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The phylogenetic relationship between populations of marginally and sympatrically located *Pinus halepensis* Mill. and *Pinus brutia* Ten. in Turkey, based on the ITS-2 region

C. Özge TOZKAR, Sertaç ÖNDE, Zeki KAYA*

Department of Biological Sciences, Middle East Technical University, 06531 Ankara - TURKEY

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Abstract: Turkish red pine (*Pinus brutia*) is a widespread and important forest tree species in Turkey, occurring mainly in southern, western, and northwestern Turkey, while the natural occurrence of Aleppo pine (*Pinus halepensis*) is restricted to 2 locations and is found sympatrically with Turkish red pine. In the present study sympatric populations of both species from Muğla and Adana provinces in Turkey were sampled, and the internal transcribed spacer 2 (ITS-2) region of ribosomal DNA was comparatively studied with sequence analysis. Analysis of molecular variance (AMOVA) demonstrated 100% of total molecular variation between the species in Muğla province, versus only 50.65% in Adana province. Construction of a phylogenetic tree with a bootstrap value of 92% revealed that Aleppo pine and Turkish red pine samples at the species level were well separated. Estimated F_{ST} values indicated that Turkish red pine and Aleppo pine were highly differentiated in Muğla province due to possible reproductive isolation, while the 2 species shared a more common genetic background due to possible natural hybridization in Adana province.

Key words: Turkish red pine, Aleppo pine, ITS region, phylogeny, genetic differentiation, molecular diversity

Türkiye'de marjinal ve örtüşük olarak yayılış gösteren doğal *Pinus halepensis* Mill ve *Pinus brutia* Ten. popülasyonlarının ITS-2 bölgesine göre filogenetik ilişkileri

Özet: Kızılçam (*Pinus brutia* Ten.) Türkiye için yaygın ve önemli bir orman ağacı türü olup yoğunlukla güney, batı ve kuzey batı bölgelerinde yayılım gösterirken, Halep çamı'nın (*Pinus halepensis* Mill.) doğal yayılışı iki alan ile sınırlı olup kızılçam ile örtüşüktür. Bu çalışmada her iki türün Muğla ve Adana illerindeki örtüşük yayılış gösteren popülasyonları örneklenmiş ve ribozomal DNA'nın "Internal Transcribed Spacer-2 (ITS-2)" bölgeleri sekans analizi yöntemi ile karşılaştırmalı olarak çalışılmıştır. Moleküler Varyans Analizi (AMOVA) Muğla ilinde toplam moleküler varyasyonun tamamının, Adana ilinde ise % 50.65 'nin türler arasında olduğunu göstermiştir. Yüzde 92 "seç-bağla (bootstrap)" değeriyle oluşturulan filogenetik ağaç Halep çamı ve Kızılçam örneklerinin tür bazında iyi bir ayrım gösterdiğini ortaya koymuştur. Hesaplanan F_{ST} değerleri; Muğla ili için Halep çamının olası bir üreme-izolasyonu sebebiyle yüksek düzeyde farklılaşma gösterdiğini, buna karşın bu iki türün Adana ilinde olası bir doğal melezleme nedeniyle daha fazla ortak bir genetik geçmişe sahip olduklarını işaret etmektedir.

Anahtar sözcükler: Kızılçam, Halep çamı, ITS bölgesi, filogeni, genetik farklılaşma, moleküler çeşitlilik

* E-mail: kayaz@metu.edu.tr

Introduction

Turkish red pine and Aleppo pine belong to the family Pinaceae, and both are included in the subsection *Sylvestres* of the section *Pinus* (*Diploxylon*) (Critchfieldt and Little, 1966; Quezel, 2000), although Klaus (1989) placed them in the subsection *halepensis* of the section *Pinus*. Although it is disputed (Frankis, 1993; Quezel, 2000), Turkish red pine is regarded by some taxonomists as a variety of *Pinus halepensis* Mill. (*P. halepensis* var. *brutia*) (Duffield, 1952) because these 2 species hybridize with each other naturally (Panetsos, 1975; Yaltırık and Boydak, 1993) and artificially (Moulalis et al., 1976). Thus, the production of natural hybrids is to be expected whenever 2 species come in contact (Panetsos, 1975).

