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RELATIONSHIP BETWEEN GEOGRAPHIC PROXIMITY AND GENETIC SIMILARITY AMONG THE NATURAL POPULATIONS OF *PINUS BRUTIA* TEN.: ITS IMPLICATION ON GENETIC CONSERVATION

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Abstract

Genetic variation in five natural populations of *Pinus brutia* Ten., was determined with isoenzyme analyses. Isozymes from nine enzyme systems extracted from haploid female gametophytes of the seeds were separated by horizontal starch gel electrophoresis. In the nine enzyme systems, 14 loci and 32 alleles were observed. The average proportion of polymorphic loci for the populations ranged from 64.3% to 78.6%. The average number of alleles per locus per populations was estimated 2.08 (± 0.2). The mean estimated expected-heterozygosity (He) of the populations was 0.276. A rather high proportion of genetic variation (95.8%) was due to within-population variation and the remaining (4.2%) was due to variation among populations. The level of gene flow (N_m) was 5.75 per generation. According to genetic variation parameters, although there is no significant differentiation among population, the populations in the western and central parts of Mediterranean region (Muğla, Isparta and Mersin) have relatively more genetic variation than populations in the eastern part. Therefore, these populations should be given a high priority in forest tree breeding, selection and for *in situ* conservation studies in the region.

Introduction

Pinus brutia TEN. subsp. *brutia* is an important forest tree species in Turkey for various economic and ecological reasons. It occurs in the eastern Mediterranean area, i.e., mainly in the eastern part of the Aegean region, on Crete and Cyprus and also sparsely along the shore of western and central parts of the Black Sea region in Turkey and in Syria, Lebanon and Iraq (Nahal, 1983; Boydak *et al.*, 2006). The largest distribution of the species is in Turkey, occupying 3.729.866 ha. of the whole forest lands (26.4%) in the country. About 88% of *P. brutia* forests are located in southern and western Anatolia, mainly in the mountains facing to the Mediterranean and Aegean Seas. In Turkey, about 47% of *P. brutia* forests are located in the Mediterranean, about 40% in the Aegean and about 10% in the Marmara regions. It grows from sea level up to 1200 m, rarely to 1500 m elevation on the Taurus Mountains along the Mediterranean Sea (Mirov, 1967; Gökşin, 1987; Neyişçi, 1987; Boydak *et al.,* 2006), under several variations of the Mediterranean climate (Emberger *et al.*, 1963), and on various bedrock formations and soil types (Arbez, 1974). Within the altitudinal and horizontal distribution range, the species exhibits considerable variation in various form and growth characteristics (Arbez ,1974; Işık, 1986; Isik & Isik, 1999; Isik *et al.*, 1999). It is an important tree species due to variety of uses of its timber. This tree can be used for afforestation of degraded areas in the Mediterranean region and similar climates due to drought resistance (Oppenheimer, 1967). Because of such properties, *P. brutia* has been selected as one of the forest tree species under breeding programs.

Genetic variation and genetic structure of forest trees should be known in order to maximize the effectiveness and efficiency of tree improving programs and gene conservation efforts (Adams, 1981; Slavov & Zhelev, 2004; Tastad *et al*., 2010). Earlier studies on *P. brutia* used various morphological, anatomical and biochemical traits to determine the extent of intra- and interpopulation genetic diversity. The results of such studies on the species established the existence of altitudinal variation in various traits including allele frequencies (Conkle *et al.*, 1988), cortex and needle resin composition (Schiller & Grunwald, 1987; Schiller & Genizi, 1993), morphological and anatomical needle characters, resistance of seedlings to water stress, and shoot morphology (Calamassi, 1982; Calamassi *et al*., 1988a, 1988b). Işık (1983 & 1986) studied altitudinal variation of seed and seedling characteristics on the populations within a narrow geographical region in southwestern Turkey. Later studies by Işık (1993), Işık & Kaya (1993), Işık & Kara (1997), Kara *et al.,* (1997) and Bilir *et al.,* (2002) yielded further evidence of higher intra- and interpopulation genetic variability.

Isozyme analysis is one of effective and rapid techniques for determination of genetic variation in forest trees (Feret & Bergmann, 1976; Weber & Stettler, 1981; Buth & Murphy, 1999). Efficient strategies for long term gene conservation programs can be formed based on the distribution of genetic diversity within and among populations by using data on patterns of allozyme variation (Adams, 1981).

