; UK: Ula-kızılyaka, G: Gökova, PK: Pos-karsantı, PS: Pos-sogukoluk, KB: Kadirli-bahadırlı

The minimum spanning tree between 35 individual sequences (16 from Turkish red pine populations, 12 from Aleppo pine populations and the other 7 sequences of *P.resinosa*, *P.sylvestris*, *P.pinea, P.pinaster*, *P.strobus*, Aleppo pine (AF037007) and *Picea rubens*) were shown in Figure 5.1. Number 1-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19-20 were samples of *P.resinosa*, *P.sylvestris*, *P.pinea, P.pinaster*, *P.strobus* and Aleppo pine populations from Muğla and Adana, respectivelly. Number 2-21-22-23-24-25-26-27-28-29-30-31-32-33-34-35 were samples of Turkish red pine populations from Muğla and Adana. The number 3 (*Picea rubens*) and the number 4 (Aleppo pine) (AF037007) were the outgroups.

Figure 5.1 : Minimum spanning tree between 35 operational taxonomical units (OTUs)

Sample-1: *Pinus resinosa*, Sample-2: Turkish red pine, Muğla-Gökova, Sample-3: *Picea rubens*, Sample-4: Aleppo pine (AF037007)



5.3. Genetic differentiation between Turkish red pine and Aleppo pine based on Fst values

Population pairwise Fst values ranged between '0' and '1'. When Fst = 0, it means that there is not any difference between compared populations or species. Fst values among Turkish red pine populations (Muğla-Gökova, Muğla-Ula-kızılyaka, Adana-Pos-Karsantı, Adana-Pos-Soğukoluk) and Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) varied between 0.52 and 1.00. Fst values within Turkish red pine populations varied between 0.00 and 0.33. While Fst values within Aleppo pine populations did not show any variation. The Fst values between Turkish red pine populations (Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Soğukoluk) and subsection *Pinus, Pinea*, *Pinaster*, section *Strobus* indicate high differentiation among them (Fst:1.00). Also, Turkish red pine population from Adana-Pos-Karsantı was different from subsection *Pinus, Pinea*, *Pinaster*, section *Strobus* since Fst values were high and ranged from 0.42 to 0.98. Aleppo pine populations were close to subsection *Pinus, Pinea*, *Pinaster* and section *Strobus* since Fst values were small and did not vary much (0.00-0.3). The outgroup *Picea rubens* was the most distant from Aleppo pine and Turkish red pine populations with Fst values being high and ranging from 0.98 to 1.00 as it was expected (Table 5.5)

Table 5.5 : Pairwise comparison of Fst values among Aleppo pine, Turkish red pine and outgroups (Population names; 1:Muğlag PB, 2:Muğlauk PB, 3:Adanapk PB, 4:Adanaps PB, 5: subsection pinus (*P.resinosa and P.sylvestris*), 6: subsection *pinea*, 7: subsection *pinaster* (*P.halepensis and P.pinaster*), 8: section *strobus*, 9: outgroup (*Picea rubens*), 10: Adanakb PH, 11: Muğlauk PH, 12:Muğlag PH) (PB and PH incoded labels stand for *P.brutia* and *P.halepensis*. kb,uk,g,ps,pk incoded labels stand for kadirli-bahadırlı,ula-kızılyaka,gökova,pos-sogukoluk and pos-karsantı)

	1	2	3	4	5	6	7	8	9	10	11	12
PB/Mugla/g	0.00											
PB/Mugla/uk	0.00	0.00										
PB/Adana/pk	0.20	0.27	0.00									
PB/Adana/ps	0.00	0.00	0.33	0.00								
Pinus	1.00	1.00	0.59	1.00	0.00							
Pinea	1.00	1.00	0.42	1.00	0.00	0.00						
Pinaster	0.34	0.32	0.22	0.37	0.00	-1.0	0.00					
Strobus	1.00	1.00	0.42	1.00	0.00	0.00	-1.0	0.00				
P.rubens	1.00	1.00	0.98	1.00	1.00	1.00	0.80	1.00	0.00			
PH/Adana/kb	0.75	0.77	0.52	0.77	0.11	-0.3	0.28	-0.3	0.99	0.00		
PH/Mugla/uk	1.00	1.00	0.60	1.00	0.00	0.00	0.32	0.00	1.00	0.00	0.00	
PH/Mugla/g	1.00	1.00	0.60	1.00	0.00	0.00	0.32	0.00	1.00	0.00	0.00	0.00

Fst values between Turkish red pine populations (Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Karsantı, Adana-Pos-Soğukoluk) and Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) were found to be significant at $P \leq 0,05$. Only, the Fst values between Turkish red pine population from Adana-Pos-Karsantı and Aleppo pine population from Adana Kadirli-Bahadırlı was not significant (Table 5.6).

Table 5.6 : Matrix of significant Fst values between populations of Turkish red pine and Aleppo pine in Muğla and Adana, significance level=0.05, number of permutations : 3024

Species		Turkish red pine			
	Population	Muğla Gökova	Muğla Ula/Kızılyaka	Adana Pos/Karsantı	Adana Pos/Soğuklouk
Aleppo pine	Muğla Gökova	*	*	*	*
	Muğla Ula/Kızılyaka	*	*	*	*
	Adana Kadirli/Bahadırlı	*	*	ns	*

* Significant at P≤0.05, ns: non-significant at P≤0.05

5.4. Molecular diversity within Turkish red pine and Aleppo pine populations

The number of the total site was 348 and the number of usable site was 343 among Turkish red pine populations (Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Karsantı, Adana-Pos-Soğukoluk) and Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova). There were no transitions, transversions, substitutions, deletions and polymorphic sites within Turkish red pine populations from Muğla and Adana Provinces, except for Adana-Pos-Karsantı population.

However, there was one transversion, one substitution and one polymorphic site within population of Aleppo pine from Adana-Kadirli-Bahadırlı, but no transitions or deletions were detected. There were one transversion and transition, 2 polymorphic sites and substitutions within population of Turkish red pine from Adana-Pos-Karsantı. No deletions were observed in either species (Table 5.7).

Table 5 .7 : Summary of molecular diversity indices within populations of Turkish red pine and Aleppo pine

Species		Turkish red pine				Aleppo pine		
Population		Muğla Gökova	Muğla Ula	Adana Pos	Adana Karsantı	Muğla Gökova	Muğla Ula	Adana Kadirli
	Haplotype frequency	3	3	2	3	2	1	3
	Loci	348	348	348	348	348	348	348
	Usable loci	343	343	343	343	343	343	343
	Transitions	0	0	0	1	0	0	0
	Transversions	0	0	0	1	0	0	1
	Substitutions	0	0	0	2	0	0	1
	Deletions	0	0	0	0	0	0	0
	Polymorphic sites	0	0	0	2	0	0	1

Comparative results about GC content, length, variable sites, parsimony informative sites, conserved sites and nucleotide pair frequencies of ITS-2 sequence data were provided in Table 5.8.

Table 5.8: Comparative results of ITS-2 sequence data analysis for Turkish red pine and Aleppo pine from Adana and Muğla region

	P.brutia vs. P.halepensis populatıons (Muğla)	*P.brutia* vs.*P.halepensis* populations (Adana)	*P.brutia* vs.*P.halepensis* populatıons vs.outgroups
GC content(%)	58.6	58.5	58.5
Length (bp)	348	348	348
Conserved sites	342	340	279
Variable sites	1	3	64
Parsimony informative sites	1	2	3
Identical pairs	340	339	334
Transitional pairs	0	0	2
Transversional pairs	1	1	3

The sequence length of all populations of Aleppo pine and Turkish red pine including outgroups was 348 bp. When Turkish red pine and Aleppo pine were compared with closely related species and outgroup species, there were 334 identical pairs, 2 transitional pairs and 3 transversional pairs, 279 conserved sites, 64 variable sites and 3 parsimony informative sites. The GC content was 58.5 % (Table 5.8)

When two closely related species Turkish red pine and Aleppo pine from both Muğla and Adana Provinces were compared, there were 340 identical pairs, one transversional pairs, 340 conserved sites, 3 variable sites and 2 parsimony informative sites. There was no transitional pairs.

When Turkish red pine and Aleppo pine populations from Muğla were compared; there were 340 identical pairs, one transversional pairs, 342 conserved sites, one

variable and one parsimony informative sites. No transitional pairs were observed.

When Turkish red pine and Aleppo pine populations from Adana were compared; there were 339 identical pairs, one transversional pairs, 340 conserved sites, three variable and two parsimony informative sites. There was no transitional pairs.

Among populations of Turkish red pine from Muğla and Adana, there were 341 identical pairs, 341 conserved sites, two variable sites and one parsimony informative sites. No transitional and transversional pairs were observed.

Among populations of Aleppo pine from Muğla and Adana, there were 340 identical pairs, 342 conserved sites,1 variable sites and 1 parsimony informative sites. Again, no transitional and transversional pairs were found.

5.5. Pairwise Differences based on nucleotide/p-distance model

According to nucleotide p-distance model, pairwise differences of Turkish red pine and Aleppo pine populations were 0.00. There were small differences between Turkish red pine from Turkey and Aleppo pine from Mexico (GeneBank accession no: AF037007) (0.01) and between Aleppo pine from Turkey and Aleppo pine from Mexico (0.02). When Aleppo pine and Turkish red pine and the outgroup *Picea rubens* were considered, the distance was large (0.18). Distance between Aleppo pine from Mexico and *Picea rubens* was also at the similar magnitude (0.18) (Table 5.9).