Turkish red pine (*Pinus brutia* Ten.) is naturally distributed mainly in the Mediterranean, western, and northwestern regions of Turkey; however, small isolated populations can be also found in some areas of the Black Sea region with a micro climate similar to that of the Mediterranean (Kayacık, 1954; Davis, 1965; Arbez, 1974; Atalay, 1982) (Figure 1A). Turkish red pine is an economically important forest tree species, having the largest distribution in Turkey, covering about 4.2 million ha of forest (Devlet Planlama Teşkilatı, 2001), whereas Aleppo pine distribution is limited to one location in the Aegean region and another location in the Mediterranean region of Turkey (Figure 1B) (Kayacık, 1954; Yaltırık and Boydak, 1989; Yaltırık, 1993).

Nuclear ribosomal DNA (nrDNA) has 2 internal transcribed spacers (ITS-1 and ITS-2) that are located between the small subunit (16s-18s) and 5.8S rRNA cistronic regions, and between the 5.8S and large subunit (23S-28S) rRNA cistronic regions, respectively. The 2 spacers and the 5.8S subunit are collectively known as the internal transcribed spacer (ITS) region and have become an important nuclear locus for systematic molecular investigations of closely related taxa. This is because the ITS region is highly conserved intraspecifically, but variable between different species (Bruns et al., 1991; Hillis and Dixon, 1991). Furthermore, the ITS region evolves much more rapidly than other conserved regions of rDNA (Baldwin et al., 1995). Thus, phylogenetic studies based on nrDNA, ITS sequences have provided novel insights into plant evolution and hybridization in various plant species (Baldwin et al., 1995; Sang et al., 1995; Wendel et al., 1995; Buckler and Holtsford, 1996a; Quijada et al., 1998; Semerikov and Lascoux, 2003); however, at least for the genus *Corylus* there is a reported case in which the ITS region failed to explain the genetic relationship between species (Erdoğan and Mehlenbacher, 2000).

On a large scale, the relationship between Aleppo and Turkish red pine species has been extensively studied using morphological, anatomical, and ecological traits, biochemical, chemical, and molecular markers, karyotype analysis, and sexual hybridization. Most of these studies reported the divergence of Turkish red pine and Aleppo pine (Mirov, 1961; Mirov et al., 1966; Panetsos, 1981; Conkle et al., 1988; Vidakovic, 1991; Acar, 1993; Schiller, 2000a), but genetic and evolutionary relationships between naturally occurring Aleppo pine and Turkish red pine populations in marginal and sympatric localities have not yet been investigated. The present study aimed to describe the taxonomic and evolutionary relationship between the natural-admixture populations of these 2 closely related species in Turkey with the use of ITS-2 region-sequence comparison.

Materials and methods

Plant material

Seeds from 4 populations of Turkish red pine (Figure 1A) and 3 populations of Aleppo pine (Figure 1B) were obtained from Muğla and Adana provinces, with the collaboration of the Turkish Forestry Tree Seeds and Tree Breeding Research Directorate, Ankara (Table 1A). The Aleppo pine population located in Adana province consists of about 178 ha of pure-natural and relatively young stands (< 40 years of age), and the remaining is mixed with Turkish red pine. There were 2 Aleppo pine populations in Muğla province and both were included in the study. One of the 2 populations, Muğla-Kızılyaka, is an Aleppo pine seed-orchard that was established with 10 clones that represent a nearby Aleppo-Turkish red pine natural-mixed stand in the same region. The second Aleppo pine population was from Muğla-Gökova, which

