Genetic Variation in Turkish Red Pine (*Pinus brutia* Ten.) Seed Stands as Determined by RAPD Markers

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Summary

Turkish red pine (*Pinus brutia* Ten.) is one of the important tree species in Turkey. The species has been heavily disturbed by anthropogenic factors and fire, making it important to understand the magnitude of the impact on genetic diversity of areas reserved for seed production. To explore the extent of genetic variation in Turkish red pine seed stands, a random amplified polymorphic DNA (RAPD) marker system was used in the study. The estimated genic diversity for seed stands was found to range from H = 0.17 (P, %of polymorphic loci = %58.7) in Çameli-Göldağ to 0.29 (P = %81.7) in Bayramiç-Karaköy seed stand though the lowest polymorphism was observed in Fındıkpınarı seed stand (P = 55.8%). The total gene diversity was calculated as $H_T = 0.34$, in Turkish red pine. About 65% of the total diversity was within seed stands. No distinct pattern of genetic diversity was found according to the geography, elevation or breeding zones. The highest genetic differentiation was found in the Western Mediterranean geographic region, however, within population genetic diversity was found to be similar among different geographic regions ($H_S = 0.22$-0.24). G_{ST} value calculated was high (0.35) indicating that genetic differentiation among the studied seed stands was substantial. Dendrogram constructed with genetic distance data did not yield a firm pattern with respect to geography, elevation and breeding zones. In fact, the most of the clusters in the dendrogram had very low bootstrap values, suggesting that the clusters could be refined if more populations and markers are included in the future studies. The implication of the results with respect to *in situ* conservation of genetic resources of the species was discussed.

Key words: Pinus brutia, RAPD markers, Seed stands, genetic diversity, *in situ* conservation.

Introduction

Turkish red pine (*Pinus brutia* Ten.) is an important commercial tree species and used widely in afforestation and reforestation programs in southern and western Turkey. The species naturally grows from sea level up to 1200 m, occasionally to 1400 m elevation in the Taurus Mountains along the Mediterranean Coast. Within its altitudinal and horizontal distribution ranges, Turkish red pine exhibits significant amount of variation in various form and growth characteristics (Arbez, 1974; Işık, 1986; Işık *et al.,* 1987; Atalay *et al.,* 1998; Kandemir, 2002). It grows on a variety of sites with very different annual precipitation and climatic conditions (Arbez, 1974; Panetsos, 1981).

Turkish red pine is considered as a fast growing conifer when compared to other native forest tree species in Turkey (Işık *et al.,* 1987). The species has been introduced to several countries in the Mediterranean region and to overseas countries such as Australia and Mexico (Palmberg, 1976; Fisher *et al.,* 1986; Weinstein, 1989a; Weinstein, 1989b).

Information on the magnitude and structure of genetic variation within a species is an integral part of planting program and conservation of genetic resources. Allozymes have been employed to estimate the genetic variation and population divergence in many plant species (Hamrick *et al.,* 1992). In recent years, random amplified polymorphic DNAs (RAPDs) have become popular molecular markers in genetic studies in conifers and in other plants (Grattapaglia *et al.,* 1992; Binelli and Bucci, 1994; Lu *et al.,* 1995; Hurme and Savolainen, 1999; Stevens *et al.,* 1999; Diaz *et al.,* 2001; Newton *et al.,* 2002; Peng *et al.,* 2003) because of certain advantages of RAPD markers over other DNA markers and isoenzymes (Isabel *et al.,* 1995; Kaya and Neale, 1995; Neale, 1998). The RAPD system has been used for linkage map construction and also studying genetic polymorphism in many conifers including Turkish red pine (Kaya and Neale, 1993 and 1995; Hurme and Savolainen, 1999; Stevens *et al.,* 1999; Diaz *et al.,* 2001; Newton et al., 2002) since the first use of the RAPD primers to reveal amplified DNA fragment variation (Williams *et al.,* 1990; Welsh and McCelland, 1990).

The Turkish red pine tree breeding program was initiated in 1994 as a part of the Turkish National Tree Breeding Program (Koski and Antola, 1993). Tree breeding zone designations and plus tree selections for each breeding zones have been completed, and progeny tests have been established on multiple sites to evaluate the genetic merits of the selected trees. There are also well distributed clonal seed orchards established to represent the breeding zones of the species. Some of these seed orchards, however, are still too young to provide sufficient seed production to meet afforestation and reforestation needs. The main seed sources for all kinds of plantation activities are "seed stands", which are reserved natural stands of Turkish red pine.

The objectives of this study were to use RAPD markers to investigate the genetic composition and differentiation of 19 seed stands of Turkish red pine representing mainly three different geographical regions of the country (*Figure 1*) and to provide a genetic reference data for effective gene conservation activities concerning Turkish red pine in the future.

