

The Impact of Over-Exploitation on the Genetic Structure of Turkish Red Pine (*Pinus brutia* Ten.) Populations Determined by RAPD Markers

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To determine the possible impact of over-exploitation on the genetic structure of Turkish red pine (*Pinus brutia* Ten.) populations, three natural and three over-exploited (human degraded) populations of the species in the Mediterranean region of Turkey were investigated with Randomly Amplified Polymorphic DNA (RAPD). With the 80 RAPD primers tested, 12 of them yielded 137 polymorphic RAPD fragments. Four of the studied populations maintained unique fragments. The mean proportion of polymorphic fragments for all populations ranged from 89.8 to 98.9% and there were no significant differences between natural (94.8%) vs. over-exploited populations (92.7%). The estimated heterozygosity values suggested that Turkish red pine maintains high levels of genetic diversity (range 0.24–0.28) though studied populations and grouped ones as natural ($H_e=0.28$) vs. over-exploited (0.27) did not differ significantly. The mean F_{ST} value indicated that the large portion of the total genetic diversity was within populations (93%), but this value was lower in the natural populations (92%) than in the over-exploited ones (94%). In over-exploited populations, excess of homozygosity was observed (about 6% higher) as compared to natural populations, indicating impacts of inbreeding in *P. brutia*.

Keywords *Pinus brutia* Ten., RAPD-PCR, human over-exploitation, genetic structure, inbreeding

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1 Introduction

Turkish red pine (*Pinus brutia* Ten.) is naturally found mainly in the Mediterranean, western and north-western parts of Turkey and in small isolated populations in the Black Sea Region with a micro climate similar to the Mediterranean region (Arbez 1974, Atalay 1982). Turkish red pine is the most widespread forest tree species in Turkey with a total coverage area of over 3.1 million ha which account for 15% of the total forest land. Turkish red pine grows from the sea level up to 1300 m in pure stands and to 1500 m as individuals.

For centuries, natural forests of Turkey have been exploited or destroyed by men-caused or natural fires and grazing for years in the past. Additionally, economical needs of local forest communities lead to over-utilization of Turkish red pine natural resources through disgenic selection by cutting of good-quality and superior tree phenotypes, in turn, degrade natural forests to meet wood demand or to expand agricultural lands. Degradation manifests itself by reducing the population size and crown closure and causes isolation of populations which leads fragmentation. Together with ongoing natural stresses (drought, cold, insect infestation etc), the maintenance of genetic resources of forest trees with high genetic diversity may be seriously threatened in Turkey (Kaya 1998). Especially, Turkish red pine is one of the most affected tree species from deforestation and other human related factors and many populations are being lost or endangered, taking with them much of the genetic diversity. However, the magnitude of the impact of these human factors on the loss of genetic resources is not well documented (Kaya and Isik 1997).

In forest tree-improvement programs, selections are based on a few desired phenotypic traits. Thus, the genetic base of plantations with improved stocks might be expected to be narrower. However, studies related with direct comparison of genetic variation in seed orchards and natural populations generally confirm similar results; genetic diversity is maintained (Knowles 1985, Savolainen and Kärkkäinen 1992, Chaisurisri and El-Kassaby 1994, El-Kassaby and Ritland 1996, Schmidling and Hipkins 1998, Godt et al. 2001). On the other hand, reduced allelic richness in

white spruce (Cheliak et al. 1988, Rajora 1999, Godt et al. 2001) and jack pine (Godt et al. 2001) phenotypic selections and reduced allelic richness and heterozygosity in seed orchard clones of interior spruce (Stoehr and El-Kassaby 1997) were also reported in the literatures.

Two recent studies involving Turkish red pine indicate that impact of humans on genetic resources of the species are significant (Kandedmir et al. 2004, İçgen et al. 2006). Kandedmir et al. (2004) reported that there is large amount of genetic diversity within and among Turkish red pine seed stands, but no distinct pattern of genetic diversity according to the geography, elevation or breeding zones, implying the occurrence of past human disturbances such as forest fires, in turn, artificial regeneration. Furthermore, İçgen et al. (2006) studied the potential impact of forest management and breeding practices on established Turkish red pine plantations. They reported that the genetic relationships between seed sources (seed stands, orchards and plantations originating from the same locality) varied with respect to seed source locations. In general, seed stands were genetically distant to seed orchards and plantations, demonstrating that some genetic changes have taken place during the course of seed orchard (plus tree selection) and plantation (seed and/or seedling production) establishment.