Table 5.9 : Average of pairwise differences between populations according to nucleotide/p-distance model

	Aleppo pine 1 (Mexico)	Aleppo pine 2	*P.brutia* 3	*P.sylvestris* 4	*P.Pinaster* 5	*P.Strobus* 6	*P.Pinea* 7	*Picea* 8
1								
2	0.02							
3	0.01	0.00						
4	0.02	0.00	0.00					
5	0.02	0.00	0.00	0.00				
6	0.02	0.00	0.00	0.00	0.00			
7	0.02	0.00	0.00	0.00	0.00	0.00		
8	0.18	0.18	0.18	0.18	0.18	0.18	0.18	

5.6. Phylogenetic Trees

A phylogenetic tree was constructed by using Neighbour-joining method (Nei and Kumar, 2000), the unrooted tree with branch lengths was shown in Figure 5.2 The phylogenetic tree revealed two major groups; one with only Aleppo pine populations along with subsection *Pinus,*subsection *Pinea*, subsection *Pinaster*, section *Strobus,* while the other with Turkish red pine populations from Adana and Muğla (Figure 5.2).

There were also small clusters formed within two major groups, but it is diffucult to make firm conclusions about the relationships because of low bootstrap values (below %50). The length of the branches corresponds to the distance which is the number of the nucleic acid substitutions that have taken place along the branch.

Subsection *Pinus,* subsection *Pinea*, subsection *Pinaster*, section *Strobus* and Aleppo pine (populations from Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) were closely related with a branch length of 0.00. Turkish red

pine populations were grouped together in the same cluster, but apart from Aleppo pine group. Aleppo pine from Mexico formed another branch away from Turkish red pine and Aleppo pine from Turkey. The outgroup *Picea rubens* was formed another branch which was distant from the Aleppo pine from Mexico, Turkish red pine and Aleppo pine from Turkey as it was expected.

This branching pattern has a bootstrap value of %91 for neigbour-joining method. But the topology within Turkish red pine and Aleppo pine population samples had low bootstrap support (below %50). Interior branch test of phylogeny also supported the topology between Turkish red pine and Aleppo pine populations with a value of %85.

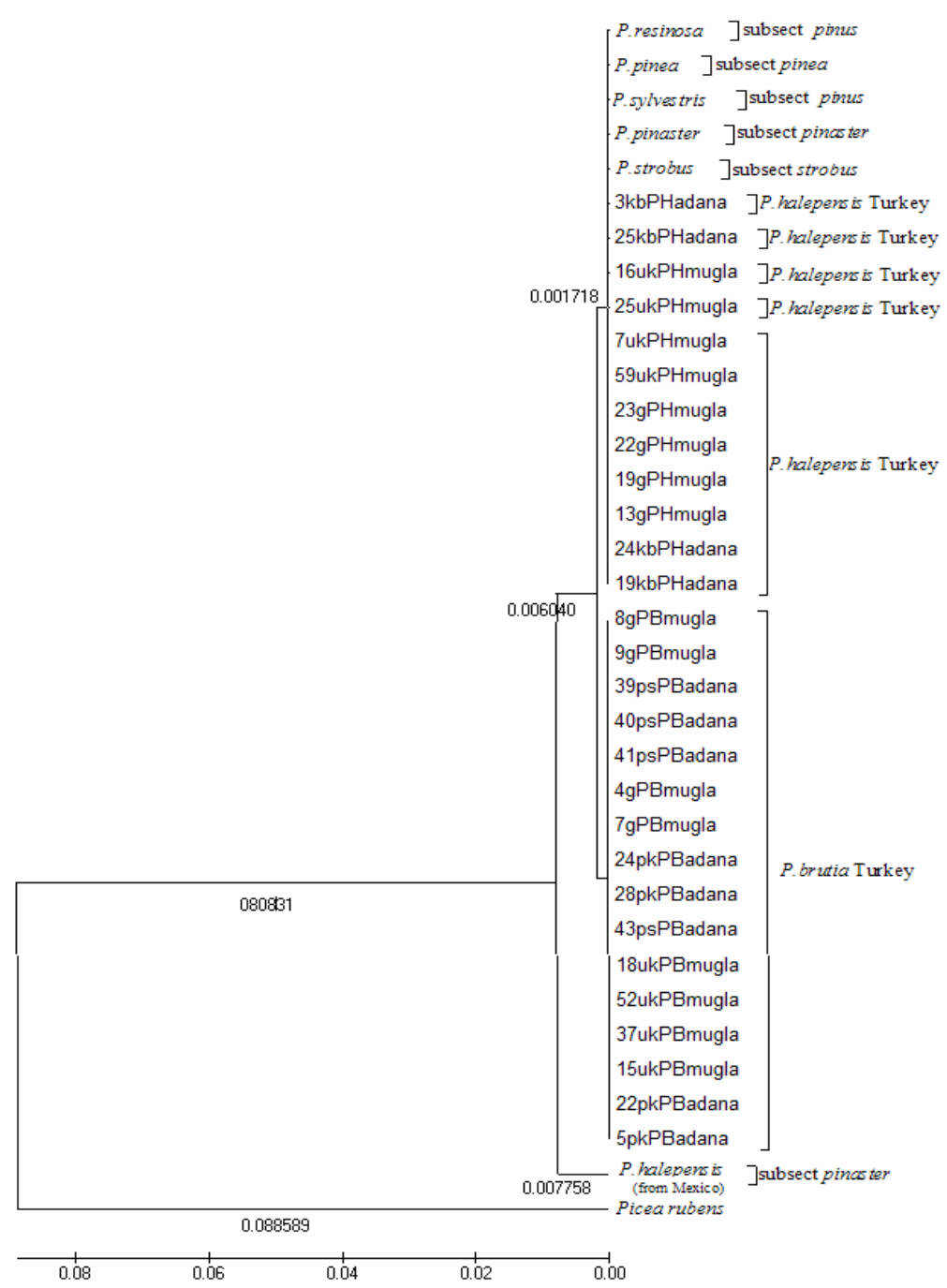


Figure 5.2 : Phylogenetic tree constructed by using Neighbour-joining method (Nei and Kumar, 2000)

CHAPTER VI

DISCUSSION

6.1. Genetic and Evolutionary Differences between Aleppo pine and Turkish red pine in Turkey

The results of AMOVA carried out at three categories (between species from both Muğla and Adana locations, between species in Muğla and between species in Adana) indicated that the great majority of variance (81.23%-100%) was between species except for Adana location where significant amount of variation was attributed to among populations within species (about 50%) and that indicated the possibility of gene flow occuring between the species or physical mixing of individuals within this location. In a study (Quijada *et al*., 1998), that detected the variation in the nrDNA internal transcribed spacer (ITS) region of *Pinus rzedowskii*; most of the variation was found within, rather than among populations, 34% of the variation due to differences among populations and 66% due to differences among individuals within populations. However, in other molecular diversity studies dealing other than ITS region showed that within population component of total variation was quite high. In genetic analysis of *Pinus cembra* L. subsp. *cembra*, hierarchical analyses of sequence difference under AMOVA indicated that great portion of molecular variance was atributed to the difference within populations, whereas, very small portion of molecular variance attributed to difference among populations (Höhn *et al*, 2005). Similarly, Dvornyk *et al*. (2002) studied nucleotide polymorphism in Scots pine (*Pinus sylvestris*) in the gene (*pal1 l*ocus) encoding phenylalanine ammonialyase and detected the differences within

and between populations with the analysis of molecular variance (AMOVA) by arlequin software (Excoffier, 2000). According to the results, interpopulation variability was very low (11%) while intra-population variability was 89%. In the study about the nuclear and cytoplasmic variation within and between Eurasian *Larix* (*Pinaceae*) species, AMOVA analysis indicated that differentiation among species as well as among populations within species was much more pronounced for mtDNA than for either cpDNA or nuclear DNA (Semerikov and Lascoux, 2003).

6.2 Haplotype comparison between Turkish red pine and Aleppo pine

35 individual sequences (16 from Turkish red pine populations from Turkey; 12 from Aleppo pine populations from Turkey, 7 sequences from related species and outgroup of *Picea rubens*) revealed two distict haplotypes. Haplotype-1 was specific to Aleppo pine populations from Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka and Muğla-Gökova, while the second haplotype was characteristics of Turkish red pine populations from Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Soğukoluk, Adana-Pos-Karsantı. The taxonomically related species to Aleppo pine (*P.resinosa*, *P.sylvestris, P.pinea, P.pinaster* and *P.strobus*) shared the same haplotype; that was haplotype-1. It should be noted that no shared haplotypes were observed between Turkish red pine and Aleppo pine populations. This is probably an implication of limited genetic divergence between these two species. Natural or artificial selection could maintain the observed haplotype distribution. One haplotype would be favoured under different conditions (for example, cooler conditions could favour haplotype-2 that was specific to Turkish red pine populations, thus affect their geographical distribution). In the study of Provan *et al*. (1998), between 30 and 37 haplotypes were found in each population of Caledonian and European Scots pine (*P.sylvestris* L.).

Between 47.1% and 80% of these were unique to populations (private haplotypes). 48.8% of individual trees contained a private haplotype.

6.3. Genetic differentiation between Turkish red pine and Aleppo pine based on F_{st} values

F_{st} values between Turkish red pine populations (Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Karsantı, Adana-Pos-Soğukoluk) and Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) varied between 0.52 and 1.00.

In this study statistically significant pairwise differentiation was found between Turkish red pine and Aleppo pine populations from Adana and Muğla. In Muğla province, genetic differentiation between Aleppo pine and Turkish red pine populations were high, suggesting that the magnitude of gene flow between these species may not be existing. On the other hand, the F_{st} values between Turkish red pine and Aleppo pine populations in Adana province were quite low and significant, however the F_{st} value between Turkish red pine population from Adana-Pos-Karsantı and Aleppo pine population from Adana Kadirli-Bahadırlı was not significant (F_{st} = 0.52, $P \leq 0.05$). The lack of detectable differentiation between these populations is likely due to incomplete seperation of haplotypes among this location that was caused by migration among these populations or recent seperation of the populations. This observation corroborated the moderate level of gene flow between these populations in the third AMOVA analysis where molecular variance was between species in Adana Province.