Materials and Methods

Plant Materials

Open-pollinated bulk seeds were collected in 1997 from 19 Turkish red pine seed stands representing natural distribution of the species in Turkey to study the genetic structure of seed stands (*Figure 1, Table 1*). From these collections, 30 seeds per seed stand were randomly chosen for DNA extraction.

DNA isolation procedure

Seeds were soaked in distilled water at 4 °C for 24 hours to help separate the megagametophytes from seed coat and

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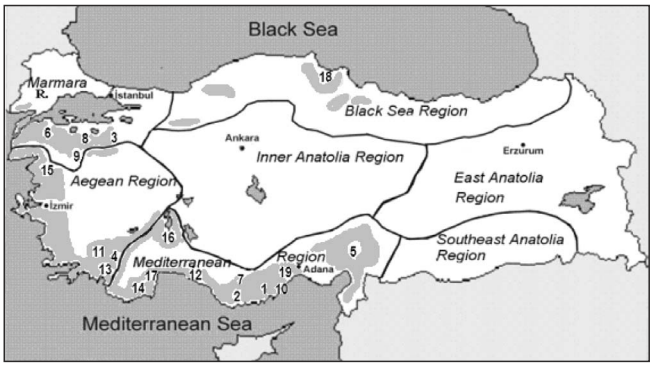

Figure 1. – Map showing the natural distribution of Turkish red pine and location of studied seed stands (numbers). Please see *Table 1* for seed stand names corresponding to the numbers.

Table 1. – Geographic location, breeding zone designations, aspects, altitudes, and core area of *Pinus brutia* seed stands (from KOSKI and ANTOLA, 1993).

Code	Name	Latitude	Longitude	Altitude (m)	Aspect	Area (ha)
1	Gülnar-Pembecik	36 14	33 15	650	N	4.5
2	Bozyazı-Tekmen	36 09	33 05	250	SE	8.5
3	Orhaneli-Göktepe	40 00	28 55	650	S	49
4	Çameli-Göldağ	37 06	29 07	800	N	40
5	Pos-Soğukoluk	37 35	35 21	735	S	27
6	Bayramiç-Karaköy	39 50	28 55	450	Variable	118.5
7	Bozyazı-Toldağ	36 13	33 06	350	N	38.5
8	M.Kemalpaşa-Çaltılıbük	39 58	28 40	250	NE	44
9	Sındırgı-Seydan	39 12	28 08	557	W-N	50
10	Silifke-Akdere	36 13	33 42	100	SW	50
11	Yılanlı-Boyalı	37 17	28 34	750	N-SE	21
12	Serik-Pınargözü	37 16	30 59	500	W-SW	112
13	Köyceğiz-Ağla	37 01	28 44	650	W	51
14	Kaş-Karaçay	36 24	29 32	975	S	86
15	Bergama-Kozak	39 14	27 06	650	N	33
16	Bucak-Melli	37 24	30 33	800	E	84
17	Düzlerçamı-Antalya	36 59	30 37	275	Flat	128.5
18	Bafra-Alaçam	41 38	35 26	100	N	10.5
19	Fındıkpınarı	36 56	34 26	825	E	23

embryo. DNA extraction from megagametophytes was conducted according to KREIKE (1990) and DELLAPORTA *et al.* (1983). Megagametophytes were homogenized with disposable pestle in 400 μl of extraction buffer [10 *mM* Tris-HCl pH 8.0; 50 μM Ethylenediamine tetra aceticacid (EDTA); 500 μM NaCl; 10mM β-mercaptoethanol] in 1.5 *ml* eppendorf centrifugation tubes. Additional extraction buffer of 400 μl (+2% SDS) was added and incubated at 65 °C for at least 30 minutes. Then, 250 μl 5M potassium acetate was added and incubated at 0 °C for a minimum of 30 minutes. The mixture was centrifuged for 15 minutes at 0 °C at 13000 rpm, and the supernatant was transferred to a new tube for use in DNA analyses.

Polymerase chain reaction (PCR) steps optimized for Turkish red pine (KAYA and NEALE, 1995) were used for amplification of RAPD fragments. The PCR steps were as follows; 85 °C for 15 sec, 95 °C for 5 sec, 92 °C for 1 min 55 sec, 45 cycle of "95 °C for 5 sec, 92 °C for 55 sec, 37 °C for 1 min, 72 °C for 2 min" and 72 °C for 7 min. Prescreening of 80 random decamer primers (UBC, Vancouver, British Columbia, Canada and Operon, Alameda, CA, USA) revealed that 20 primers could be useful for further study and data collection (*Table 2*).

Gels were run in 1X Tris-aceticacid-EDTA buffer at 75 volts for 2.5 hours. The resulting gel was stained with running buffer (0.4 M Tris Acetate with 1 *mM* EDTA) containing 0.5 $\mu g/l$ ethidium bromide. Loading dye was 25% Ficoll. A 100 base pair ladder was used as a size standard marker (DNA Ladder Plus, Fermentas, Ukraine). To secure the reproducibility of PCR product, negative controls were included in primer screening. During the primer screening and data collection

Table 2. – List of the best yielding primers and corresponding base sequences.