In both Kandedmir et al. (2004) and İçgen et al. (2006), studied populations were managed ones such as seed stands, seed orchards, or plantations originating materials from respective seed stands or seed orchards. There were no references made in sampling of populations with respect to distance from coast, elevation or naturalness vs. fragmentedness. By applying a nested sampling of populations as natural vs. degraded from coastal plain in south to inland in the north, in the current study, the magnitude and pattern of genetic variation existing in 6 populations (3 natural and 3 degraded populations) of Turkish red pine in the Mediterranean Region of Turkey was determined by using RAPD markers. Also, the impact of degradation and fragmentation of natural populations on the genetic structure of future forests was discussed.

2 Materials and Methods

2.1 Plant Material

Open-pollinated seeds from 6 Turkish red pine populations (Table 1A and Fig. 1) were collected from the Mediterranean region of Turkey. Among these populations, two were from high elevations, two from inland and two from coastal areas. One of the two populations in each geographic location represents relatively good stands, which has maintained natural stand structure and continuous distribution due to absence of human interference including intensive forestry practices. The other population represents over-exploited (human degraded) stand, which has low tree stocking, highly fragmented structure due to excessive human activities (i.e., dysgenic-selective cuttings, intensive forestry practices such as clear cuttings, conversion to agricultural lands and past forest fires) (Table 1A). The descriptive information in Table 1A was obtained from the forest management plans prepared by the Turkish Forest Service (personal communication, Mr. H. Serdar Kip, General Directorate of Forests, Department

of Forest Planning and Management). Based on available data from the forest management plans of these stands, it could be said that degradation (or over-exploitation) has been the case for at least last 5 generations. Here on, the former and later populations have been referred as natural and over-exploited populations, respectively.

From each population, 25 parent trees (families) were chosen randomly by considering separation (at least 100 m), elevation (max. 300 m difference) and location of cones (upper 1/3 of the trees). Seeds were extracted from cones in open air and kept in cool (+4 °C) storage until they were used.

2.2 DNA Isolation and Quantification

From each of 6 populations, megagametophyte tissues from 20 seeds from each family (mother tree) of 150 families (25 families per population) were used for DNA extraction. The procedures described in Kaya and Neale (1995) were used as the DNA extraction method. To determine the number of megagametophytes needed for accurate identification of mother tree genotypes, the

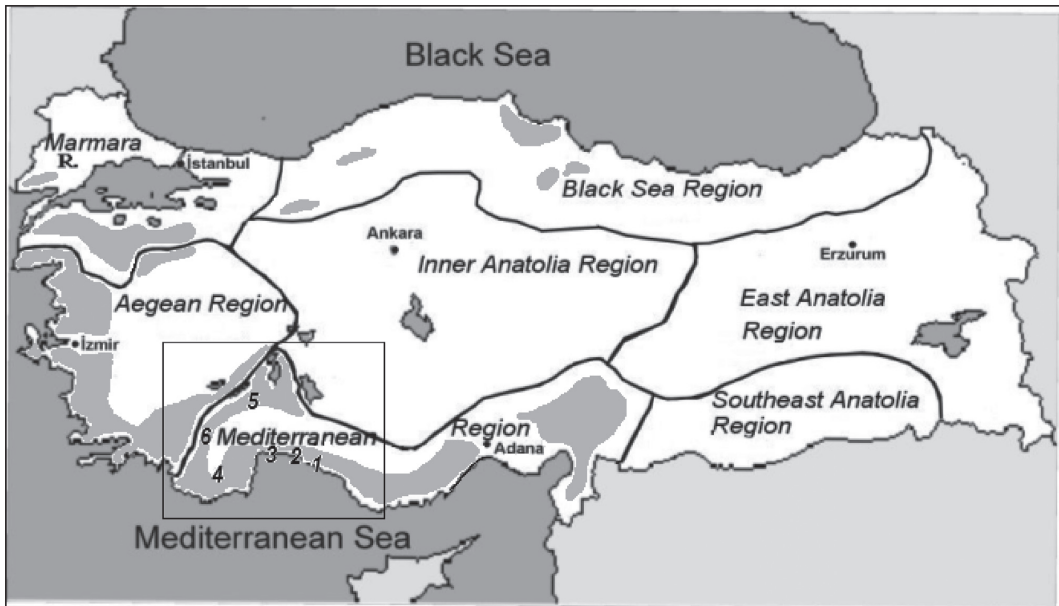


Fig. 1. Natural distribution of Turkish red pine and locations of studied populations (see Table 1 for codes and description of populations).