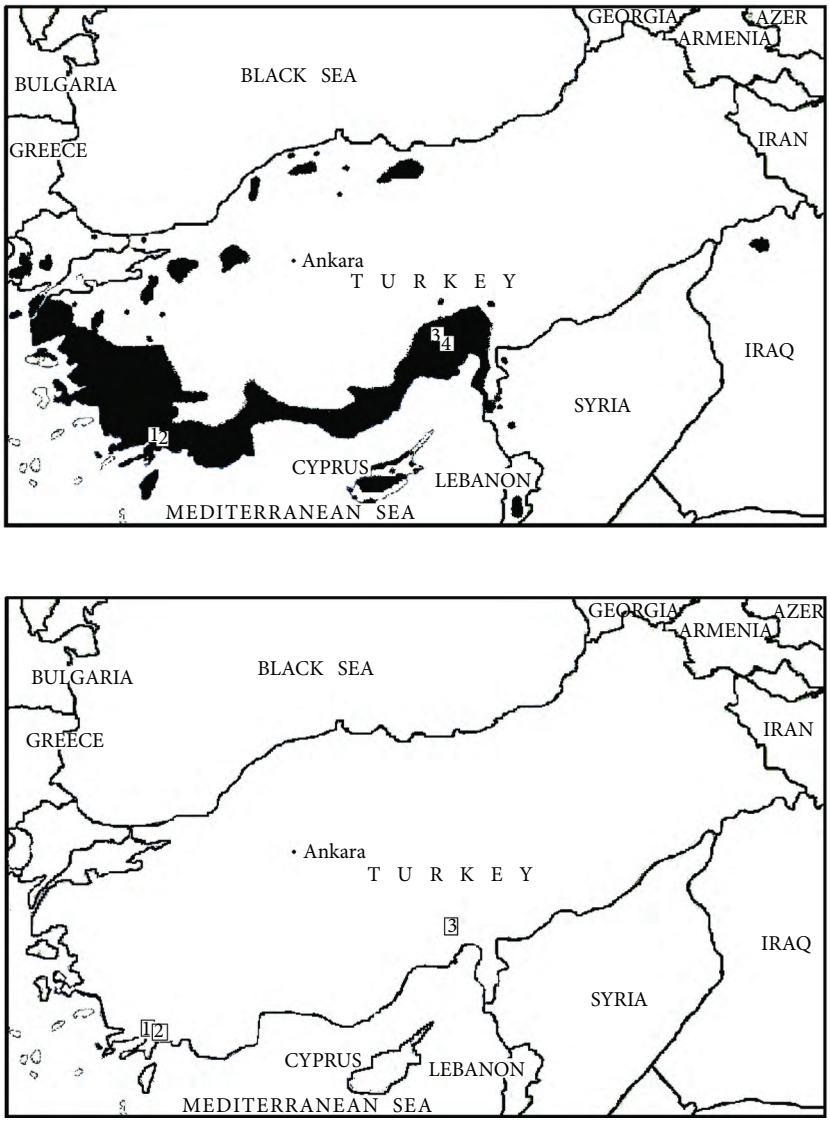

Figure 1. A. Map showing the natural distribution of Turkish red pine (shaded area) in Turkey and locations of the sampled populations. The populations used in the study were as follows: 1. Muğla-Gökova, 2. Muğla-Ula-Kızılyaka, 3. Adana-Pos-Karsantı, and 4. Adana-Pos-Soğukoluk.
B. Map showing the locations of naturally occurring Aleppo pine populations. The populations are as follows: 1. Muğla-Gökova, 2. Muğla-Ula-Kızılyaka, and 3. Adana-Kadirli-Bahadırlı.

consists of pure-natural (84.5 ha) stands > 40 years of age and mixed stands with Turkish red pine. The Turkish red pine populations were further away from the Aleppo pine populations in Adana province than they were in Muğla province. Detailed topographic and geographic data for the study populations are provided in Table 1A.

DNA extraction

For each population of Turkish red pine and Aleppo pine megagametophyte tissue from at least 36 seeds was used for DNA extraction. A combination of DNA extraction methods from Dellaporta et al. (1983) and Kreike (1990) were adapted (Kaya et al., 1995) to our laboratory conditions.