Primer	Sequence 5' to 3'	Number of polymorphic bands (i.e. segregating in seed stands)
OP-V18 170	TGG TGG CGT T	5
UBC-782 159	GGG AAG AGA G	3
UBC-682 144	CTG CGA CGG T	4
UBC-571 149	GCG CGG CAC T	1
UBC-189 124	TGC TAG CCT C	5
UBC-103 121	GTG ACG CCG C	7
UBC-105 122	CTC GGG TGG G	12
UBC-499 190	GGC CGA TGA T	6
OP-V08 168	GGA CGG CGT T	12
OP-N05 184	ACT GAA CGC C	4
OP-K09 179	CCC TAC CGA C	6
UBC-413 131	GAG GCG GCG A	9
UCG-80 119	GTG CTC TAG A	1
UCG-757 187	GGA AGG GAG G	4
UCG-431 138	CTG CGG GTC A	4
UCG-790 162	GGG TGT GGT T	3
OP-G16 178	AGG GTC CTC C	4
UCG-570 148	GGC CGC TAA T	2
UBC-200 126	TCG GGA TAT G	4
OP-L19 165	GAG TGG TGA C	8

with 20 primers, there was no amplification of artifacts and all PCR produced bands were resulted from template DNA.

Data collection and evaluation

After screening seed stands with 80 RAPD primers, 75 percent of the primers were discarded due to poor or no PCR amplification. Data collection with only 20 primers was carried out. Amplification products were scored as discrete, binary states (present/absent) for each of 30 megagametophytes from each seed stand and labeled by primer and estimated band size. Since haploid data were collected from megagametophytes, bands were scored as "0" and "1" for the absence and presence of the fragment, respectively.

The bands initially displayed had very low frequencies and poor staining in seed stands were dropped out from the data set. Genetic diversity statistics applied to RAPD data would underestimate the genetic diversity due to the dominant character of RAPD markers when compared to genetic diversity estimates derived from codominant markers such as isozymes (FRITSCH and RIESEBERG, 1996). Therefore we included a large sample of seeds per seed stand in our analyses and avoided the bands that were monomorphic and bands with frequency higher than 1-(3/n, where n is the number of individuals in analysis (LYNCH and MILLIGAN, 1994). The final data set contained only polymorphic bands (segregating 1:1 ratio at least in one of the 19 seed stands). The data files were constructed so that they could be analysed with *Popgene* Software (YEH *et al.,* 1999) and *Populations* 1.2.28 *(http://www.pge.cnrs-gif.fr/bioinfo/populations).*

For each seed stand, number of observed alleles (*Na*), number of effective alleles (*Ne*) (KIMURA and CROW, 1978), NEI's (1973) genetic diversity (H) and SHANNON's Information Index (*I*) (LEWONTIN, 1972) were calculated. In addition, the observed heterozygosity of an individual in a population (H_I), the expected heterozygosity of an individual in a population (H_S) and, the expected heterozygosity of an individual in overall populations (H_T) were estimated in accordance with the Hardy-Weinberg expectations (NEI, 1987).

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The relative magnitude of genetic differentiation among subpopulations (G_{ST}) was obtained according to NEI (1987). We used G_{ST} estimates to calculate the average number of immigrants per generation for each locus using the formula, $N_m = 0.5(1 - G_{ST})/G_{ST}$) and the mean value across loci (MCDERMOTT and MCDONALD, 1993). According to the geographical location, altitude, and breeding zones, 19 seed stands used in this study were grouped and the above genetic parameters were also estimated for these groups.

The estimates of NEI's (1972) standard genetic identity (I_{xy}) and standard genetic distance (D_{xy}), unbiased for sample size (NEI, 1978) for all pair-wise seed stands were calculated to show the genetic relationships between studied seed stands. All the estimations were calculated with the POPGENE software (YEH *et al.,* 1999). A dendrogram was also constructed by using boot-strap values (*Populations* 1.2.28 *http://www.pge.cnrs-gif.fr/bioinfo/populations*) and genetic distance matrix based on NEI (1972) (YEH *et al.,* 1999).

The nineteen seed stands were grouped with respect to geographic (climatic) regions, elevational ranges and current breeding zones in which seed stands are located, to explore pattern of genetic variation further. In the geographic grouping, the climatic conditions of seed stand localities were taken into consideration and four geographic regions were identified as the Marmara, eastern Mediterranean, western Mediterranean and Black Sea regions.