In the study about the variation in the nrDNA internal transcribed spacer (ITS) region of *Pinus rzedowskii* (Quijada *et al*., 1998); F_{st} estimates using both ITS and isozyme data coincided with diversity estimates, indicating significant

differentiation among populations. Fst for ITS was significantly different from zero (P< 0.05). In another study on the genetic analysis of *Pinus cembra* L. subsp. *cembra* (Höhn *et al.*, 2005), low differentiation between populations were detected (Fst = 0.02, P≥0.05). Also Dvornyk *et al.* (2002) measured the differences between populations of *Pinus sylvestris* from different locations by Fst and statistically significant estimate of Fst was reported between the most distantly located populations (Fst = 0.310). In the study of Provan *et al.* (1998), significant (p≤0.001) portions of the variation existed between the populations but there were no significant difference between Scotish and mainland European populations of *P.sylvestris* L. In another study about the genetic structure of *P.sylvestris* L. in a Mediterrenean refugial area, genetic variation among populations was low, and most of the total variation attributable to within-population differentiation (Robledo-Arnuncio *et al*., 2005).

6.4. Molecular diversity Indices Within populations

In this study, the sequence length of Turkish red pine populations (Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Karsantı, Adana-Pos-Soğukoluk) and Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) was about 348 bp. The length of ITS-2 region in our study was somewhat longer than the length of ITS-2 in other phylogenetic studies. In our study, products were sequenced in both 5' to 3' and 3' to 5' direction and the 5' and 3' ends of the alignment were trimmed to remove missing data from the analysis. Thus, approximately 100 bp of length difference could include nucleotide pairs from 5.8S nrDNA cistronic region or 28s (large subunit). The length of the ITS-2 varied from 241 to 243 bp in a phylogenetic study dealing with 47 *Pinus* species based on nrDNA ITS region (Liston *et al*., 1999). The length of the ITS-2 region for *Pinus* subgenus *Strobus* was 242; for subsection *Cembroides* (pinyon pines) was 162 (Gernandt *et al*., 2001).

ITS-2 of *Larix* (Pinaceae) had a conserved length of 231-233 bp, respectively and most of the length variations came from the ITS-1 region (Wei and Wang, 2004). The length of ITS-2 region of *Pseudotsuga* (Pinaceae) was 232 bp (Gernandt and Liston, 1999). The length of the ITS-2 for *Fagus* was 228-229 bp. (Denk *et al*., 2002).

ITS-2 divergence was low in closely related species like Turkish red pine and Aleppo pine, revealing few fixed differences using a direct sequencing approach. According to the comparison between Turkish red pine and Aleppo pine in Muğla, there were 340 identical pairs and one transversional pairs. When Turkish red pine and Aleppo pine in Adana were compared, there were 339 identical pairs, one of which was transversional pairs. When related species and outgroup *Picea rubens* were included to these comparisons; the nucleotide changes slightly increased as 2 transitional pairs and 3 transversional pairs. In our study, there was not any transitional bias in ITS-2 region of Turkish red pine and Aleppo pine populations in Muğla and Adana. However; the ITS region showed a transitional bias in *Pinaceae*, but no trend was discernable in relative transitional bias in ITS-1, 5.8S, and ITS-2 within *Larix, Pseudotsuga*, and subgenus *Strobus* (Gernandt *et al*., 2001).

In our study, the sequence statistics for ITS-2 region of both studied species was lower than the findings in other phylogentic researches. Thus, low amount of species-specific differentiation in ITS-2 region provided limited resolution of phylogenetic relationship between Aleppo pine and Turkish red pine. In general the values for transitions in the ITS-2 region of *Pinus* was 90; the value for transversions in ITS-2 was 87 (Liston *et al*., 1999). The substitutions per site, total transitions and total transversions were 0.0074, 24 and 12 for subsection *Cembroides,* respectively; the substitutions per site was 0.0070 for *Pinus* subgenus *Strobus*, 0.0012 for *Larix* and 0.026 for *Pseudotsuga*; total transitions were 34 for

Strobus, 3 for *Larix* and 6 for *Pseudotsuga*; total tranversions were 10 for *Pinus* subgenus *Strobus,* and 2 for *Larix* and *Pseudotsuga* (Gernandt *et al*., 2001). Also according to this study; substitution rates and distribution of insertions and deletions (indels) indicated that ITS-1 was evolving with faster rate than ITS-2 and the 5.8S in *Pinaceae*. In all three genera, substitution rates in ITS-2 were approximately 50% lower than those in ITS-1, but because ITS-1 was much longer than ITS-2, most of the variable sites (94% in subgenus *Strobus*) occured in ITS-1. According to nuclear ribosomal DNA ITS region sequence statistics for *Larix* and *Pseudotsuga,* there were 27 transitions and 20 transversions in ITS-2 (Gernandt and Liston, 1999). In the study of Conkle *et al* (1988); variation of the loci analyzed in the research, was grouped into six classes for comparing species and subspecies and diversity parameters provided ample evidence to conclude that *P.halepensis* was derived from *P.brutia*-like progenitors

Long lived, wind-pollinated, geographically wide-spread plant species characteristically maintain high levels of intrapopulation genetic variation (Brown, 1978, 1979; Hamrick *et al*., 1979). Very few studies have evaluated within-population variability of nrDNA in pines (i.e. Quijada, 1996). In this study, low sequence divergence in ITS-2 region was adequate to resolve relationships between Turkish red pine and Aleppo pine in Muğla and Adana, but it appeared too low to address fully the relationships within these species. There were no transitions, transversions, substitutions, indels and polymorphic sites within Turkish red pine populations of Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Soğukoluk and Aleppo pine populations of Muğla-Gökova, Muğla-Ula-Kızılyaka. However, there was one transversion, one substitution and one polymorphic site within population of Aleppo pine from Adana-kadirli-bahadırlı, but there were no transitions or deletions. One transversion and transition, 2 polymorphic sites and substitutions within population of Turkish red pine from Adana-Pos-Karsantı were also observed, but there were no deletions.

These nucleotide changes within these two populations occured because of the moderate amount of gene flow between Aleppo pine from Adana-Kadirli-Bahadırlı and Turkish red pine from Adana-Pos-Karsantı.

In this study, ITS-2 was slightly variable. When Turkish red pine and Aleppo pine populations in Muğla and Adana were compared; there were total of 340 conserved sites and 3 variable sites of which 2 were parsimony informative. When we compare Turkish red pine and Aleppo pine populations in Muğla; there were 342 conserved sites, one variable and one parsimony informative sites. On the other hand, Turkish red pine and Aleppo pine populations from Adana were compared; there were 340 conserved sites, three variable and two parsimony informative sites. These values lower than those obtained for the other species from Pinaceae by other research groups. Amount of samples per species and cloning could be the reasons for these differences. In other researches, large number of samples per species and numerous clones from different PCR products were analysed. In the study about phylogenetics of 47 *Pinus* species based on nrDNA ITS region; there were 163 variable sites (63.7%) and 63 informative sites (24.6%) in ITS-2 region of *Pinus* (Liston *et al*., 1999). The number of variable sites were 35, number of informative sites were 12 for *Pinus* subgenus *Strobus* (Gernandt *et al*., 2001). In our study, the inclusion of the related species and an outgroup introduced to the data set created much more additional variation in ITS-2 region.

The GC content was 58.5% for all of the populations included in our study. This value fit within the known ranges for *Pinus* species as reported in study of Liston *et al*.(1999) and for *Pinus pinea*, *Larix, Pseudotsuga* and *Pinus* subgenus *Strobus* as reported in study of Gernandt *et al*. (2001). In the study of Liston *et al*. (1999), a relatively broad range of G+C content was observed for the ITS-1 among *Pinus* species. In contrast, G+C content was fairly stable across all taxa for the 5.8s

rDNA and ITS-2. G+C content of ITS-2 region was high (range between 0.559-0.615). The relatively high ITS-2 G+C content is also characteristic of angiosperms (Baldwin *et al*., 1995; Hershkovitz and Zimmer, 1996).

6.5. Pairwise Differences and Constructed Phylogenetic Tree

According to nucleotide/p-distance model, the distance is the proportion (p) of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. According to nucleotide p-distance model, pairwise differences between Turkish red pine and Aleppo pine populations were 0.00. Distances between each Turkish red pine populations and Aleppo pine from Mexico (AF037007) was 0.01. But distances between our Aleppo pine populations and Aleppo pine from Mexico (AF037007) was slightly higher (0.02). The largest distance between Aleppo pine, Turkish red pine populations and the outgroup *Picea rubens* was found out to be 0.18.

According to nucleotide/number of differences model; the distance is the number of sites at which the two compared sequences differ. As a result of this nucleotide model; subsection *Pinus*, subsection *Pinea*, subsection *Pinaster*, section *Strobus* were related more closely to all Aleppo pine populations. Within Aleppo pine and Turkish red pine, the populations from Adana and Muğla showed no divergence with pairwise difference. Pairwise differences between our Aleppo pine populations and Aleppo pine from Mexico (AF037007) was 5.00; between Turkish red pine populations and Aleppo pine from Mexico (AF037007) was 4.00. Surprisingly Aleppo pine from Mexico (AF037007) was closer to Turkish red pine populations than Aleppo pine populations. Turkish red pine and Aleppo pine populations differed from each other with pairwise differences value of 1.00. Also, the differences between subsection *Pinus*, subsection *Pinea*, subsection *Pinaster*, section *Strobus* and Turkish red pine populations were 1.00.

However, pairwise differences between subsection *Pinus*, subsection *Pinea*, subsection *Pinaster*, section *Strobus* and all Aleppo pine populations were 0.00. According to these results; subsection *Pinus*, subsection *Pinea*, subsection *Pinaster*, section *Strobus* were closer to Aleppo pine populations than Turkish red pine populations.