Table 1. A. Detailed description of Turkish red pine and Aleppo pine populations used in the study.

Species	Regional Forest Directorate	Forest Management Directorate	Forestry Unit	Latitude	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°00′17	28°28′11	100
Turkish Red pine	Muğla	Ula	Kızılyaka	37°05′33	28°32′22	680
Aleppo pine	Muğla	Muğla	Gökova	37°01′45	28°06′25	50

Polymerase chain reaction (PCR) primers

Specifically designed PCR primers were used to amplify the internal transcribed spacers (ITS-1 and ITS-2) of Turkish red pine and Aleppo pine. For ITS-1 region primers ITS Plant1 (TCCGTATGTGAACCTGCGG, forward primer) and ITS-Gym2 (GGGGAATCCTGGTTAGTTC, reverse primer) were used. For the ITS-2 region the primers ITS-Gym3 (GCACCGATGAAGAATGTAGC, forward primer) and ITS-Plant4B (GGGGAATCCTGGTTAGTTTC, reverse primer) were utilized. These primers were specifically designed by Rogers and Kaya (2006) for conifers.

The optimized PCR reaction mixture consisted of 1 pmol of each primer (primer pairs ITS Pant1/ITS-Gym2 and ITS-Gym3/ITS Plant4B, and all were used separately in reactions), 5 mM of dNTPs, 25 mM of $MgCl_2$, 10× reaction buffer, 5 units μL^{-1} of *Taq* DNA polymerase, BSA (1.8 $\mu g\ \mu L^{-1}$), 0.13 µL of Tween 20, and 3 ng μL^{-1} of DNA. 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). For all the primer-pairs, after an initial denaturation step at 94 °C for 5 min, the PCR cycles involved 30 cycles of amplification, including denaturation at 94 °C for 30 s, annealing at 55 °C for 1.5 min, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 5 min. The PCR amplified products were then stored at −20 °C until they were used. Each PCR reaction was subjected to electrophoresis on agarose gel. When electrophoresis was completed, DNA fragments were stained with 5 $\mu g\ mL^{-1}$ of ethidium bromide. After staining, the bands were visualized by direct examination of the gel under UV light and were photographed using a gel visualization system (Vilber Lourmat, France). A θX174 DNA/BsuRI (HaeIII) marker (MBI Fermentas) was used to determine the size of the ITS-2 bands. Although the primers designed for both the ITS-1 and ITS-2 regions were tested, primers for ITS-1 did not yield any PCR amplification. As such, hereafter only information for the ITS-2 region will be provided. ITS amplicons from PCR amplification with ITS-2 primers were about 420 bp long. All PCR products were purified before sequencing using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions.

DNA sequencing

Purified PCR products were sequenced with the amplification primers by a biotechnology company (Refgen Biotechnology, METU Teknokent, Ankara), according to the primer strategy outlined in the *ABI 310 Genetic Analyser User's Manual*. In the sequencing, the Big Dye Cycle Sequencing Kit (Applied Biosystems) and ABI 310 Genetic Analyser (PE Applied Biosystems) automatic sequencer were used. For each sample, forward and reverse sequencing reactions were obtained and compared for sequence confirmation. Resulting ITS-2 sequences were proof-read with CHROMAS Lite v.2.01 software (Chromas software, Technelysium Pty Ltd. [1998-2005]; Gene Codes Corporation, Ann Arbor, MI, USA [(2000]). Comparison of the data from each sequence was performed using Sequencer Software (demo version) (http://www.genecodes.com) and a consensus sequence for each sample was formed.

Data analysis

In all, 28 proof-read ITS-2 regions were subjected to sequence alignment processes, which were performed with Clustal W (Thompson and Higgins, 1994). The 5' and 3' ends of the alignment were trimmed to remove missing data or unreliable readings from the sequences with BioEdit v.5.0.6 (Hall, 2001). Then, edited sequences were submitted to the NCBI database (Table 1B). These sequences of the ITS-2 region of the nuclear genome were organized so that they could be analyzed with MEGA v.3.1 software (Kumar et al., 2005) and used to construct input data for analysis by Arlequin v.2.0 software (for population genetics data analysis) (Schneider et al., 2000).