Table 1. A) Description of studied populations. Al=altitude, Lo=longitude, La=latitude, Rn=mean annual rainfall, AnInc=annual increment and B) variation parameters. n_a =observed number of alleles, n_e =effective number of alleles, N =sample size H_o =observed heterozygosity, H_e =expected heterozygosity, P =polymorphic loci, N_m =gene flow, F_{IS} =fixation index within subpopulations, F_{IT} =total fixation index and F_{ST} =differentiation among populations. The numbers in parenthesis following n_a , n_e , H_o , and H_e are the standard errors of the estimates.

Population name	Population type	Al (m)	Lo (E)	La (N)	Rn (mm)	Number of trees/ha	AnInc (m ³ /ha)	Site index ^{a)}	Stand type	Crown closure ^{b)}	Stand age
1 Alanya-Kargı	Natural	350	31°57'	36°36'	1103	343	5.07	1	Pure <i>P. brutia</i>	3	83 years
2 Manavgat-Yaylaalan	Over-exploited	500	31°30'	36°57'	1050	160	7.12	1	Mixed with deciduous trees and <i>Cupressus sempervirens</i>	2	>60
3 Antalya-Çalkaya	Over-exploited	50	30°50'	36°55'	1060	183	3.54	2	Mixed with deciduous trees	2	>60
4 Fethiye-Yapraktepe	Natural	800	29°28'	36°44'	993	425	7.17	1	Pure <i>P. brutia</i>	3	52
5 Burdur-Gölnhisar	Over-exploited	1100	29°32'	37°40'	634	283	2.94	2	Pure <i>P. brutia</i>	2	110
6 Çameli-Göldag	Natural	800	29°07'	37°06'	1222	306	3.01	2	Pure <i>P. brutia</i>	3	67

Population name	Population type	N (number of mother trees)	n_a	n_e	H_o	H_e	No. of P	% of P	F_{IS}
1 Alanya-Kargı	Natural	25	1.86 (.06)	1.46 (.07)	0.27 (.04)	0.27 (.03)	118	86.1	-0.01
2 Manavgat-Yaylaalan	Over-exploited	25	1.86 (.06)	1.46 (.07)	0.25 (.04)	0.28 (.03)	118	86.1	0.09
3 Antalya-Çalkaya	Over-exploited	25	1.79 (.08)	1.40 (.07)	0.24 (.04)	0.25 (.03)	108	78.8	0.01
4 Fethiye-Yapraktepe	Natural	25	1.85 (.07)	1.41 (.07)	0.27 (.04)	0.25 (.03)	117	85.4	-0.10
5 Burdur-Gölnhisar	Over-exploited	25	1.81 (.07)	1.39 (.06)	0.23 (.04)	0.25 (.03)	111	81.0	0.06
6 Çameli-Göldag	Natural	25	1.82 (.06)	1.40 (.07)	0.22 (.04)	0.24 (.03)	113	82.5	0.07

^{a)} Site index (SI)-1 for *Pinus brutia* at age of 80: 20.05 m, SI-1 at age 60: 18.25, SI-2 at age 60: 14.38-18.24, SI-1 at age of 50: 16.92 m, SI-2 at age of 110: 17.09-21.67 m and SI-2 at age of 65: 14.82-18.79

^{b)} Crown closure 2: 40%-70%, Crown closure 3: 70%-100%

B)

formula suggested in Morris and Spieth (1978) was used. If 8 megagametophytes are used, the probability of error is less than $0.0078 ((1/2)^7)$. Considering cost and labor involved in large sample size, it seemed that 6 megagametophyte DNAs per family were adequate to address genotyping of families (incorrect identification probability is less than 1.56%). Due to the dominant nature of the RAPD primers, embryo tissues were not used in the study.

The determination of DNA concentration of all isolated DNA samples was performed by using a fluorometric assay (Cesarone et al. 1979). The first 6 of 20 megagametophytes with the highest DNA yield were selected and diluted to a 3 ng/ μ l for PCR applications and data collection.