The aims of this study were (1) to obtain additional information on allele genotype frequencies of nine enzyme systems from five different natural populations of *P. brutia*; (2) to estimate genetic variation parameters to determine level of genetic variation within and among populations; (3) to determine if any geographical variation appears in allozyme frequencies in association with geographical gradients.

Materials and Methods

Plant Material: Bulked seed materials from five natural *P. brutia* stands from mid-elevation zone (650-800 m) were obtained via the Forest Tree Seeds and Tree Breeding Research Directorate, Ankara, Turkey (Fig. 1, Table 1). These stands have been used by the Forest Service as seed sources for afforestation purposes within the respective altitudinal zones in the region. The seeds were air dried and stored at 5°C until they were used in the isozyme analysis. A random sample of 90 to 100 seeds was taken for each population to examine isozyme patterns of all enzyme systems. Haploid megagametophytes of seeds were used for the analyses.

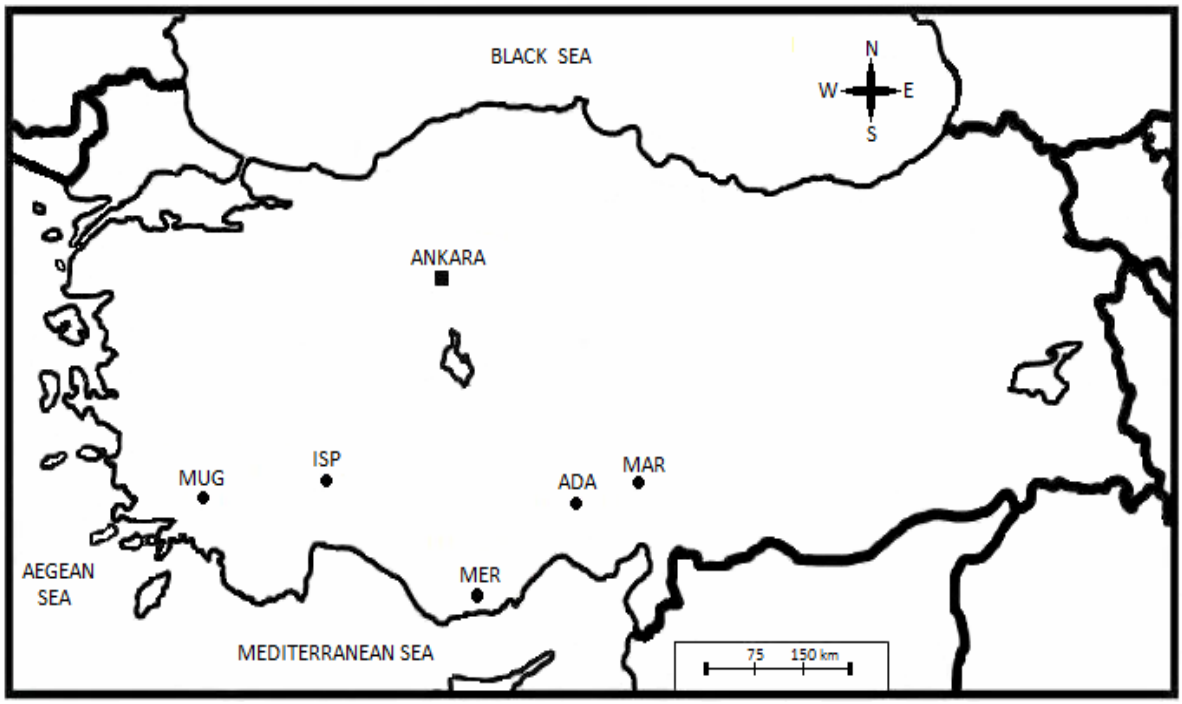

Fig. 1. Location (black dots) of *Pinus brutia* populations included in the study (See Table 1 for the details on populations).