In the elevational grouping, three elevational groups were formed as low (0–350 m), medium (350–600 m) and high elevation (600–1000 m). These are also the elevational groupings used in seed transferring. Studied seed stands were arranged according to the breeding zones to which they belong. The breeding zones were designated, by the Turkish Forest Tree Seeds and Tree Breeding Research Directorate, as a part of the *National Tree Breeding Program* (KOSKI and ANTOLA, 1993). With respect to the breeding zones, seed stands were assigned to six groups, namely 1.1, 1.2, 1.3, 2.2, 3.1 and 6.1. The second number of the breeding zone correspond the elevational range. For example, 1.1 describes the low elevation of breeding zone 1 while 1.2 and 1.3 stand for medium and high elevations of breeding zone 1, respectively.

Results

The 20 RAPD primers initially revealed a total of 417 polymorphic bands. However, poorly stained, unique and very low frequency-bands were discarded from the data set for further analysis. As a result, 104 polymorphic bands (loci) with 2 alleles were used in the analysis. The proportion of polymorphic loci (using the 0.99 criteria) ranged between 55.8% in Fındıkpınarı and 81.7% in Bayramiç-Karaköy seed stands, with a mean value of 66.4%. The mean observed number of alleles (*Na*) was 1.66. The range was between 1.56 in Fındıkpınarı and 1.81 in Bayramiç-Karaköy. The highest number of effective alleles (*Ne*) was observed in Bayramiç-Karaköy population (1.49), whereas, the lowest *Ne* value was found in Çameli-Göldağ (1.27). When all populations were considered, the mean *Ne* value was 1.38 (*Table 3A*). NEI's genetic diversity or heterozygosity (H) was the lowest in Çameli-Göldağ (0.17) and the highest in Bayramiç-Karaköy (0.29). For all populations, the mean observed heterozygosity was calculated as 0.23 (*Table 3A*).

The calculated G_{ST} value (genetic differentiation) was high (0.35) indicating that genetic differentiation among the studied seed stands was substantial. The total gene diversity was high in Turkish red pine ($H_T = 0.34$), but 22% of this was within population variation ($H_S = 0.22$) (*Table 3B*). Estimated gene flow (N_m) for Turkish red pine seed stands was 0.94, indicating that the genetic exchange between seed stands is not very high.

Table 3. – **A)** Summary of genic variation statistics for all loci. (N: sample size, Na: observed number of alleles, Ne: effective number of alleles, H: NEI's gene diversity, I: SHANNON's Information Index, P: number of polymorphic loci) **B)** NEI's Analysis of gene diversity in subdivided populations.

A)

Seed stand	N	Na	Ne	H	I	# of P	% of P
1-Gülnar –Pembecik	18	1.72±0.11	1.40±0.08	0.24±0.04	0.37±0.06	75	72.1
2-Bozyazı-Tekmen	18	1.63±0.11	1.38±0.09	0.22±0.05	0.33±0.07	66	63.5
3-Orhaneli-Göktepe	17	1.75±0.11	1.43±0.09	0.26±0.05	0.39±0.07	78	75.0
4-Çameli-Göldağ	19	1.59±0.11	1.27±0.08	0.17±0.04	0.26±0.06	61	58.7
5-Pos-Soğukoluk	18	1.69±0.11	1.42±0.09	0.25±0.05	0.37±0.07	69	66.4
6-Bayramiç-Karaköy	18	1.81±0.09	1.49±0.08	0.29±0.04	0.43±0.06	85	81.7
7- Bozyazı-Toldağ	18	1.67±0.11	1.38±0.08	0.23±0.04	0.35±0.06	70	67.3
8-M.Kemalpaşa-Çaltılıbük	20	1.60±0.11	1.34±0.08	0.20±0.04	0.30±0.06	62	59.6
9-Sındırgı-Seydan	19	1.74±0.10	1.41±0.08	0.24±0.04	0.37±0.06	71	68.3
10-Silifke-Akdere	18	1.58±0.12	1.32±0.08	0.19±0.04	0.29±0.07	60	57.7
11-Yılanlı – Boyalı	15	1.65±0.12	1.44±0.10	0.25±0.05	0.37±0.07	68	65.4
12-Serik–Pınargözü	18	1.59±0.12	1.32±0.09	0.19±0.05	0.28±0.07	60	57.7
13-Köyceğiz-Ağla	18	1.74±0.10	1.40±0.09	0.23±0.04	0.36±0.06	77	74.0
14-Kaş–Karaçay	19	1.69±0.11	1.38±0.08	0.22±0.04	0.34±0.06	72	69.2
15-Bergama-Kozak	17	1.67±0.11	1.37±0.09	0.22±0.05	0.33±0.07	70	67.3
16-Bucak-Melli	18	1.60±0.12	1.32±0.08	0.20±0.04	0.30±0.06	62	59.6
17-Düzlerçamı-Antalya	16	1.75±0.11	1.49±0.09	0.28±0.05	0.42±0.07	79	75.9
18-Bafra–Alaçam	19	1.67±0.11	1.36±0.08	0.21±0.04	0.32±0.06	70	67.3
19-Fındıkpınarı	19	1.56±0.11	1.32±0.08	0.19±0.05	0.28±0.06	58	55.8
Mean	**18**	**1.66±0.02**	**1.38±0.01**	**0.23±0.01**	**0.34±0.01**	**69±1.72**	**66.4±1.65**

B)

Number of markers	Sample size	H_T	H_S	G_{ST}	Nm	# P loci	% P loci
104	341	0.34± 0.02	0.22±0.01	0.35	0.94	103	99.04

H_T: Total gene diversity; **H_S**: Gene diversity within populations; **G_{ST}**: Genetic differentiation; **Nm**: Gene flows.