2.3 RAPD Primers, PCR Amplification and Electrophoresis

Initially, 80 primers, each composing of 10 oligonucleotides were selected for this study by considering the results of previous studies on conifers (Kaya and Neale 1993, Kaya and Neale 1995, Kandedmir et al. 2004, İçgen et al. 2006). All primers were initially screened against a set of 6 megagametophyte DNAs from each of 6 populations to identify segregating loci. Primers giving the highest number of polymorphic loci were then screened against 900 samples (6 populations \times 25 families/population \times 6 megagametophytes/families = 900 samples) to obtain single locus segregation data, using one of the well working and simple banding pattern primer (UBC179) as positive control for repeatability of RAPD data. The fragments which initially displayed very low frequencies and poor staining in seed stands, were dropped out from the data set. Also, the fragments that were monomorphic and bands with extremely low frequencies (that is less than $3/n$, where n is the total number of scores in the data set) were excluded from further analysis, as recommended by Lynch and Milligan (1994).

Although RAPDs do not address gene loci and corresponding alleles, polymorphic RAPD fragments produced by a given primer were treated as loci with two alleles (one with the presence of the polymorphic fragment and the other with the absence) to be able to estimate population genetic

parameters. All of the reaction conditions and the PCR amplification cycles optimized by Kaya and Neale (1995) were adopted in the present study. PCR products were loaded with 25% formamide loading dye and visualized in 1.7% agarose gel. Gels were run in 1X TAE (0.4 M Tris Acetate) buffer at 100 volts for 2.5 hours. The resulting gel was stained with 0.5 μ g/l ethidium bromide and running buffer 1X TAE (0.4 M Tris Acetate) with 1mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA) for 30 minutes and destained with distilled water for 5 minutes. The 12 best yielding primers were used to screen 900 DNA samples and the data were collected with these primers.

2.4 Evaluation of RAPD Data as a Diploid Data

With two possible states for a haploid RAPD fragment, presence (marker allele) or absence (null allele), a RAPD locus was defined as a fragment that would segregate or be monomorphic among the haploid megagametophyte samples within a family. Genotypes of mother trees determined from the segregation pattern of six haploid megagametophytes were converted to diploid genotypes for each of the 150 trees at each locus. For each locus, all dominant homozygotes (AA) and heterozygotes (AB) were scored as individuals possessing the fragment, whereas recessive homozygotes (BB) were scored as individuals with no fragment in order to better understand the genotype of the mother tree. Then, the data file was organized, so that it could be analyzed with POPGENE (POPGENE version 1.32), Microsoft Windows-based freeware program for population genetic analysis (Yeh et al. 1999). The following parameters were estimated: Polymorphic RAPD fragment (for convenience, it was treated as an allele) frequencies, allelic richness, proportion of polymorphic loci, heterozygosity, F-Statistics (Nei 1987), genetic distance and identity (Nei 1972, Nei 1978), and effective number of alleles at a locus (Kimura and Crow 1978). The bootstrap values for the constructed dendrogram (Langella 2002) and statistical test of F-statistics (Belkhir et al. 2004, Genetix 4.05) were also estimated for the studied populations.

3 Results

3.1 RAPD Primer and Polymorphism

A total of 12 RAPD primers revealed 245 polymorphic fragments, but the fragments with very low frequencies and poor staining were dropped out of the data set. After that, 137 polymorphic and 5 unique fragments (markers) were available. It is interesting that 2 of the unique fragments were found in the same population, Manavgat-Yaylaalan which is an over-exploited population. On the average, 14 RAPD fragments were produced per primer.

3.2 Genetic Diversity Statistics

The mean number of observed alleles (n_a) within the populations varied between 1.79 to 1.86 (Table 1B). Mean number of effective alleles (n_e) was always lower than n_a as expected. The highest n_e value was observed in over-exploited Manavgat-Yaylaalan and natural Alanya-Kargi populations, whereas the lowest n_e value was found in over-exploited Burdur-Göhlhisar population. Nevertheless, considering the standard errors of estimates, these n_e values are not statistically significant.

Manavgat-Yaylaalan, Antalya-Çalkaya and Burdur-Göhlhisar populations are considered to be over-exploited populations, whereas Alanya-Kargi, Fethiye-Yapraktepe, and Çameli-Göldag are relatively intact natural populations. There was no significant difference between over-

exploited versus natural populations for number of observed (n_a) and effective alleles (n_e) per locus (Table 2).

The proportion of polymorphic loci (0.99 criteria) ranged from 81.0% in over-exploited Burdur-Göhlhisar population to 86.1% in natural Alanya-Kargi and over-exploited Manavgat-Yaylaalan populations (Table 1B). In general, the over-exploited and natural populations did not vary significantly in proportion of polymorphic loci (Table 2).