In this study, phylogenetic tree was constructed by using Neighbour-joining method (Nei and Kumar, 2000). Species were divided into two well-supported groups with a bootstrap value of 92%. The first group included all Turkish red pine populations from Adana and Muğla and the second clade included only Aleppo pine populations from Adana and Muğla, as well as subsection *Pinus* ,subsection *Pinea*, subsection *Pinaster*, section *Strobus.* The topology between Turkish red pine and Aleppo pine populations were also supported by interior branch test of phylogeny with a value of 85%. Relationships among species within these two clades were not well resolved (with a bootstrap value below 50%). The branches from different locations were highly mixed, indicating efficient gene flow between them.

Subsection *Pinus,* subsection *Pinea*, subsection *Pinaster*, section *Strobus* and Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) were closely related populations which were clustered together. Aleppo pine from Mexico (AF037007) was a sister species to all the remaining taxa; closer to Turkish red pine populations. The root was outgroup *Picea rubens;* it was placed on a seperate long branch; distant from Aleppo pine from Mexico (AF037007), Turkish red pine and Aleppo pine populations from Adana and Muğla.

According to the results of a study regarding the phylogenetic relationships of Eurasian Pines (Wang *et.al*., 1999); the 32 *Pinus* species were split into two distict groups corresponding to the subgenera *Pinus* and *Strobus*. In the subgenus *Pinus*

clade, the species were split into two distinct clades. One of these two clades included pines occuring in the Mediterreneean. In this clade, *Pinus halepensis* and *Pinus brutia* formed a strongly supported (98%) group. The second clade of the subgenus *Pinus* consisted of species from subsection *Sylvestres*. Frankis (1993) combined *P.pinaster, P.canariensis, P.halepensis* and *P.brutia* into one subsection, *Pinaster*, but *P.pineae* was placed in a seperate subsection.

Studying the paleobotany of the *P.brutia-P.halepensis* complex, Nahal (1962) came to the conclusion that in the tertiary era, the ancestors of *P.halepensis* and *P.brutia* occupied a much larger common geographic range in the north of the Mediterrenean. It is suggested that continious gene flow between *P.halepensis* and *P.brutia* would at that time remove or reduce considerably all distinction among the pines (Conkle *et al.,* 1988). At the end of the tertiary era and at the beginning of the quaternary era, the cooling of the European continent resulted in the migration of *P.halepensis* in the Occidental part of Mediterrenean, *P.brutia* being more resistant to colder conditions favoured the east (Prus-glowacki *et al*., 1985).

The findings of the present study revealed that Turkish red pine and Aleppo pine populations did not show great differentiation for ITS-2 region. Especially, populations of two species in Adana province appeared to have exchanged genetic material in the past through natural hybridization. Aleppo pine and Turkish red pine revealed more differentiation due to reproductive isolation in Muğla province. These findings were well-adjusted with the idea that phylogenetically, *P.halepensis* and *P.brutia* have emerged from a common ancestor evolving independently (Prus-glowacki *et al*., 1985). According to Price (1998), the ability to hybridize is generally indicative of a close phylogenetic relationship in pines, despite the fact that it may be a plesiomorphic trait. Klaus (1989) noted several morphological characters shared between these two species that were also other indications of close evolutionary link between them.

Evolution and differentiation of taxa are related with levels of genetic variation (Gottlieb, 1977; Ledig 1986a). In the present study, we were able to test the phylogenetic utility of ITS-2 to determine the relationship between Turkish red pine and Aleppo pine by comperative sequence analysis. Our results showed that nrDNA ITS-2 region revealed insufficient informative sites for the generation of robust phylogenetic hypothesis for Turkish red pine and Aleppo pine populations. Further studies dealing with ITS-1 and 5.8s of ribosomal DNA are needed.

CHAPTER VII

CONCLUSION

The general objective of this study was to reveal genetic and evolutionary relationship between natural Aleppo pine and Turkish red pine populations from Adana and Muğla provinces (where Aleppo pine was naturally found) by comparing the sequence divergence of nrDNA ITS-2 region.

Based on the results of Analysis of Molecular Variances, the differences between Turkish red pine and Aleppo pine populations from Muğla and Adana, 81.23% of total variation was between species. It was also found that the highest genetic differentiation between Turkish red pine and Aleppo pine were observed in Muğla with 100% percentage of total variation. On the other hand, Turkish red pine and Aleppo pine populations in Adana indicated the possibility of low-level migration between the species at this location since only 50.65% of total molecular variance was due to species.

Haplotype comparison revealed that there were two major haplotypes, one being only in Aleppo pine samples, whereas the second was specific to the samples of Turkish red pine. The taxonomically related species; *P.resinosa*, *P.sylvestris, P.pinea, P.pinaster* and *P.strobus* were characterized haplotype-1 showing affinity to Aleppo pine cluster.

The significant genetic differentiation was detected between Aleppo pine and Turkish red pine populations in Muğla province. More genetic divergence between

the species in Muğla province may be accounted for the reproductive isolation. Also, the Fst values between Turkish red pine and Aleppo pine populations in Adana Province were significant, but the lack of detectable differentiation between Turkish red pine population from Adana-pos-karsantı and Aleppo pine population from Adana Kadirli-Bahadırlı suggest that the efficient amount of gene flow may have occured in the past. In Adana Province, the two species shared more common genetic background due to possible hybridization.

In this study, the sequence length of Turkish red pine populations and Aleppo pine populations was 348 bp which was longer than the length of ITS-2 in other phylogenetic studies of Pinaceae. Approximately 100 bp of length difference could include nucleotide pairs from 5.8S nrDNA cistronic region or 28s (large subunit). The GC composition was at moderate level of 58.5% for all of the populations included to the present study. This value fit within the known ranges for *Pinus* species as reported previously.

According to ITS-2 region molecular diversity results, sequence divergence values were low as it is expected among closely related species such as Turkish red pine and Aleppo pine, revealing few fixed differences. Thus, the relationships within populations of these two species were poorly resolved. According to the comparison between Turkish red pine and Aleppo pine populations in Muğla and Adana, there were 340 identical pairs and 1 transversional pairs and there was no transitional bias in ITS-2 region as in Pinaceae which was detected by related phylogenetic studies.

In this study, phylogenetically informative characters were found in ITS-2, but the region was slightly variable. When Turkish red pine and Aleppo pine populations in Muğla and Adana were compared; there were total of 340 conserved sites and 3 variable sites of which 2 were parsimony informative. These values lower than those obtained for the other species from Pinaceae.

This may result from low number of species or insufficient amount of samples per species and not testing a cloning strategy in the study.

According to phylogenetic tree constructed with Neighbour Joining procedure, the species including outgroups were divided into two well-supported groups with a bootstrap value of 92%. All Turkish red pine populations were grouped together in the same cluster, but apart from Aleppo pine group. This branching pattern was also supported by Interior Branch Test of phylogeny with a value of 85%. To resolve relationships among species within these two clades was difficult because of highly mixed branches from different locations.

Based on the results of ITS-2 region sequence analysis, Aleppo pine and Turkish red pine populations could not be fully differentiated in our study. In Muğla province Turkish red pine and Aleppo pine revealed more differentiation due to reproductive isolation. But in Adana province, populations of two species appeared to have exchanged genetic material in the past through natural hybridization. However, this suggestion need to be tested by expanding the study to whole ITS region combined with reproductive biology of studied populations of Turkish red pine and Aleppo pine in Muğla and Adana. Since ITS-2 region of nuclear ribosomal DNA revealed a few variable and parsimony informative sites for both species, thus, ITS-2 region of ribosomal DNA appears to be insufficient for clearly resolving genetic relationships between Turkish red pine and Aleppo pine populations in Turkey. Further studies dealing with ITS-1 and 5.8s of ribosomal DNA and populations included from major Aleppo pine distribution areas will be useful to understand the evolutionary relationship between Aleppo pine and Turkish red pine populations in Turkey.

REFERENCES

Acar, İ. 1993. Genetic structure of *P.brutia* forests. Uluslararası Kızılçam Sempozyumu (18-23 Ekim 1993, Marmaris), Orman Bakanlığı Yayını, pp. 228-234, Ankara.

Agundez, D., Degen, B., Von Wuehlısch, G., Alia, R. 1999. Multilocus analysis of *P.halepensis* Mill. from Spain: Genetic diversity and clinal variation. Silvae Genetica 48: 173-178.

Akıncı, M., Y. 1963. Distribution of natural *Pinus brutia* forests in the Northern Black Sea Region. Orman Mühendisliği Dergisi, pp. 28-35.

Anonymous: Mediterrenean Pine seed collection. 1973. FAO For. Occas. paper, For. Genetic. Resour. Info. 2: 64.

Anonymous: The Turkish Forestry. 1989. The Ministry of Forestry, OGM Publication, Ankara, Turkey. Publication No. 673, 126 p.

Anonymous, 2001. The structure and features of Forestry Sector in Turkey. Sekizinci Beş Yıllık Kalkınma Planı, Ormancılık Özel İhtisas Komisyonu Raporu, DPT Yayın No. 2531- OİK: 547, pp. 1-26, Ankara.

Arbez, M, and Miller C. 1971. Contribution a l'etude de la variabilitè geographique de *Pinus nigra* ARN. Anna. Sci. For., 28, 23-49.

Arbez, M. 1974. Distribution, ecology and variation in *Pinus brutia* in Turkey. FAO For. Occas. paper, For. Genetic Resour.Info. 3: 21-33.

Arianoutsou. M. And Ne'eman, G. 2000. Post-fire regeneration of natural *Pinus halepensis* forests in the east Mediterrenean basin: In: Ne'eman, G. And Trabaud. L. (eds.), Ecology, Biogeography and Management of *Pinus halepensis* and *Pinus brutia* forest systems in the Mediterrrenean basin. Backhuys publishers. Leiden. pp. 269-289.