The genetic distances between populations were ranged from 0.29 (Bergama-Kozak and Gülnar-Pembecik) to 0.11 (Bucak-Melli and Bozyazı-Toldağ). Thus, the most genetically distant populations were Bergama-Kozak and Gülnar-Pembecik (genetic distance = 0.292), whereas the most similar populations were Bucak-Melli and Bozyazı-Toldağ (0.114). The genetic identities did not follow completely the genetic distance pattern (or geographic distribution patterns). For example, as expected, Bergama-Kozak and Gülnar-Pembecik were the least similar (0.747) whereas the geographically distant Fındıkpınarı and Köyceğiz-Ağla were unexpectedly the most similar (0.910) seed stands to each other (data shown in *Figure 2*).

Pattern of genetic diversity

Geographic diversity

The level of genetic diversity (H) did not vary significantly among the Marmara, eastern and western Mediterranean regions (ranging from 0.32–0.34 (*Table 4A*). In contrast, the Black Sea region had the lowest genetic diversity though this region included only one seed stand. The Marmara region has the lowest G_{ST} (0.29) and the highest H_T (0.34) together with western Mediterranean region while western Mediterranean region has the highest G_{ST} (0.35). The partition of gene diversity as a total (H_T; 0.32–0.34) within populations (H_S; 0.22–0.24) was similar among geographic regions. However, the Marmara region had the highest H_S (0.24) and the gene flow (1.25) (*Table 4A*).

Elevational pattern

The gene diversity (H) among elevational groups did not vary significantly, ranging from 0.32 to 0.34. The 600–1000 m elevational group had high G_{ST} (0.35) and H_T (0.34) values and the lowest gene flow (0.92). The lowest G_{ST} (0.27) and the highest gene flow (1.39) were estimated for mid-elevational group

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Table 4. – Summary of genetic diversity statistics for all loci, with respect to geographical region (**A**), altitudes (**B**) and breeding zones (**C**).

A)

Geographical regions	N	Na*	Ne	H	I	H_T	H_S	#P	%P	G_{ST}	Nm
Marmara region (seed stands 3,6,8,9,15)	90	1.96±0.02	1.58±0.03	0.33±0.02	0.50±0.02	0.34±0.02	0.24±0.02	100	96.2	0.29	1.25
Western Mediterranean region (seed stands 4,11-14,16,17)	123	1.97±0.02	1.58±0.03	0.34±0.01	0.50±0.02	0.34±0.02	0.22±0.01	101	97.1	0.35	0.92
Eastern Mediterranean region (seed stands 1,2,5,7,10,19)	110	1.94±0.02	1.53±0.03	0.32±0.01	0.48±0.02	0.32±0.02	0.22±0.01	98	94.2	0.31	1.10
Black Sea region (seed stand 18)	19	1.67±0.11	1.36±0.08	0.21±0.04	0.32±0.06	-	-	70	67.3	-	-

B)

Elevational Groupings	N	Na	Ne	H	I	H_T	H_S	#P	%P	G_{ST}	Nm
Low elevation (350m) (seed stands 2,8,10,17,18)	91	1.97±0.02	1.55±0.03	0.32±0.02	0.49±0.02	0.33±0.02	0.22±0.01	101	97.1	0.33	1.02
Mid-Elevation (350-600m) (seed stands 6,7,9,12)	72	1.95±0.03	1.54±0.04	0.32±002	0.48±0.02	0.32±0.03	0.24±0.02	99	95.2	0.27	1.39
High elevation (600-1000m) (seed stands 1,3,4,5,11,13-16, 19)	179	1.98±0.01	1.58±0.02	0.34±0.01	0.51±0.01	0.34±0.02	0.22±0.01	102	98.1	0.35	0.92

C)