Observed (unbiased) heterozygosity was highest (0.27) in natural Fethiye-Yapraktepe and Alanya-Kargi populations while it was lowest (0.22) in the natural Çameli-Göldag population. On the other hand, expected heterozygosities ranged from 0.24 for Çameli-Göldag to 0.28 for the over-exploited Manavgat-Yaylaalan (Table 1B). Observed heterozygosity in over-exploited populations was lower (0.24) than in natural populations (0.26) though the difference was not significant. However, the difference between expected and observed heterozygosities was slightly higher in over-exploited populations than that of natural ones (Table 2).

3.3 F-Statistics

F_{IS} value for over-exploited populations was higher (0.05) than the estimate for natural populations (-0.01%), showing excess in homozygotes (Table 2). This was supported with negative estimated F_{IS} for natural populations in general (except for Burdur-Göhlhisar, Table 1B) and positive estimated F_{IS} values for all over-exploited

Table 2. Genetic diversity parameters for pooled “over-exploited” and “natural” populations. For notation, see Table 1.

Population type	N (number of mother trees)	n_a	n_e	H_o	H_e	No. of P	% of P	F_{IS}	F_{IT}	F_{ST}	N_m
Over-exploited pop.	75	1.93 (.02)	1.44 (.02)	0.24 (.01)	0.27 (.01)	127	92.7	0.05*	0.11*	0.06*	4.1
Natural pop.	75	1.95 (.02)	1.46 (.02)	0.26 (.01)	0.28 (.01)	130	94.8	-0.01*	0.07*	0.08*	2.8
Mean	150	1.98 (.01)	1.45 (.01)	0.25 (.02)	0.27 (.01)	135	98.5	0.02*	0.09*	0.07*	2.9

* The estimates are significant at $p < 0.05$; Tests were done by estimating bootstrap confidence intervals (Number of permutation = 1000), according to Weir (1996)

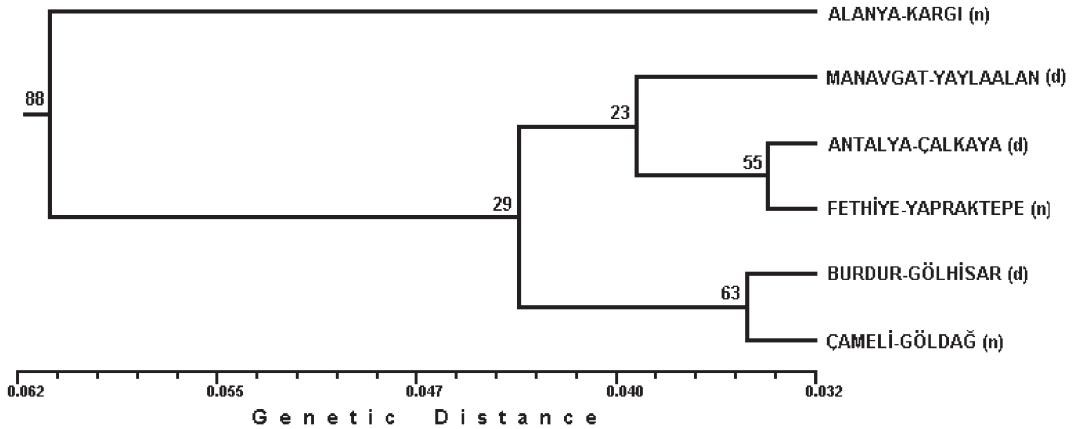


Fig. 2. Dendrogram based on Nei's (1978) genetic distances of 6 Turkish red pine populations was constructed by using UPGMA method modified from NEIGHBOR procedure of PHYLIP Version 3.5. The numbers on clusters indicate the boot strap values. (n) and (d) denote natural and degraded populations, respectively.

populations (Table 2). Mean F_{ST} value of over-exploited populations ($F_{IS}=0.06$) was about 2% lower than it was in natural populations ($F_{IS}=0.06$, Table 1B). This shows that 6% of the total variation could be attributed to between populations in over-exploited group while it was 8% in natural group. Furthermore, 94% of total variation was within populations for the over-exploited group while it was 92% for natural group. The estimate for the gene (N_m) flow was 4.1 for the over-exploited and 2.9 for natural populations, suggesting higher gene flow in over-exploited populations than natural populations (Table 2).