Arnheim, N., Krystal, M., Schmickel, R., Wilson, G., Ryder, O., and Zimmer, E. 1980. Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and apes. Proceedings of the National Academy of Sciences, U.S.A. 77: 7323-7327.

Arnheim, N. 1983. Concerted evolution in multigene families. pp. 38-61 in M. Nei and R. Koehn, eds. Evolution of genes and proteins. Sinauer, Sunderland, Mass.

Atalay, İ. 1982. A general survey of the vegetation of northeastern Anatolia: Ege Coğrafya Dergisi (Aegean Geographical Journal), 1, 14-39

Atalay, İ., Sezer, L. İ. And Çukur, H. 1998. Ecological features of *Pinus brutia* forest and dividing its natural distribution areas to seed transfer regions. Orman Ağaçları ve Tohumları Islah Araştırma Müdürlüğü, Yayın No. 6, 108 p., Ege Üniversitesi Basım Evi, İzmir.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, J.S., and Donoghue, M.J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden 82:247-277.

Baradat, P.H., Lambardi, M. And Michelozzi, M.1989. Terpene composition in four Italian provenances of Aleppo pine. Journal of Genetics and Breeding. 43: 195-200

Baradat, P.H., Michelozzi, M., Tognetti, R.,Khouja, M.L., Khaldi, A. 1995. Geographical variation in the terpene composition of *Pinus halepensis* Mill. Population Genetics and Genetic Conservation of Forest Trees. pp.141-158.

Barbero, M., Loisel., Quezel, P., Richardson, D.M. et Romane, F. 1998. Pines of the Mediterrenean Basin. In D.M Richardson (ed.), Ecology and biogeography of Pinus. P. 153-170. Cambridge University Press, Cambridge, Royaume-Uni.

Bridge, P.D., Arora, D.K. 1998. Interpretation of PCR methods for species definition. In: Bridge PD, Arora DK, Reddy CA, Elander RP, eds. Applications of PCR in Mycology. CAB International. P 63-84.

Brown, A.H.D. 1978. Isozymes, plant population genetic structure and genetic conservation. Theor.Appl.Genet. 52:145-157.

Brown, A.H.D. 1979. Enzyme polymorphism in plant populations. Theor. Popul. Biol. 15:1-42.

Bruns, T.D., White, T.J. and Taylor, J.W. 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. 22: 525-564.

Boydak, M. 2005. Plant diversity, Phoenix theoprasti and *Pinus brutia* in Turkey. In: A.J.Karamanos and C.A. Thanos (eds.), Proceedings of the conference (Theophrastus 2000, 5-8 July 2000, Lesbos), pp.251-277, Athena.

Boydak, M. 2006. Biology and silviculture of Turkish Red Pine (*Pinus brutia* Ten.)

Bucci, G., Anzidei, M., Madaghiele, A. And Vendramin, G.G. 1998. Detection of haplotypic variation and natural hybridization in halepensis-complex pine species using chloroplast SSR markers. Molecular Ecology 7: 1-11.

Buckler IV, E.S., Holtsfoot, T.P., 1996a. *Zea* systematics ribosomal ITS evidence. Mol. Biol. Evol. 13, 612-622.

Calamassi, R., Falusi, M., and Mugni, L. 1980a. Shoot morphology and growth pattern in seedlings of *Pinus brutia* provenances. Can. J. For. Res. 18: 188-194.

Calamassi, R., Falusi, M.,Tocci, A. 1980b. Geographic variation and resistance to hydric stress in seeds of *P.halepensis*, *P.brutia* and *P.elderica*. Ann. Ins. Sper. Selv. Arezzo 11:194-230.

Calamassi, R. 1982. Light and temperature effects on seed germination of some provenances of *P.halepensis* MILL. and *P.brutia* TEN. L'Italia Forestale e Montana. 37: 174-187.

Campbell, C.S., Wright, A.W, Cox, M., Vining, T.F., Major, C.S.,Arsenault, M.P., 2005. Nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) in *Picea* (*Pinaceae*): sequence divergence and structure. Molecular Phylogenetics and Evolution 35 :165-185.

Cesarone, C., Bolognesi, C. and Santi, L.1979. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. Anal Biochem. 100: 188-197.

Critchfieldt, W.B. and Little E.L., Jr. 1966. Geographic distribution of the pines of the world. U.S.D.A Forest Service, Miscellaneous Pub. 991. 97 pp.

Cockerham, C.C. 1969. Variance of gene frequencies. Evolution, 23: 72-83.

Cockerham, C.C. 1973. Analysis of gene frequencies. Genetics, 74: 679-700.

Conkle, M.T., Hodgskıss, P.D., Nunnally, B.L., and Hunter, S.C. 1982. Starch Gel Electrophoresis of Conifer Seeds: a labratory manual. USDA Forest Service, Pasific Southwest Forest and range Experiment Station, General Technical Report PSW-64.

Conkle, M.T., Schiller, G., Grunwald, C. 1988. Electrophoretic analysis of diversity and phylogeny of *Pinus brutia* and closely related taxa. Systematic Botany, Vol. 13, No.3,pp. 411-424.

Davis, P.H. 1965. Flora of Turkey and East Agean Islands. Volume 1, pp. 74.

Dellaporta, S.L., Wood, J. And Hicks, J.B. 1983. A plant DNA minipreparation: version II. Plant Mol. Biol Rep. 1:19-21.

Denk, T., Grimm., G., Stögerer, K., Langer, M., Hembleben, V. 2002. The evolutionary history of *Fagus* in Western Eurasia: Evidence from genes, morphology and the fossil record. Plant Syst. Evol. 232:213-236.

Dover, G.A. 1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111-116.

Duffield**,** J.W. 1952. Relationship and species hybridization in the genus *Pinus.* Zeitschrift für Forstgenetik 1: 93-97.

Dvornyk, V., Sirviö, A., Mikkonen, M., and Savolainen, O. 2002. Low nucleotide diversity at the *pal1* Locus in the widely distributed *Pinus sylvestris*. Mol. Biol. Evol. 19(2):179-188.

Efron, B. 1982. The Jackknife, the Bootstrap and Other Resampling Plans. CBMS-NSF Regional Conference Series in Applied Mathematics, Monograph 38, SIAM, Philadelphia.

Enright N.J., Marsula, R., Lamont B.B and Wissel, C. 1998. The ecological significance of canopy seed storage in fire-prone environments: a model for non-sprouting shrubs. Journal of Ecology, 86: 946-959.

Emberger, L., Gaussen, H., Kassas, A., Philipis, A. 1963. Bioclimatic map of the Mediterrenean zone; Ecological study of the Mediterrenean zone. Arid zone Research, UNESCO-FAO, Paris. 58 pp.

Ersöz, E. 2001. The pattern of genetic variation of *P.brutia* TEN. populations from southern Turkey determined by nuclear SSR markers. A master's thesis. Middle East Technical University, Graduate School of Natural and Applied Sciences, Ankara, Turkey. 162 pp.

Excoeffier, L., Smouse, P.E. and Quattro. 1992. Analysis of molecular variance inferred from Metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479-491.

Excoeffier, L., Smouse, P.E. 1994. Using allele frequencies and geographic subdivision to reconstruct gene genealogies within a species. Molecular Variance Parsimony. Genetics, 136: 343-359.

Farjon, A. 1984. Pines: drawings and description of the genus. E.J Brill and W. Backhuys, Leiden.

Feinbrun N. 1959. Spontaneous Pineta in the Lebanon. Bulletine Research Council Israel 7D, 132-153.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fischer, J.T., Neumann, R.W. and Mexal, J.G. 1986. Performance of *Pinus halepensis/brutia* group pines in southern New Mexico. Forest Ecology and Management 16: 403-410.

Frankel, O.H., Brown, A.H. and Burdon, J.J. 1995. The conservation of plant diversity. Cambridge University Press.

Frankis, M., P. 1993. Morphology and Affinities of *Pinus brutia*. Uluslararası Kızılçam Sempozyumu (18-23 Ekim 1993, Marmaris), Orman Bakanlığı Yayını, pp.11-18, Ankara.

Gallis, A.T; Lang, K.J and Panetsos, K.P 1998. Bud monoterpene composition in *Pinus brutia (*TEN.), *Pinus halepensis (*MILL.) and their hybrids. Silvae Genetica 47, 2-3 .

Gallis AT., Panetsos K.P. 1997. Use of cortical terpenes to discriminate *Pinus brutia* (Ten.), *Pinus halepensis* (Mill.) and their hybrids. Silvae Genetica, 46, 82-88.

Gauthier, S. Bergeron, Y. and Simon, J.P. 1996. Effects of fire regime on the serotiny level of jack pine. Journal of Ecology 84: 539-549.

Gemici, Y., Akyol, E., Seçmen, Ö., Akgün, F. 1990. Macro et microflore du bassin neogene d' Eskihisar (Yatağan-Muğla). Journ. of Fac. Of Sci., E. Ü., Seri B, Vol. 12, 1, pp.29-41

Gemici, Y., Akyol, E., Akgün, F., Seçmen, Ö. 1991. Macro and micro flora of Soma charcoal mines. MTA, No. 112.

Gernandt, D.S., Liston, A. 1999. Internal transcribed spacer region evolution in *Larix* and *Pseudotsuga* (*Pinaceae*). American Journal of Botany. 86(5):711-723.

Gernandt, D.S., Liston, A., Pinaro, D. 2001. Variation of the nrDNA ITS of *Pinus* subsection *Cembroides*: implications for molecular systematic studies of Pine species complexes. Mol. Phylogenet. Evol. 21, 449-467.

Germano**,** J., Klein, A.S., 1999. Species specific nuclear and chloroplast single nucleotide polymorphisms to distinguish *Picea glauca, P.mariana* and *P.rubens*. Theor. Appl. Genet. 99, 37-49.