Table 1. Geographic locations and some genetic parameters in five natural populations of *Pinus brutia.*

Population and abbreviation	Altitude (m)	Latitude	Longitude	Mean sample size per locus	Mean number of alleles per locus (A)	% of polymorphic loci (P)	Mean expected heterozygosity (He)*
Muğla, MUG	750 m	37º 06′	28º 32′	95.1 (±0.6)	2.1 (±0.2)	64.3	0.280 (±0.058)
Isparta, ISP	800 m	37º 30′	30º 41′	96.2 (±0.6)	2.0 (±0.2)	78.6	0.293 (±0.054)
Mersin, MER	650 m	36º 14′	33º 15′	95.1 (±0.5)	2.1 (±0.2)	78.6	0.291 (±0.058)
Adana, ADA	745 m	37º 32′	35º 23′	94.6 (±0.5)	2.1 (±0.2)	71.4	0.258 (±0.061)
K. Maraş, MAR	800 m	37º 47′	36º 40′	77.7 (±5.5)	2.1 (±0.2)	64.3	0.260 (±0.063)
Overall mean	-	-	-	91.7	2.08	71.4	0.276

* Unbiased estimate
± Standard error

Electrophoretic analysis: For the analysis, seeds were germinated on moistened Whatman N3 filter paper in Petri dishes at 22°C. Horizontal starch gel electrophoresis was used to obtain information on enzyme mobility variants in 14 loci encoding nine enzyme systems. The haploid megagametophyte tissue was homogenized in a grinding plate with 75 µl of 0.2 M phosphate buffer pH 7.5, 0.1% Triton X-100, 1% BSA and 0.1% β-mercaptoethanol for all enzyme systems (Kara *et al.*, 1997). The resulting homogenates were subjected to starch gel (12% starch) using four different buffer systems according to slightly modified methods of Conkle *et al.,* (1982), as described in Kara *et al.,* (1997). Gels were sliced and stained for each enzyme system according to Conkle *et al.,* (1982). The enzymes assayed are given in Table 2.

Table 2. The assayed enzymes, their abbreviations (Abbr.), enzyme commission numbers (E.C.No.), buffer systems used for electrophoresis and number of loci scored.

Enzyme	Abbr.	E.C. No.	Buffer*	Loci scored
Aconitase	ACO	4.2.1.3	$MC_{6.1}$	1
Alcohol dehydrogenase	ADH	1.1.1.1	TBE	1
Glutamate dehydrogenase	GDH	1.4.1.2	$MC_{8.3}$	1
Glutamate oxaloacetate-transaminase	GOT	2.6.1.1	TC	3
Malate dehydrogenase	MDH	1.1.1.37	$MC_{8.3}$	1
Menadione reductase	MNR	1.6.99.2	TBE	2
6-Phosphogluconate dehydrogenase	6PGD	1.1.1.44	$MC_{6.1}$	2
Phosphoglucoisomerase	PGI	5.3.1.9	$MC_{8.3}$	2
Shikimate dehydrogenase	SDH	1.1.1.25	$MC_{6.1}$	1

* $MC_{6.1}$ = Morpholine Citrate (pH 6.1), $MC_{8.3}$ = Morpholine Citrate (pH 8.3), TBE = Tris-Borate-EDTA, TC = Tris-Citrate. Details on the gel buffers are reported in Kara *et al.,* 1997

Statistics: Genetic variation was described by three parameters: 1) percentage of polymorphic loci (P) at the 0.95 criterion; 2) average number of alleles per locus (A); 3) expected heterozygosity (He) (Nei, 1973). Genetic differentiation among the populations was analyzed by using Nei's genetic diversity indices (Nei, 1973, 1978). The total genetic variation at polymorphic loci (H_T) was partitioned into within (H_S) and among (D_{ST}) components, with latter also expressed as a partition of total variation, i.e., $G_{ST} = D_{ST} / H_T$. Cluster analysis resulting in a phylogenetic tree was based on Edwards' (1971, 1978) Euclidean distance procedure of rooting at the midpoint of longest path. BIOSYS-1 computer program was used for all computations (Swofford & Salender, 1981). Wright's (1951) equation was used to estimate the amount of gene flow (N_m) among populations, *i.e.*, $N_m = [(1-G_{ST}) / 4G_{ST}]$; where G_{ST} is the coefficient of genetic differentiation.

Table 3. Allele frequencies for polymorphic loci in five populations of *Pinus brutia*.