3.4 Genetic Distance and Identities

Antalya-Çalkaya and Fethiye-Yapraktepe were the most closely related populations (genetic identity $I=0.967$). On the other hand, Çameli-Göldag and Alanya-Kargı were the most distant populations ($I=0.929$). The cluster analysis carried out with POPGENE software (Yeh et al. 1999) which uses the "Unweighted Pair Group Method with Arithmetic Means" procedure indicated the clusters of two large branches. The first branch covered just Alanya-Kargı, showing that it is the most distant population. The second one covered three sub-groups. In this branch, Fethiye-Yapraktepe and Antalya-Çalkaya formed a group, Man-

avgat-Yaylaalan was joined to this group alone, and Burdur-Göhlhisar and Çameli-Göldag formed the last group (Fig. 2).

4 Discussion

The estimated values for proportion of polymorphic loci and allelic richness in natural and degraded populations revealed the existence of high levels of genetic variation in Turkish red pine. The estimated values for these parameters were comparable with the results of previous studies regarding conifers and using RAPD markers (Isabel et al. 1995, Szmidt et al. 1996, Rajora 1999, Kandemir et al. 2004, Igen et al. 2006). However, higher allelic richness was reported in studies related with old and unmanaged forests (Rajora 1999, Macdonald et al. 2001, Lee et al. 2002, Rajora and Pluhar 2003).

When the over-exploited populations are compared with natural populations regarding the expected heterozygosities, no significant differences between two groups were observed (0.27 in degraded vs. 0.28 in natural populations). Obviously, for years of dysgenic selection practices and other anthropogenic factors causing fragmentation, reducing stocking and crown closure in stands did not result in any drastic changes in

the amount of genetic diversity. Many studies on forest fragmentation, management and habitat degradation in other species report observations of inbreeding immediately after impact, but genetic diversity is gradually diminished over subsequent generations, which may take decades for forest trees (Lowe et al. 2005).

Based on estimated F_{ST} values, differences among populations explained 7% of total variation, whereas within population differences accounted for 93%. These results are consistent with the results of similar studies in conifers (mean F_{ST} for conifers = 0.06; Hamrick et al. 1992). Previous allozyme studies (Isık and Kara 1997, Kara et al. 1997, Panetsos et al. 1998, Gülbaba and Özkurt 1998, Dogan 2000) also report little genetic differentiation among populations. The constructed dendrogram, based on the genetic distances among populations, did not yield a clear clustering pattern between over-exploited and natural populations. Only two inland populations (Çameli-Göldag and Burdur-Göhlhisar) grouped with high bootstrap values. The grouping probabilities were low among coastal populations. However, Kandedmir et al. (2004) also reported a maintained high genetic variation and less differentiation in low elevation coastal populations of red pine seed stands despite of their highly disturbed nature due to over-exploitation, forest fires and increased plantation activities.

Inbreeding coefficient (F_{IS}) varied considerably among populations. The coefficient was six-fold higher in over-exploited populations ($F_{IS}=0.05$) than in natural populations ($F_{IS}=-0.01$), suggesting a higher deficiency of heterozygotes within over-exploited populations than those of natural populations. In contrast, there was excess of heterozygotes in natural populations except for Çameli-Göldag which is a large population with continuous distribution. This inland population, being away from fire sensitive and settlement areas, probably received very little or no seed or seedling introduction and therefore displayed a relatively lower heterozygosity. Similar low heterozygosity values were also reported for other conifer species (Guries and Ledig 1981, Conkle et al. 1988, Hamrick and Godt 1989, Sproule and Dancik 1996, Thomas et al. 1999). Loss of heterozygosity in small, isolated populations is linked to inbreeding depression (Charlesworth

and Charlesworth 1987, Aldrich et al. 1998, Lowe et al. 2005), and restriction of gene flow due to deforestation and habitat fragmentation (Dayanandan et al. 1999).

Turkish red pine has semi-serotinous cones, which only release seed during forest fires or under high temperatures. According to Neyisci (2001) trees might keep these semi-serotinous cones as long as 9 years, thus, creating ample opportunity to disperse several generations of seeds for natural regeneration, usually after forest fires. As a common silvicultural practice, naturally germinated seedlings from local stands and seeds sources previously collected from other stands are used to reforest burnt areas. This practice may considerably increase the richness of the gene pool of new stands, but at the same time reduce the population differentiation (Lee et al. 2002, Kandedmir et al. 2004, Rogers 2004). However, in order to elaborate the effects of serotinous cone maintenance and fire on genetic composition of pine forests such as Turkish red pine forests, further studies are needed.

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