Geogoulis, E.B. 1964. Cultivation of Aleppo pine , together with evergreen hardwoods and possible influence on gum yield in composite forest. For. Res. Inst., No:11, Athens.

Goncharenko, G.G., Bolsun, S.I., Nevo, E. And Zehavi, A. 1998. Genetic taxonomic relations between *Pinus pithyusa*, *P.stankewiczii* and *P.brutia*. In: Report of the Academy of Sciences of Russia 359: 565-568.

Gottlieb, L.D. 1977. Electrophoretic evidence and plant systematics. Ann. Missouri. Bot. Gard. 64: 161-180.

Grunwald, C., Schiller, G., Conkle, M.T 1986. Isozyme variation among native stands and plantations of Aleppo pine in Israel. Israel Journal of Botany. 35: 161-174.

Hamrick, J.L., Linhart, Y.B. and Mitton, J.B. 1979. Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. Annual Rev. Ecol. Syst. 10:173-200.

Hamby, R.K. and Zimmer, E.A. 1992. Ribosomal RNA as a phylogenetic tool in plant systematics. Pp. 50-91 in *Molecular systematics of plants*, eds, P.S. Soltis, D.E. Soltis, and J.J. Doyle. New York: Chapman and Hall.

Hershkovitz, M.A., and Zimmer, E.A. 1996. Conservation patterns in angiosperms rDNA a ITS-2 sequences. Nucleic Acids Res. 24:2857-2867.

Hillis W. 1987. The future of forest chemicals. Chinese Academy of Forestry. Chemistry and Industry of Forest Products 7(1): 1-13.

Hillis, D.M., Moritz, C., Porter, C.A., Baker, R.J. 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science 251: 308-310.

Hillis, D.M and Dixon, M.T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. The Quat Rev of Biol 66:411-453.

Höhn, M., Abran, P., Vendramin, G.G. 2005. Genetic analysis of Swiss stone pine populations (*Pinus cembra* L. subsp. *cembra*) from the Carpathians using chloroplast microsatellites. Acta Silv. Lign. Hung. Vol. 1:39-47.

Işık, K. 1986. Altidunal variation in *Pinus brutia* TEN: Seed and seedling characteristics. Silvae Genetica 35 (2-3): 58-66.

Işık, K., Topak M. and Keskin, A.C. 1987. Genetic variation among and within six *Pinus brutia* Ten. stands in southern Turkey: six-year results at five common garden plantations. Institute of Forest Tree Seeds and Improvement Publ. No 3, Ankara, 139pp.

Işık, K. 1993. Genetic differences among 60 open-pollinated *P.brutia* TEN. families at four test sitesin southern Turkey. In: Proceedings of the FAO-IUFRO and the Turkish Ministry of Forestry, Turkey.pp. 235-242.

Işık, F. And Kaya, Z. 1993. Genetic variation in *P.brutia* TEN. natural populations. In: Proceedings of the FAO-IUFRO and the Turkish Ministry of Forestry, Turkey pp. 243-253.

Kara, N., Korol, L., Işık, K. and Schiller, G. 1997. Genetic diversity in *P.brutia* Ten.: Altitudinal variation. Silvae Genetica, 46, 155-161.

Karvonen, P., Karjalainen, M. And Savolainen, O. 1993. Ribosomal RNA genes in Scots pine (*Pinus sylvestris* L.): chromosomal orginazation and structure. Genetica 88: 59-68.

Kaya, Z. and Işık, F. 1997. The pattern of genetic variation in shoot growth of *Pinus brutia Ten*. populations sampled from Toros Mountains in Turkey. Silvae Genetica. 46: 73-81.

Kaya, Z., Kün, E. and Güner, A. 1997. National plan for in situ conservation of genetic diversity in Turkey. Submitted to the Republic of Turkey, The Ministry of Environment, Ankara. 125p.

Kaya, Z., Raynal, D.J. 2001. Biodiversity and conservation of Turkish Forests. Biological Conservation.

Kayacık, H. 1954. Pines in Turkey and an invesigation about their geographical distribution. İ.Ü. Orman Fakültesi Dergisi, 4: 44-46.

Kayacık, H. 1954. Turkish pines and investigations of their natural distribution. İ.Ü. Orman Fakültesi Dergisi, Cilt 4, Sayı 1-2, pp. 44-64.

Keely, J.H. and Zedler, P.H. 1998. Evolution of life histories in pines. In: Richardson D.M. (ed.). Ecology and Biogeography of *Pinus*. Cambridge University press. Cambridge, pp. 219-249.

Klaus, W. 1989 . Mediterrenean pines and their history. Plant Systematics and Evolution. 162: 133-163.

Korol, L. and Schiller, G. 1996. Relations between native Israeli and Jordanian Aleppo pine based on allozyme analysis. Forest Genetics 3: 197-202.

Korol, L., Shklar, G. And Schiller, G. 2002 b. Genetic variation within and among *Pinus brutia* Ten. seed stands in Turkey in their isoenzymes. Forest Genetics 9(3): 233-242.

Kreike, J. 1990. Genetic analysis of forest tree populations: isolation of DNA from spruce and fir apices. Plant Molecular Biology, 14: 877-879.

Kruskal, J.R. 1956. On the shortest spanning subtree of a graph and the travelling salesman problem. Proc. Amer. Math. Soc. 7: 48-50.

Kumar, S., Tamura, K. and Nei, M. 2005. MEGA3: Integrated software for molecular Evolutionary Genetics Analysis and sequence alignment; Brief.Bioinform. 5:150-163.

Laban, A. 1972. Mountain soils and growth of *Pinus brutia* in West Turkey. A 'Meryemana Report', pp 67.

Ledig, F.T. 1986a. Heterozygosity, heterosis, and in outbreeding plants. pp. 77-104 in Conservation biology; the science of scarsity and diversity, chap. 5,ed. M.E. Soule. Sunderland, Massachusetts: Sinauer Associates, Inc.

Lepart, J. and Debussche, M. 1991. Invasion processes as related to succession and disturbance. In: Groves, R.H., di Castri, F. (eds.), Biogeography of Mediterrenean Invasions. Cambridge. Pp. 159-177.

Lise, Y. 2000. The impact of anthropogenic factors on the composition of genetic variation on *Pinus brutia* Ten. populations determined by DNA markers. Master Thesis. Middle East Technical University, Ankara.

Liston, A., Robinson, W.A., Oliphant, J.M., 1996. Length variation in the nuclear ribosomal DNA internl transcribed spacer region of non-flowering seed plants. Syst. Bot. 21, 109-120.

Liston, A., Gernandt, D.S., 1999. Internal Transcribed Spacer Region Evolution in *Larix* and *Pseudotsuga* (*Pinaceae*). American Journal of Botany, 86(5):711-723.

Liston, A., Robinson, W.A., Pinero, D. and Alvarez-Buylla, E.R. 1999. Phylogenetics of *Pinus* (*Pinaceae*) based on nuclear ribosomal DNA internal transcribed spacer region sequences. Molecular Phylogenetics and Evolution, Vol. 11, No.1,pp.95-109.University of Edinburgh Press, Edinburgh.

Long, J.C. 1986. The allelic corelation structure of Gainj and Kalam speaking people. The estimation and interpretation of Wright's F-statistics. Genetics, 112: 629-647.

Maggini, F., Frediani, M., Gelati, M.T., 2000. Nucleotide sequence of the internal transcribed spacers of ribosomal DNA in *Picea abies* Karst. DNA sequence 11, 87-89.

Marrocco, R., Gelati, F., Maggini, F., 1996. Nucleotide sequence of the internal transcribed spacers and 5.8S region of ribosomal DNA in *Pinus pinea* L. DNA Sequence 6, 175-177.

Matziris, D., 1997. Variation in Growth, Flowering and Cone Production in a Clonal Seed Orchard of Aleppo Pine Grown in Greece. Silvae Genetica 46, 4.

M'Hirit, O. 1999. Mediterrenean Forests: ecological space and economic and community wealth. Unasylva, 197, 3-15.

Mirov, N.T. 1952-53. Chemical aspects of diploxylon pines. Zeitshrift für Forstgenetik und Frostpflanzenzüchtung, 2. Band, Heft 5, pp.93-96.

Mirov, N. T. And Iloff, P. M. Jr.1955. Composition of gum turpentines of pines. J. Of the American Pharmaceutical Ass. Sc. Edition. XLIX: 186-189.

Mirov, N.T. 1955. Relationships between *Pinus halepensis* and other insignies pines of the Mediterrenean region. Bultin Research Council, Israel 5D: 65-72.

Mirov, N.T. 1961. Composition of gum terpentines of pines. U.S.D.A. Forest Srevice, Pasific South West Forest and Range Experimant Station, Technical Bulletin, No. 1239.

Mirov, N.T., Zavarin, E. And Snajberk, K. 1966. Chemical composition of the terpentines of some eastern Mediterrenean pines in relation to their classification. Phytochemistry 5: 97-102.

Mirov, N.T. 1967. The Genus *Pinus*. The Ronald Press Company, New York, N. Y.pp. 252-254.

Morgante, M., Felice, N. And Vendramin, G.G. 1998. Analysis of hyper-variable chloroplast microsatellites in *Pinus halepensis* reveals a dramatic genetic bottleneck. In: Karp, A., Isaac, G.P. and Ingram D.S.(eds), Molecular Tools for Screening Biodiversity. Chapman and Hall, London, pp.407-412.

Moulalis D**.** 1991. Heritability of resin yield in *Pinus halepensis*. Forstwissenschaftliches Centralblatt 110 (6): 380-392.

Moulalis D., Bassiotis, C., Mıtsopoulos, D. 1976. Controlled pollinations among Pine species in Greece. Silvae Genetica 25(3-4): 95-107.