Locus	Allele		Populations[a)]				
			MUG	**ISP**	**MER**	**ADA**	**MAR**
Aco		♦N=	96	96	95	93	67
	1		0.885	0.885	0.726	0.903	0.851
	2		0.083	0.104	0.274	0.075	0.104
	3		0.031	0.010**	0.000	0.022	0.045
Adh2		N=	96	96	96	94	96
	1		0.021	0.083	0.083	0.053	0.052
	2		0.979	0.917	0.917	0.947	0.948
Gdh		N=	95	96	96	96	96
	1		0.968	0.823	0.885	0.917	0.927
	2		0.032	0.177	0.115	0.083	0.073
Got1		N=	93	96	95	92	64
	1		0.624	0.604	0.400	0.522	0.563
	2		0.376	0.396	0.600	0.478	0.437
Got2		N=	96	96	96	96	61
	1		0.594	0.854	0.938	0.958	0.984
	2		0.406	0.146	0.062	0.042	0.016
Got3		N=	96	96	96	96	64
	1		01.00	1.000	1.000	1.000	0.984
	2		0.000	0.000	0.000	0.000	0.016*
Mdh1		N=	96	100	94	92	96
	1		0.281	0.280	0.383	0.315	0.438
	2		0.719	0.720	0.617	0.685	0.562
Mnr2		N=	96	73	96	96	96
	1		0.771	0.760	0.823	0.917	0.990
	2		0.229	0.240	0.177	0.083	0.010**
6Pgd2		N=	96	960	95	94	96
	1		0.688	0.427	0.284	0.404	0.344
	2		0.312	0.573	0.716	0.596	0.656
6Pgd3		N=	96	96	95	95	96
	1		0.688	0.646	0.611	0.674	0.615
	2		0.177	0.062	0.031	0.094	0.000
	3		0.135	0.292	0.347	0.232	0.385
	4		0.000	0.000	0.011*	0.000	0.000
Pgi2		N=	95	100	96	96	64
	1		0.726	0.780	0.781	0.792	0.609
	2		0.242	0.220	0.219	0.198	0.313
	3		0.032	0.000	0.000	0.010**	0.078
Sdh1		N=	88	91	89	92	32
	1		0.409	0.429	0.270	0.359	0.281
	2		0.523	0.429	0.404	0.380	0.563
	3		0.068	0.142	0.281	0.152	0.063
	4		0.000	0.000	0.045	0.109	0.093

a) See Table 1 for abbreviation of populations
♦ N= Number of megagametophytes analyzed
* Private or unique alleles
** Rare alleles (i.e., those with frequencies ≤ 0.01)

Results and Discussion

The allozyme analysis provided important information about the geographic pattern and genetic variation in *P. brutia*. Five *Pinus brutia* populations at this study exhibits considerable variation at allozyme loci examined. Of the 14 loci analyzed, two (*Mnr1* and *Pgi1*) were monomorphic for all populations. Estimates of allelic frequencies for each polymorphic locus are presented in Table 3. The data of gene frequency from our analysis indicate that in total 32 alleles were observed at 14 loci for all populations. Two of 32 alleles were private at K. Maraş (*Got3-2*) and Mersin populations (*Pgd3-4*). *Aco-3*, *Mnr2-2* and *Pgi2-3* were observed as rare (that is, their frequencies are ≤0.01) at Isparta, K. Maraş and Adana populations, respectively (Table 3). Observation of rare and private alleles may be due to strong selection pressure that operates similarly on these loci. Another possibility is that these alleles might have been formed recently, thus may not have enough time to be dispersed through populations within a relatively short period, so their frequencies could not have been increased. Rare alleles may not directly contribute to the genetic distance coefficient value, but they may be an indicator for microevolutionary events occurring in populations (Goncharenko *et al.*, 1994).

Genetic variability parameters were presented in Table 1. The overall mean number of alleles per locus was 2.08 (ranging from 2.0 to 2.1); the overall mean percentage of polymorphic loci was 71.4 (ranging from 64.3 to 78.6); and the overall mean expected heterozygosity was 0.276 (±0.016) (ranging from 0.258 to 0.293). The mean expected heterozygosity was the highest in Isparta population (0.293±0.054), the lowest in Adana population (0.258±0.061). The mean percentage of polymorphic loci in Isparta and Mersin populations was higher than the others. Genetic variability parameters of the populations in our study are close to the values typical for gymnosperms. Hamrick *et al.*, (1981) reported that among 20 species of conifers, the mean number of alleles