BLAST searches and phylogenetic analysis

To compare our Aleppo pine and Turkish red pine ITS-2 sequence data with data for other related pine species from the same region, an outgroup and the most closely related species were chosen on the basis of previous phylogenetic studies within *Pinus* (Liston et al., 1999). BLAST searches of the international sequence database (NCBI [National Center for Biotechnology Information] BLASTN search [http://www.ncbi.nlm.nih.gov/]) were used to include the sequences of the outgroup (*Picea rubens*), and the species most closely related to Turkish red pine and Aleppo pine sequences. In all, 28 sequences (Table 1B), along with 7 conifer rDNA ITS-2 sequences from NCBI (*Pinus resinosa* (subsection *Pinus*)-Genebank

Table 1. B. Number of sequences submitted to the NCBI GeneBank database and their codes.

Species	Location	Codes given in the paper	NCBI GeneBank Codes
Turkish red pine	Muğla	4gPBmugla	EU647182
		7gPBmugla	EU647183
		8gPBmugla	EU647184
		9gPBmugla	EU647185
		18ukPBmugla	EU647203
		37ukPBmugla	EU647204
		52ukPBmugla	EU647205
		15ukPBmugla	EU647202
	Adana	5pkPBadana	EU647194
		22pkPBadana	EU647195
		24pkPBadana	EU647196
		28pkPBadana	EU647197
		39psPBadana	EU647198
		40psPBadana	EU647199
		41psPBadana	EU647200
		43psPBadana	EU647201
Aleppo pine	Muğla	13gPHmugla	EU647186
		19gPHmugla	EU647187
		22gPHmugla	EU647188
		23gPHmugla	EU647189
		7ukPHmugla	EU647206
		16ukPHmugla	EU647207
		25ukPHmugla	EU647208
		59ukPHmugla	EU647209
	Adana	3kbPHadana	EU647190
		19kbPHadana	EU647191
		24kbPHadana	EU647192
		25kbPHadana	EU647193

accession number AF37002, *Pinus sylvestris* (subsection *Pinus*)-AF37003, *Pinus pinea* (subsection *Pinea*)-PPITS12RN, *Pinus pinaster* (subsection *Pinaster*)-AF037024, *Pinus halepensis* (subsection *Pinaster)*-AF037007, *Pinus strobus* (section *Strobus*, subsection *Strobi*-AY430069.1, and the most outgroup *Picea rubens*-AF136611), were comparatively analyzed.

Analysis was performed to determine ITS-2 polymorphism in marginally distributed populations of Aleppo pine that occur naturally and sympatrically to Turkish red pine populations. Arlequin software (Schneider et al., 2000) and MEGA v.3.1 software (Kumar et al., 2005) were used to estimate the molecular diversity indices (such as variable and parsimony sites, transitional and transversional pairs, and deletions and substitutions), perform analysis of molecular variance (AMOVA), and determine F_{ST} values and haplotype distribution between the populations. Construction of a phylogenetic tree was carried out using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap testing of phylogeny and interior branch testing of phylogeny were performed according to Nei and Kumar (2000).

Results

Molecular diversity

Unfortunately, although 4 different PCR cycling conditions were tested and 3 different primers (ITS105/ITS594, ITS105/ITS1467 [*Picea*], and ITS-Plant1/ITS-Gym2 [Fungi]), which were previously screened for *Picea* and Fungi, were used, no DNA amplification was detected.

The ITS-2 region-sequence length for Aleppo pine and Turkish red pine populations was 348 bp, which consists of 340 identical, 1 transversional pair, and 3 variable and 2 parsimony informative sites. When the populations of Aleppo and Turkish red pine within each province were compared regarding molecular diversity, the pine populations in Adana province were more diverse than those in Muğla province (Table 2). For example, 1 transversion, 3 variable, and 2 parsimony informative sites were observed among the populations of both Aleppo and Turkish red pines from Adana province, while only 1 variable and 1 parsimony informative site were observed in the populations from Muğla province (Table 2).