Breeding zones (BZ)	N	Na	Ne	H	I	H_T	H_S	#P	%P	G_{ST}	Nm
BZ:1.1 (seed stands 2,7,10,17)	70	1.98±0.02	1.53±0.04	0.32±0.02	0.48±0.02	0.33±0.02	0.23±0.01	100	96.2	0.29	1.20
BZ:1.2 (seed stands 1,4,5,12,16)	91	1.96±0.02	1.56±0.04	0.32±0.02	0.48±0.02	0.32±0.03	0.21±0.02	100	96.2	0.36	0.91
BZ:1.3 (seed stands 14,19)	38	1.85±0.07	1.46±0.07	0.27±0.03	0.42±0.04	0.28±0.03	0.21±0.02	89	85.6	0.26	1.44
BZ:2.2 (seed stands 11,13,15)	50	1.97±0.02	1.54±0.05	0.32±0.02	0.48±0.03	0.32±0.03	0.23±0.02	101	97.1	0.26	1.45
BZ:3.1 (seed stands 3,6,8,9)	72	1.95±0.03	1.56±0.04	0.33±0.02	0.49±0.02	0.33±0.03	0.25±0.02	99	95.1	0.26	1.40
BZ:6.1 (seed stands 18)	19	1.67±0.11	1.36±0.08	0.21±0.04	0.32±0.06	0.21±0.03	0.21±0.04	70	67.3	-	-

* For legends to the abbreviation, please see *Table 3.*

(350–600 m). The rest of the genetic parameters like *Na*, *Ne*, H, I, H_T, H_S were more or less similar between low and mid elevational groups (*Table 4B*).

Breeding zones related pattern

Breeding zone 3.1 had the highest genetic diversity, H, (0.33) while breeding zone 6.1 has the lowest gene diversity (0.21). In terms of HT, all the breeding zone groups have more or less the same value except for breeding zone 6.1, which has the lowest (0.21). The lowest gene flow (N_m) and the highest G_{ST} were observed in breeding zone 1.2 (*Table 4C*).

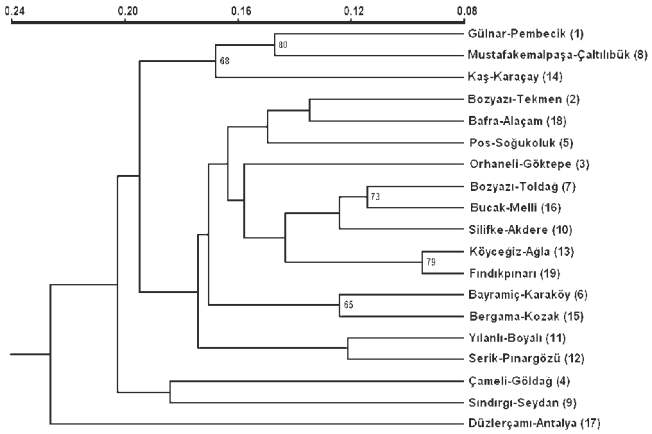

Figure 2. – Dendrogram depicting the genetic relationship among the studied 19 seed stands of Turkish red pine. The numbers on clusters indicate the boot strap value (only boot -strap values of clusters which are greater than 50 percent were provided).

Relationships among Turkish red pine seed stands

The dendrogram did not produce any significant clusters that could be related to geographic distribution of Turkish red pine seed stands (*Figure 2*). The dendrogram contained five clusters; the first group consisted of Gülnar-Pembecik and M. Kemalpaşa-Çaltılıbük with a bootstrap value of 80, then Kaş-Karaçay was joined to the group with a bootstrap value of 68. The second group was formed with nine seed stands. Within the second cluster, Bozyazı-Toldağ and Bucak-Melli were connected together with bootstrap value of 73. Additionally, Köyceğiz-Ağla and Fındıkpınarı seed stands in the same cluster group also showed a high bootstrap value (79). The third group consisted of Bayramiç-Karaköy and Bergama-Kozaklı with a moderate bootstrap value of 65. The forth and the fifth groups only contained two seed stands with a much smaller bootstrap values, and the Düzlerçamı-Antalya seed stand was segregated by itself.

Discussions

The present study revealed that there is large amount of genetic diversity within and among Turkish red pine seed stands. There was, however, no distinct pattern of genetic diversity according to the geography, elevation or breeding zones. From the G_{ST} and N_m values among geographic, elevational groups and breeding zones, it appears that substantial gene flow may be occurring. According to WRIGHT (1969), the critical value for Nm is 0.5. When Nm value is below 1, it means that population began to differentiate due to genetic drift. Nm value below 0.5 indicates that populations will diverge extensively as a result of drift (MCDERMOTT and MCDONALD, 1993). Overall, the calculated gene flow (Nm) indi-

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cates that the seed stands are in the process of differentiation due to genetic drift. Especially, the breeding zone designation seems to be appropriate because G_{ST} values are lowered and Nm values are increased among seed stands within a breeding zone. The highest G_{ST} and the lowest gene flow in the Mediterranean breeding zone 1.2 indicate that these seed stands are about to differentiate with each other. Within this region, the group of seed stands is close to each other geographically so that one can expect gene flow among them. Alternately, the lowest G_{ST} and the highest Nm values in breeding zones 1.3, 2.2, 3.1 and 6.1 indicate that the groups are isolated from gene flow and have subsequently differentiated (see *Table 4C* and *Figure 1*). Frequent forest fires (natural and man caused), forestry practices such as long distance seed transfers before the *National Tree Breeding Program* initiated in 1993 may have contributed for maintaining high genetic diversity among Turkish red pine seed stands.