Moulopoulos, CH. And Bassiotis, C.1961**.** Artificial hybrids of *Pinus halepensis* and *Pinus brutia*. Annals of the Agricultural and Forestry Faculty, Aristotelian University, Salonica, 161-179.

Muthoo, K.M. 1997. Forests and forestry in Turkey. FAO, Ankara.

Nahal, I.1962. Le pin D'alep (*Pinus halepensis* MILL.) Etude taxonomique, Phytogeographique ecologique et sylvicole. Annales de I'ecole Nat. Des eaux et Forets. XIX (4): 475-686.

Nathan, R., Safriel, U.N., Noy-Meir, I., Schiller, G. 1999. Seed release without fire in *Pinus halepensis*, a Mediterrenean serotinous wind-dispersed tree. Journal of Ecology 87: 659-669.

Nei, M. and Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Oppenheimer, H.R. 1967. Mechanism on drought resistance in Conifers of the Mediterrenean zone and the arid west of the U.S.A. Part I. Physiological and Anatomical Investigations. Final on project no. A 10-FS 7, Grant No.FG-Is-119. Herbew University of Agriculture, Rehovot, Israel.pp.73.

Özer, H. 1997. The patterns of genetic variation in *P.brutia* TEN. seed stands: implications for *in situ* conservation. A master's thesis. Middle East Technical University, Graduate School for Natural adn Applied Sciences. Ankara, Turkey. 60 pp.

Palmberg, C. 1976. Geographic variation and early growth in south-eastern semi-arid Australia of *Pinus halepensis* Mill. and the *P.brutia* Ten. species complex. Silvae Genetica. 24: 150-160.

Panetsos, K. P.1975. Natural hybridization between *Pinus brutia* and *Pinus halepensis* in Greece. Silvae Genetica **24**, 5-6: 163-168.

Panetsos, K. P.1981. Monograph of *Pinus halepensis* (MILL.) and *Pinus brutia* (TEN.). Annales Forestales **9,** No. 2: 39-77, Zagreb.

Panetsos, K.P., Moulalis, D., Mitsopoulos, D. 1983. Artificial hybrids between *Pinus brutia* and *Pinus halepensis* in Greece. Growth Adaptation Lab. of Forest Genetics and Forest Plant Breeding, Univers. of Thessaloniki, pp. 18.

Panetsos, K. P.1986b. Genetics and breeding in the group halepensis. Foret mediterraneenne VIII, No. 1: 5-12.

Panetsos, K. P.1989. Hybridization and its implications on Forest Tree Breeding. Lab. of Forst Genetics and Forest Plant Breeding. Ar. Univers. Of Thessaloniki. pp. 19.

Panetsos, K. P.1990. Artificial hybrids F1 and advanced generations between the species *Pinus brutia* (TEN.) X *Pinus halepensis* (MILL.). Scien. Annals of Dept. of Forestry and Natural Environment, Ar. Univers. Of Thessaloniki. Vol. LC/1(8): 287-298.

Papajoannou, J. 1936**.** Über Artbastarde zwischen *Pinus brutia* Ten. und *Pinus halepensis* Mill. in Nordost-Chalkidiki (Griechenland). Forstwissenschaftiches Centralblatt 58: 194-205.

Papajoannou, I.1954. Hybridization of Mediterrenean pines and its influence on resin production and especially in Greece. To Dassos. 25-28: 104-116 (in Gr, Fr. Sum.).

Price, R.A., Liston A., and Strauss, S.H. 1998. Phylogeny and systematics of Pinus. pp. 49-68 in: Richardson, D.M. (e.d.), Ecology and Biogeography of Pinus. Cambridge University Press.

Prim, R.C. 1957. Shortest connection networks and some generalizations. Bell. Syst. Tech. J. 36: 1389-1401.

Provan, J., Soranzo, N., Wilson, J., McNicol, W., Forrest, G.I, Cotrell, J., Powell, W. 1998. Gene-Pool variation in Caledonian and European Scots Pine (*P.sylvestris* L.) revealed by chloroplast simple-sequence repeats. Proceedings: Biological Sciences, Vol.265, pp. 1697-1705.

Prus-Glowacki, W., Szweykowski, J. and Nowak, R. 1985. Serotaxonomical investigation of the European pine species. Silvae Genetica. 34: 162-170.

Puglisi, S., Lovreglio, R., Cifarelli, S. and Leon, V. 1999. Allozyme variations in five populations of *P.halepensis* Mill. in southern Italy. Forest Genetics 6: 241-246.

Qu, L.H., XI, B.Q, SHI, S.H., and Zhang, H.D. 1993. A characteristic size of the ITS in gymnosperm rDNA. P.201 in *XV International Botanical Congress Abstracts* Yokohama, Japan: International Union of Biological Sciences.

Quezel, P. 1985. Definition of the Mediterrenean region and the origin of it's flora. In: Gomez-Campo (ed.), Plant conservation in the Mediterrenean area. Junk, Dordrecht, pp. 9-24.

Quezel, P. 2000. Taxonomy and biogeograhy of mediterrenean pines (*Pinus halepensis* and *Pinus brutia*). In: G. Ne'eman and L. Trabaud (eds). Ecology, Biogeography and Management of *Pinus halepensis* and *P. brutia* Forest Ecosystems in the Mediterrenean Basin, pp. 1-12, Backlahuys Publisher, Leiden.

Quijada, A. 1996. La región de los espaciadores internos transcritos (ITS) del DNA ribosomal del nùcleo (nrDNA): detección de hibridación y varición intraspecifica en pinos. MSc thesis, Universidad Nacional Autónoma de Mexico, Mexico.

Quijada, A., Liston, A., Delgado, P., Lobo, A.V., Alvarez-Buylla, E.R. 1998. Variation in the nuclear ribosomal DNA internal transcribed spacer (ITS) region of *Pinus rzedowskii* revealed by PCR-RFLP. Theor. Appl. Genet. 96:539-544.

Reynolds, J., Weir, B.S, and Cockerham, C.C. 1983. Estimation for the coancestry coefficient: basis for a short term genetic distance. Genetics 105: 767-779.

Robledo-Arnuncio, J., Collada, C., Alia, R., Gil, L. 2005. Genetic structure of montane isolates of *Pinus sylvestris* L. in a Mediterrenean refugial area. Journal of Biogeography. 32, 595-605.

Rogers S.O., Kaya Z., 2006. DNA from ancient Cedar wood from King Midas'Tomb, Turkey, and Al-Aksa Mosque, Israel. Silvae Genetica 55,2 (54-62).

Rohlf, F.J. 1973. Algorithm 76. Hierarchial clustering using the minimum spaning tree. The computer Journal. 16:93-95.

Saatçioğlu, F. 1971. Application possibilities of clearcutting in Turkish forests. Orman Mühendisliği Dergisi Sayı 2, pp. 6-10.

Saitou, N. and Nei., M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sang, T., Crawford, D.J., Stuessy, T.F., 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. (USA) 92, 6813-6817.

Saracino, A. Pacella, R. Leone, V. And Borghetti, M. 1997. Seed dispersal and changing seed characteristics in a *Pinus halepensis* Mill. forest after fire. Plant Ecology 130: 13-19.

Schiller, G., Conkle, M.T., Grunwald, C.1986. Local differrentiation among Mediterrenean populations of Aleppo pine in their isoenzymes. Silvae Genetica 35: 11-19.

Schiller, G. and Grünwald, C. 1987. Resin monoterpenes in range-wide provenance trials of *Pinus halepensis* Mill. in Israel. Silvae Genetica 36 (3-4): 109-114.

Schiller, G. and Grünwald, C. 1987. Cortex resin monoterpene composition in *P.brutia* provenances grown in Israel. Biochem. Sys. And Ecol. 15(4): 389-394.

Schiller, G. and Genizi, A. 1993. An attempt to identify the origin of *P.brutia* TEN. plantations in Israel by needle resin composition. Silvae Genetica. 42: 63-68.

Schiller, G. 1994. Diversity among *Pinus brutia* subsp. *brutia* and related taxa- a review. İ.Ü Orman Fakültesi Dergisi, Seri A, Cilt 44, pp. 133-147, İstanbul.

Schiller, G., Karschon, K. And Weinstein, A. 1981. Growth of Aleppo pine in the Indian hills as affected by geological substrate. Israel Forestry Division. The Volcani Center, Bet Dagan Publ. No. 200. pages 7.

Schiller, G. 2000 a. Inter and intra specific genetic diversity of *Pinus halepensis* Mill. and *Pinus brutia* Ten. In: G. Ne'eman and L. Trabaud (eds). Ecology, Biogeography and Management of *Pinus halepensis* and *P.brutia* Forest Ecosystems in the Mediterrenean Basin, pp. 13-35, Backhuys Publisher, Leiden.

Schirone, B., Piovesan, G., Bellarose, R. And Pelosi, C. 1991. A taxonomic analysis of seed proteins in *Pinus* spp. (Pinaceae). Plant systematic and Evolution 178: 43-53.

Schmida, A., Lev-Yadun, S., Goubitz, S. and Ne'eman G. 2000. Sexual allocation and gender segregation in *Pinus halepensis, Pinus brutia, Pinus pinea*. In: G. Ne'eman and L.Trabaud (eds). Ecology, Biogeography and management of *Pinus halepensis* and *Pinus brutia* Forest Ecosystems in the Mediterrenean Basin, Backhuys Publisher, pp.91-104, Leiden.

Schneider, S., Roessli, D., and Excoeffier, L. 2000. ARLEQUIN ver 2.000. A software for population genetics data analysis. Department of Anthropology and Ecology. University of Geneva, Switzerland.

Selik, M. 1958. Botanical investigation on *Pinus brutia* especially in comparison with *P.halepensis*. 1st Univ. Orm. Fab. Derg. 8a: 161-198.