Genetic differentiation and the evolutionary relationship between Turkish red pine and Aleppo pine species

The first AMOVA was performed to estimate the portion of total molecular diversity attributed to between species, regardless of the origins of the populations of Aleppo and Turkish red pine species. That is, all populations of each species were pooled. The results indicated that great variation (81.23%) exists between the species. When the second AMOVA was performed between Turkish red pine and Aleppo pine populations from Adana province half of the variation (50.65%) was observed between Turkish red

Table 2. Comparative results of ITS-2 sequence data analysis for Turkish red pine and Aleppo pine from the Adana and Muğla regions.

	P. brutia vs. *P. halepensis* populations (Muğla)	*P. brutia* vs. *P. halepensis* populations (Adana)	*P. brutia* vs. *P. halepensis* in both Muğla and Adana populations	*P. brutia* vs. outgroups	*P. halepensis* vs. outgroups
Length (bp)	348[a]	348[a]	348[a]	348[a]	348[a]
Variable Sites	1	3	3	64	64
Parsimony sites	1	2	2	3	2
Identical pairs	340	339	340	332	329
Transitional pairs	0	0	0	3	3
Transversional pairs	1	1	1	4	4

[a] Normally, the total of number of variable and parsimony sites, and identical, transitional, and transversional pairs should be equal to the length of the ITS-2 region, but due to the ambiguity of the base designation in each group some bases were left out of the data evaluation by the software

pine and Aleppo pine, whereas that between populations of the same species was estimated as 14.72% of the total variation (Table 3). A third AMOVA was performed to differentiate Turkish red pine and Aleppo pine populations from Muğla province, indicating that 100% of the total variation was attributed to between species and was therefore not presented here.

F_{ST} values between the Turkish red pine populations and subsection *Pinus, Pinea*, *Pinaster*, section *Strobus* indicate high differentiation (F_{ST} values ranged from 0.42 to 1.00), as expected; however, Aleppo pine populations were closer to subsection *Pinus, Pinea*, *Pinaster*, and section *Strobus* than to Turkish red pine populations, as F_{ST} values were low and did not vary greatly (0.00-0.3).

Differences between F_{ST} values of Turkish red pine populations (Muğla-Gökova, Muğla-Ula-Kızılyaka, Adana-Pos-Karsantı, and Adana-Pos-Soğukoluk), as well as between Aleppo pine populations (Adana-Kadirli-Bahadırlı, Muğla-Ula-Kızılyaka, Muğla-Gökova) were significant at $P \leq 0.05$. Only the F_{ST} values between the Turkish red pine population from Adana-Pos-Karsantı and Aleppo pine population from Adana Kadirli-Bahadırlı were not significant (Table 4).

In all, 36 individual sequences (16 sequences from Turkish red pine populations, 12 from Turkish Aleppo pine populations, 7 sequences from related species, and 1 outgroup sequence of *Picea rubens*) revealed 2 distinct haplotypes. Haplotype-1 was specific to Aleppo pine populations, while the second haplotype was characteristic of Turkish red pine populations from Muğla and Adana. Species taxonomically related to Aleppo pine, that is *P. resinosa*, *P. sylvestris* (subsection *Pinus*), *P. pinea* (subsection *Pinea*), *P. pinaster* (subsection *Pinaster*), and *P. strobus* (section *Strobus*, subsection *Strobi*), were all characterized with haplotype-1, showing affinity to the Aleppo pine cluster.

Phylogenetic relationship between Turkish red pine and Aleppo pine

A phylogenetic tree was constructed using the neighbor-joining method (Nei and Kumar, 2000) and the unrooted tree with branch lengths is shown in Figure 2. The phylogenetic tree revealed 2 major groups; 1 with only Aleppo pine populations, along with subsection *Pinus,* subsection *Pinea*, subsection *Pinaster*, and section *Strobus,* while the other contained only Turkish red pine populations from Adana and Muğla provinces (Figure 2). There were also small clusters formed within 2 major groups, but

Table 3. AMOVA results. A. AMOVA conducted with data from 2 species, regardless of location. B. AMOVA conducted with data from 2 species in Adana.