The percent of polymorphic loci reported using RAPD markers for black spruce and *Pinus sylvestris* ranged from 82.1 to 90.9 percent (ISABEL *et al.,* 1995; SZMIDT *et al.,* 1996), which were higher than that was found (66.4) in this study. In general, the polymorphism within seed stands was lower than other conifers. Such a low level of genetic polymorphism usually occurs in species with very narrow geographic distribution, but Turkish red pine has wide-spread distribution in Turkey. The low polymorphism could be explained by number of trees contributing to bulk seed collection, small seed stand size and mating systems of the species. LISE (2000), working on natural populations (relatively intact and degraded populations) of Turkish red pine, reported that observed heterozygosity ranged 0.28 in degraded and 0.30 in natural populations. In highly degraded populations, there was slightly loss of genetic diversity – e.g., 7% more heterozygosity deficiency in degraded populations than natural populations.

The heterozygosity (H) values (0.17–0.29) obtained for each seed stand, were similar to the value calculated for other conifers. ISABEL et al. (1995) reported that the heterozygosity obtained for black spruce was 0.31 and 0.356 for *Pinus sylvestris* based on RAPD markers (SZMIDT *et al.,* 1996). Similar heterozygosity values (ranging from 0.12 to 0.21) were found in *Cedrus libani* populations by using RAPD markers (KAYIHAN, 2000). In another study, the mean genetic diversity values (H) calculated across the 10 populations of *Pinus oocarpa* was 0.358 (DIAZ *et al.,* 2001). PENG et al. (2003) found that the overall average genetic diversity of *Pinus massoniana* based on RAPD data was 0.245 and ranges were between 0.212 and 0.262. NEWTON et al. (2002) reported the lowest G_{ST} of 0.19 and polymorphic loci of 24.5 percent for *Pinus chiapensis.* The results from the present study, however, indicate that Turkish red pine seed stands used in this study have considerably high overall heterozygosity and G_{ST} values 0.34 and 0.35, respectively.

When bulk seeds, and small population sizes are involved in studies, estimated G_{ST} values appear to be high, especially in those studies used utilizing RAPD markers. For example, the differentiation among *Cedrus libani* A. Rich seed stands was estimated to be 0.54; where the seed stands were geographically isolated and seed collection was made in poor seed year (KAYIHAN, 2000). In another study, isolated populations of Douglas-fir had a G_{ST} of 0.76 with RAPD and 0.21 with isozymes (AAGARD, 1995). AAGARD (1995) proposed that RAPD markers reveal higher polymorphism since RAPD markers screen noncoding regions, point mutation in priming sites, and repetitive DNA sequences whereas isozymes can detect product of a gene only. Nevertheless, high G_{ST} estimates in Turkish red pine forests should be taken into consideration since they have been disturbed for many years and highly fragmented (LISE, 2000; KANDEMIR, 2002). The fragmentation might have lowered genetic exchange among subpopulations of a meta-population and thereby, lead to increasing genetic isolation of small populations. The genetic diversity within the subpopulation was within the ranges of similar conifer species.

The dendrogram constructed with genetic distance data yielded no firm geographic pattern of groupings of seed stands which probably reflects the heavy disturbance over time. The more populations studied and more markers included, the pattern of grouping will be more clear since some of the clusters are not very likely the ones because bootstrap values are very low.

Although Bafra-Alaçam is the most geographically distant population, it is in the group with other seed stands such as Bozyazı-Toldağ and Pos-Soğukoluk. Isolation of populations and long distance seed or seedling material transfers during plantations of large burnt areas after big forest fires are factors which may contribute the formation of the unexpected clusters in the study. Also, seed collection years and number of genetically distant seed trees included to seed stands may be variable and they influence the genetic structure of the studied seed stands.

It is also interesting to note that Düzlerçamı-Antalya seed stand formed an independent branch. The separation of Düzlerçamı-Antalya is not expected, considering that Düzlerçamı-Antalya is located in a highly disturbed low elevation area. Over-exploited due to human activities, forest fires and intensive plantation activities are frequent in the area. Thus, one should expect that the above features favor the maintenance of high genetic diversity in the area. The other explanation may be that an exotic origin with different genetic background was the establishing seed source after a major disturbance such as a forest fire. Additionally, Düzlerçamı-Antalya seed stand is with one of the largest core area from where seeds are collected for the study so more genotypes compared to the other seed stands with smaller core areas may have contributed to the presence of high genetic diversity. These factors may be the reason for separate cluster of the source.