Semerikov, L.V., Lascoux, M. 2003. Nuclear and Cytoplasmic variation within and between Eurasian *Larix* species. American Journal of Botany 90(8): 1113-1123.

Slatkin, M. 1991. Inbreeding coefficients and coalescence times. Genet. Res. Camb. 58: 167-175.

Slatkin, M. 1995. A measure of population subdivision based on micro-satellite allele frequencies. Genetics: 139: 457-462.

Soltis, P.S., and Kuzoff. 1993. ITS sequence variation within and among populations of *Lomatium grayi* and *L. laevigatum*. Molecular Phylogenetics and Evolution 2: 166-170.

Suh, Y., Thien, B., Reeve H.E, and Zimmer E.A. 1993. Molecular evolution and phylogenetic implications of internel transcribed spacer sequences of ribosomal DNA in Winteraceae. American Journal of Botany 80: 1042-1055.

Teisseire, H., Fady, B., Pichot, C. 1995. Allozyme variation in five French populations of Aleppo pine. Forest Genetics 2: 225-236.

Thanos, C.A and Daskalakou, E.N. 2000. Post-fire regeneration of *Pinus brutia* forests. In: G. Ne'eman and L. Trabaud (eds). Ecology, Biogeography and Management of *Pinus halepensis* and *P.brutia* Forest Ecosystems in the Mediterrenean Basin, pp.291-301, Backhuys Publisher, Leiden.

Thampson, J.D., Higgins, T.J. 1994. Clustal W: improving the sensivity of progressive multiple sequence alignment through sequence weighting, positions specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.

Trabaud L. 1987. Fire and survival traits of plants. In: Trabaud, L. (ed.), The role of fire in ecological sysytems. SPB Academic publishing. The Hague, pp. 65-89.

Trabaud L., Christensen N.L. and Gill. A.M. 1993. Historical biogeography of fire in temperate and Mediterrenean ecosystems. In: Crutzen, P.J. and Goldammer, J.G. (eds.), Fire in the environment: its ecological and atmospheric importanc. New York, pp. 277-295.

Vendramin, G.G., Anzıdei, M., Autino, V.A., Madaghiele, A., Sperisen, C., Morgante, M., Zıegenhangen, B. 1998. Chloroplast microsatellites reveal high levels of genetic diversity in conifers: A new tool for biodiversity analysis in forest ecosystem. In: Scannerini, S.(ed.). Proc. Plant Biotechnology as a Tool for the exploitation of Mountain Lands. Acta Horticultura. 457: 395-401.

Vidakoviç, M. 1991. Conifers: morphology and variation. Graficki Zavod Tlrvatske, Zagreb, Crotia, 755 p.

Vildrich, V., Michelozzi, M., Franci, M. And Fusi, P. 1993. Chemical from different provenances of *Pinus brutia* Ten. Uluslararası Kızılçam Sempozyumu (18-23 Ekim 1993, Marmaris), Orman Bakanlığı Yayını, pp. 324-330

Wang, X-R, Tsumura, Y., Yoshimaru, H., Nagasaka, K., and Szmidt, A. 1999. Phylogenetic relationships of Eurasian pines (*Pinus, Pinaceae*) based on chloroplast *RBCL, MatK, RPL20-RPS18* spacer, and *TrnV* intron sequences. American Journal of Botany, 86(12): 1742-1753.

Wei, X., Wang, X. 2004. Recolonization and radiation in Larix (*Pinaceae*): evidence from nuclear ribosomal DNA paralogues. Molecular Ecology. 13: 3115-3123.

Weinstain, A. 1989a. Provenance evaluation of *Pinus halepensis*, *Pinus brutia* and *P.elderica* in Israel. Forest Ecology Management, 26: 215-225.

Weinstain, A. 1989b. Geographic variation and phenology of *Pinus halepensis*, *Pinus brutia* and *P.elderica* in Israel. Forest Ecology Management, 27: 99-108.

Weir, B.S. and Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. Evolution, 38: 1358-1370.

Wendel, J.F., Schnabel, A., Seelanan, T., 1995. Bi-directional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci. (USA) 92, 280-284.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes, M., Gelfand, D., Sninsky, J., White, T. (Eds), PCR Protocols: A guide to Methods and Applications. Academic Press, San Diego, CA, pp. 315-322.

Wright, S. 1951. The genetical structure of populations. Annals of Eugenics, 15, 323-354.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regards to systems of mating. Evolution, 19, 395-420.

Yaltırık, F., Boydak, M. 1989. A new variety and variations of *Pinus brutia* Ten. in Turkey. İ.Ü. Orman Fakültesi Dergisi, Seri A, Cilt 39, sayı 2, pp. 42-64.

Yaltırık, F., Boydak, M. 1993. Türkiye Kızılçamlarında Genetik Çeşitlilik (Varyasyon). In: Proceedings of the FAO-IUFRO and the Turkish Ministry of Forestry International Symposium on *Pinus brutia* Ten., Marmaris, Ministry of Forestry, Turkey, pp. 1-10. (Turkish with English abstract)

Yaltırık, F., Efe A. 1994. Dendrology, Gymnospermae-Angiospermae. İ.Ü Orman Fakültesi Yayını, No. 3836/431, 382p., İstanbul.

Yahyaoğlu, Z., Ayaz, F.A., and Genç, M. 1993. Provenance seperation by using isoenzyme analysis in *P.brutia* TEN. In : Proceedings of the FAO-IUFRO and the Turkish Ministry of Forestry, Turkey.pp. 254-263.

Yıldırım, T. 1992. Genetic variation in shoot growth patterns in *P.brutia* TEN. A master's thesis (unpublished). Middle East Technical University, Graduate School of Natural and Applied Sciences, Ankara, Turkey. 53 pp.

Zohary, M. 1962. Plant Life of Palestine; Israel and Jordan. The Ronald Press, New York.

Zohary, M. 1973. Geobotanical Foundation of the Middle East. Gustav Fischer Verlag, Stuttgart.

APPENDIX A

BUFFERS, CHEMICALS and EQUIPMENTS

Buffers and Solutions for Polymerase Chain Reaction (PCR)

10X PCR Buffer ($MgCl_2$ free) (BIORON):

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

dNTPs (LAROVA) : 5 mM

Taq DNA polymerase (BIORON): 5U/µl

Sterile Water: dH_2O

Primer-pairs: 20 µM

Electrophoresis Buffers and Gel Systems

***Agarose Gel Electrophoresis**

Running Buffers: X TAE prepared in distilled H_2O

Agarose, (SIGMA): 1,5 or 2 percent (w/v) Agarose gel

Ethyidium Bromide, (SIGMA): 4mg/ml

Loading Buffer: 9.5 ml Formamide, (SIGMA)

500 µl EDTA (0.5 M)

15 mg Bromophenolblue, (SIGMA)

15 mg Xylene cyanol, (SIGMA)

Equipments

Autoclave: Kermanlar – İSTANBUL

Centrifuge: Sigma 113

Deepfreezer: Sanyo – Medical Freezer

Vertical Electrophoresis System: Hoefer SE 600 Series Elect. Unit

Horizontal Electrophoresis System: Maxicell EC360M Elect. Unit

Thermocyclers: Eppendorf- Mastercycler, Techne-genius

Magnetic Stirrer: Labor Brand/Hotplate L-81

Ovens : Dedeoğlu

PH meter : Hanna Inst.

Power Supplies: EC135-90 E-C

Refrigerator: AEG

UV Transilluminator : Vilbor Lourmant

Vortex : Nüve NM110

Water Bath: Memmert

Micropipettes: GILSON

Hamilton Syringe: Microliter syringe

APPENDIX B

A PART OF THE MEGA DATA FILE

Sequence data for Turkish red pine and Aleppo pine populations
Number of populations = 10
Number of samples = 28

>3-Aleppo pine/Adana-Kadirli-Bahadırlı

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTYGAGCGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA
CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGR
CCAGCTCCGCWGCTCCCTAGCATATTGCAGGGAGCGCGTATGAATGTG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC

>16- Aleppo pine/Muğla-Ula-Kızılyaka

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTCGAGCGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA

CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGA
CCAGCTCCGCTGCTCCCTAGCATATTGCAGGGAGCGCGTWTGAATGTG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC

>23-Aleppo pine/Muğla-Gökova

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTCGAGCGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA
CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGA
CCAGCTCCGCTGCTCCCTAGCATATTGCAGGGAGCGCGTWTGAATGTG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC

>8-Turkish red pine/Muğla-gökova

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTCGAGCGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA
CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGA
CCAGCTCCGCTGCTCCCTAGCATATTGCAGGGAGCGCGTWTGAATGGG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC

>24-Turkish red pine/Adana-Pos-karsantı

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTCGAGSGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA
CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGA
CCAGCTCCGCTGCTCCCTAGCATATTGCAGGGAGCGCGTATGAATGGG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC

>43-Turkish red pine/Adana-Pos-Soğukoluk

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTCGAGSGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA
CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGA
CCAGCTCCGCTGCTCCCTAGCATATTGCAGGGAGCGCGTATGAATGGG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC

>18-Turkish red pine/Muğla-Ula-Kızılyaka

AAGTTGGAGCTAAATCCGCCCTACCCGATGGGAGGACAAGAGAGAGC
AAAGCAAGTTCGAGSGATGCCACACAAAGCCCGCATCAGCTAACGCCG
ACT-
GCCCATCCAAGGGGACAAGGTCACCGCTCGCCGATGCACGCCACGAGA

CATCGCCTGAGGCATTTCAGCCGACCGCACCGCATGGGGCACGGACGA
CCAGCTCCGCTGCTCCCTAGCATATTGCAGGGAGCGCGTATGAATGGG
ATGCGACGCCCAGACAGACGTGCCCTCGACCGAGGCCTCGGGCGCAAA
TTGCGTTCAAAAACTCGATGATTCACGGGATTCTGCAATTCACACTAAG
TATC