3A.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percent of total variation
Between species	1	7.122	0.51536	81.23
Between populations within species	5	0.271	0.00	0.00
Within populations	21	2.500	0.119	19.26
Total	27	9.893	0.618	

3B.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percent of total variation
Between species	1	2.917	0.406	50.65
Between populations within species	1	0.750	0.118	14.72
Within populations	9	2.500	0.278	34.63
Total	11	6.167	0.802	

Table 4. Estimated F_{ST} values between populations of Turkish red pine and Aleppo pine in Muğla and Adana provinces.

Species	Population	Turkish red pine			
		Muğla Gökova	Muğla Ula/Kızılyaka	Adana Pos/Karsantı	Adana Pos/Soğukoluk
Aleppo pine	Muğla Gökova	1*	1*	0.60*	1*
	Muğla Ula/Kızılyaka	1*	1*	0.60*	1*
	Adana Kadirli/Bahadırlı	0.75*	0.77*	0.52 ns	0.77*

*Significant at P ≤ 0.05. ns: Not significant at P ≤ 0.05. Number of permutations: ~3000

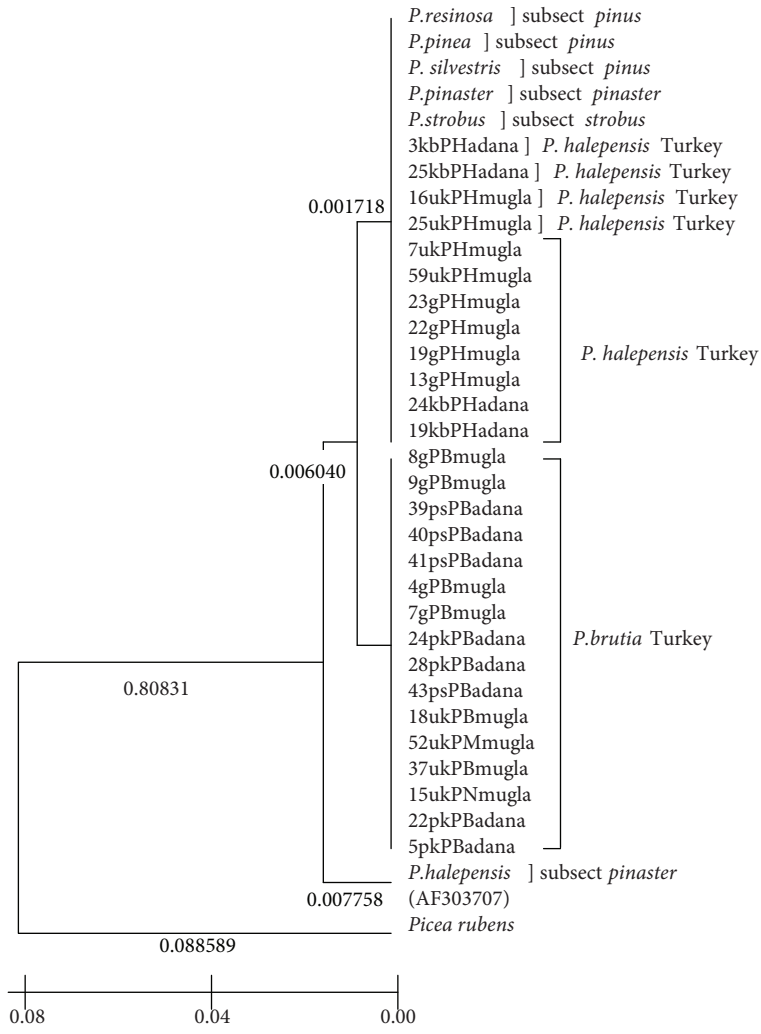

Figure 2. Phylogenetic tree constructed for Turkish red pine and Aleppo pine populations from Turkey using the neighbor-joining method (Nei and Kumar, 2000).

it is difficult to reach definitive conclusions about the relationships because of low bootstrap values (below 50).