Implications for *in situ* conservation

Genetic diversity provides a template for adaptation and evolution of populations and species; therefore, the maintenance of high genetic diversity in Turkish red pine plantations is one of the most important issues for sustainable Turkish red pine forests in the future.

Since most of Turkish red pine forests are located in forest fire sensitive areas in the Mediterranean Region and intensive plantations are common practices especially in the coastal zones of species range, it is necessary to establish new genetic reserves by using several seed stands with high genetic diversity and the representative of the current breeding zones (KOSKI and ANTOLA, 1993).

Based on the presence of high genetic diversity and the current breeding zone designations, Düzlerçamı-Antalya for coastal-low elevation (Breeding zone1.1), Pos-Soğukoluk for middle elevation (Breeding zone 1.2), and Kaş-Karaçay seed stand for high elevation (Breeding zones 1.3) breeding zones in the Mediterranean Region should be considered as *in situ* reserves. Additionally, Yılanlı-Boyalı for middle elevation breeding zone in the Aegean Region (Breeding zone 2.2), Bayramiç-Karaköy for low elevation breeding zone in the Marmara Region (Breeding zone 3.1) and Bafra-Alaçam (Breeding zone 6.1) seed stand for low elevation zone in the Blacksea Region are the most suitable candidates for *in situ* conserva-

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tion since they have the highest genetic diversity among the other seed stands of the respected breeding zones. Since Turkish red pine is a typical Mediterranean conifer, the suggested seed stands as genetic reserves should be duplicated against potential fire catastrophes in the future. In fact, Orhaneli-Göktepe seed stand in the present study was totally burnt by forest fire a few years ago after seed collections were made.

When new *in situ* genetic reserves are planned to be established in the future, size of the genetic reserves either as gene management zone or gene conservation forests (KAYA and RAYNAL, 2001), should be increased over 100 ha wherever it may be possible. This was supported by the results of the present study that those seed stands with larger core area maintained higher genetic diversity (e.g., Düzlerçamı-Antalya (genetic diversity = 0.28) and Bayramiç-Karaköy (genetic diversity = 0.29) seed stands). The large core area requirement for maintaining genetic diversity is further supported by LISE (2000) who reported that the amount of observed genetic diversity in natural populations of Turkish red pine ranged from 0.28 for degraded populations to 0.33 for natural populations. Also, when seeds are collected for afforestation or reforestation purposes, it should be kept in mind that collected seeds should be genetically good representatives of the seed stands, gene conservation forests or other seed sources.

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Genotype x Environment Interaction in Maritime Pine Families in Galicia, Northwest Spain

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Summary

The magnitude and practical importance of family x site interactions for growth and form traits in maritime pine (*Pinus pinaster* Ait.) breeding in the coastal area of Galicia (NW Spain) were analysed using several different techniques. Data were from 58 8-yr-old half-sib families planted across four sites. The analysis of variance and the ratio of interaction to family variance component showed the interactions to be quantitatively important for several traits, especially for volume and straightness. Genetic correlations between the same trait at different sites were moderate and highly variable, especially for certain pairs of sites. The results indicated that interactions are a consequence of few highly interactive families that may be particularly sensitive to environmental variation. The removal of these families from the breeding program appeared as an effective strategy to solve the interactions. Results are discussed in relation to the stability parameter considered to identify the most unstable progenies.

Key words: Stability, *Pinus pinaster*, Progeny trial, Half-sibs, Genetic correlation.

Introduction

Maritime pine (*Pinus pinaster* Ait.) is the most important forest tree species in Galicia (NW Spain). It occupies around 400,000 ha (27% of the Galician wooded area) with an annual volume increment estimated around $3 \cdot 10^6$ $m^3 \cdot year^{-1}$ (XUNTADE-GALICIA, 2001).

Genetic improvement of *P. pinaster* in the coastal area of Galicia was initiated in 1985 and has included phenotypic mass selection in wild stands and use of this material for seed production in clonal seed orchard (MERLO and FERNÁNDEZ-LÓPEZ, 2004; VEGA *et al.,* 1993). As in other countries, tree breeding objectives were focused mainly on improving growth traits (height and diameter), stem form and branch quality (ALAZARD, 2001; BUTCHER and HOPKINS, 1993). Several progeny tests of the selected material have been established in the coastal area of Galicia across a range of environments. Heritability and genetic correlation in these tests have been estimated by ZAS *et al.* (2004). Results of this study indicate that height growth, internode length, number of whorls and branch angle are under stronger genetic control than diameter growth, stem straightness and forking, and would respond to individual selection. However, several of these traits have shown important genotype x environment (G x E) interaction that must be analysed before any selection and/or recommendation is made.

The concept of G x E interaction has been defined as the varying relative performance of genotypes with environments (BURDON, 1977). An evaluation of the importance and the consequences of the G x E interaction in a breeding programme is essential for decision making about testing, deployment and selection strategies. There is substantial literature about the

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