Turkish red pine populations were grouped together in the same cluster, but apart from the Aleppo pine group; however, the topology within Turkish red pine and Aleppo pine population samples had low bootstrap support (below 50). Interior branch testing of phylogeny also supported the topology between Turkish red pine and Aleppo pine populations, with a value of 85%.

Discussion

Although the ITS-2 region is rather short, it appears to be suitably variable at the species levels (Sinclair et al., 2002). The length of the ITS-2 region in the present study was somewhat longer (348 bp) than the length of ITS-2 in other phylogenetic studies. The length of ITS-2 varied from 162 bp in subsection *Cembroides* (pinyon pines) (Gernandt et al., 2001) to 243 bp in a phylogenetic study dealing with 47 *Pinus* species (Liston et al., 1999). In the current study, products were sequenced in both the 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 the difference in length was due to inclusion of the nucleotide pairs from the 5.8S nrDNA cistronic region or 28s (large subunit).

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. Turkish red pine and Aleppo pine did not show great differentiation in the ITS-2 region and molecular diversity was lower in both species than in other studied species (Liston et al., 1999; Gernandt et al., 2001). These 2 closely related, marginally and sympatrically distributed species in Turkey could be considered as recently diverged taxa. This is also supported by the observation of common morphological characters, similar geographical ranges (Kayacık, 1954; Yaltırık and Boydak, 1989; Yaltırık, 1993) and natural hybridization between them (Panetsos, 1975, Yaltırık and Boydak, 1993). It was suggested that recent divergence in forest trees would result in lower levels of genetic variation within a clade, and poorly resolved phylogeny due to shared morphologies and interspecific hybridization (Richardson et al., 2001; Wei and Wang, 2004).

Based on the AMOVA results and pair-wise genetic differences observed between Turkish red pine and Aleppo pine populations from both Adana and Muğla provinces, gene flow between these species may not exist in Muğla; a possible genetic drift may have occurred in the past. More genetic divergence between the species in this location may be due to reproductive isolation. On the other hand, the low F_{ST} values between Turkish red pine and Aleppo pine populations in Adana province, as well as the low portion of total molecular variation attributed to species at this location, suggest that an efficient amount of gene flow may have occurred in the past. In a genetic analysis study on *Pinus cembra* L. subsp. *cembra* (Höhn et al., 2005), pair-wise genetic differentiation of populations resulted in low values, even between geographically distant populations, and the possible reasons for this result were attributed to an effective selection mechanism that eliminated inbred embryos and individuals, and/or the possibility of gene flow between populations (wind dispersed pollen and animal mediated seed transfer). The findings of the current study are in accordance with the idea that, phylogenetically, *P. halepensis* and *P. brutia* emerged from a common ancestor and evolved 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 2 species that were also indications of a close evolutionary link between them.

Conclusion

The findings of the present study show that the ITS-2 region of nuclear ribosomal DNA revealed a few variable and parsimony informative sites for both species. In particular, populations of the 2 species in Adana province were less differentiated, possibly due to an exchange of genetic material in the past via natural hybridization and gene introgression, which

could have occurred between them because of the location of plantations in close proximity to native stands. Nonetheless, Aleppo pine populations in Muğla province seem to be highly differentiated from the Turkish pine populations, in terms of the ITS-2 region. This may be due to reproductive isolation between the 2 species in Muğla province; however, this suggestion needs to be tested by expanding the study to the entire ITS region, combined with investigation of the reproductive biology of populations of Turkish red pine and Aleppo pine in Muğla and Adana provinces.

Further studies of the entire ITS region, including ITS-1, ITS-2, and 5.8s, of ribosomal DNA and populations included from major Aleppo pine distribution areas will be useful in delineating the evolutionary relationship between Turkish red pine and limited Aleppo pine populations in Turkey.

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