per locus was 2.29; the mean percentage of polymorphic loci was 67.7 and the mean expected heterozygosity was 0.207. Other studies on *P. brutia* (Doğan, 1997; Kara *et al.,* 1997; Gülbaba & Özkurt, 1998; Panetsos *et al.,* 1998) reported that the mean number of alleles per locus was between 1.6 and 2.3; the mean percentage of polymorphic loci was between 57.1 and 86.5, and the mean expected heterozygosity was between 0.193 and 0.285. Velioğlu *et al.,* (2002), using RAPD markers, found the mean number of alleles per locus was between 1.71 and 1.92 (mean 1.7), the percentage of polymorphic loci to range from 70.9% to 91.9% (mean 77%) and the mean expected heterozygosity was between 0.21 and 0.29 (mean 0.28) in *P. brutia* populations. Kandemir *et al.,* (2004), using RAPD markers, also found the mean number of alleles per locus was between 1.71 and 1.92 (mean 1.7), the percentage of polymorphic loci to range from 55.8% to 81.7% (mean 66.4%) and the mean expected heterozygosity was between 0.17 and 0.29 (mean 0.23) in *P. brutia* populations. The mean expected heterozygosity was the highest in Isparta population (0.293±0.054), the lowest in Adana population (0.258±0.061). The mean percentage of polymorphic loci in Isparta and Mersin populations was higher than other populations.

Regression equation between frequency of 1st allele in *Got2* locus and longitude was significant (y = 0.0426x – 0.5386 and r = 0.898, $p<0.05$). This indicates that frequency of 1st allele for *Got2* locus increase along the longitudinal gradient from the west to the east, which shows a clinal variation for *Got2* locus. Such a pattern of clinal variation along geographical gradients is common among forest tree species. Indeed, Kara *et al.,* (1997) found significant relations between allele frequencies and altitude of populations for five loci. Dangasuk & Panetsos (2004) reported longitudinal variations among *Pinus brutia* populations in Crete Island, Greece, based on some needle, cone and seed traits.

Gene diversity analysis in our study indicated that approximately 4.2% of the observed total genetic variation was due to differences among the populations (G_{ST}) (Table 4). These mean either that heterogeneity within populations was higher than heterogeneity among populations or that interpopulation gene diversity is not strong. The mean level of diversity estimated for the genus *Pinus* using G_{ST} is 6.5% (Hamrick *et al.,* 1992). These values fall within the range observed on other *P. brutia* populations. The G_{ST} values showed that genetic diversity among populations resulted mainly from *Got2, Mnr2* and *Pgd2* loci (Table 4). In some earlier studies, G_{ST} values ranged from 2.1% to 5.3% in populations of *P. brutia* from the Bolkar Mountain, Kazdağları & Antalya regions (Doğan, 1997; Kara *et al*., 1997; Gülbaba & Özkurt, 1998; Panetsos *et al.*, 1998). G_{ST} values for other gymnosperms varied from 1.5% to 4.6% (Kurt *et al.*, 2008, Bilgen & Kaya, 2007; Turna, 2003; Korshikov *et al.,* 2002; Dvornyk 2001).

Table 4. Selected genetic diversity parameters among five populations of *Pinus brutia.*

Locus	H_S*	H_T*	D_{ST}*	G_{ST} (%)*
Aco	0.2507	0.2605	0.0098	3.76
Adh-2	0.1089	0.1100	0.0011	0.97
Gdh	0.1689	0.1736	0.0047	2.70
Got-1	0.4838	0.4964	0.0126	2.54
Got-2	0.1923	0.2330	0.0406	17.44
Got-3	0.0063	0.0064	0.0001	1.28
Mdh-1	0.4407	0.4483	0.0076	1.69
Mnr-1	0.0000	0.0000	0.0000	-
Mnr-2	0.2363	0.2519	0.0157	6.21
Pgd-2	0.4518	0.4900	0.0382	7.79
Pgd-3	0.4871	0.4995	0.0124	2.48
Pgi-1	0.0000	0.0000	0.0000	-
Pgi-2	0.3914	0.3985	0.0071	1.78
Sdh-1	0.6266	0.6441	0.0176	2.72
Mean	0.2746	0.2866	0.0120	4.17
Standard error	0.0553	0.0569	******	******
N = Locus number	14	14	******	******

* H_S= Intrapopulation genetic diversity. H_T= Total genetic diversity in all populations. D_{ST}= Interpopulation genetic diversity. G_{ST}= Proportion of genetic diversity residing among populations within a region

The estimated level of gene flow among populations within a single generation (N_m) is calculated to be, on average, 5.75. This means that gene exchange among the *P. brutia* populations studied is high, its rate being 5.75 migrants per generation. Doğan (1997) reported that N_m was 7.54, Kara *et al.,* (1997) reported that N_m was 4.47, Gülbaba & Özkurt (1998) reported that N_m was 10.27. In our study, the highest gene flow value was estimated to be among Mersin, Adana and K. Maraş populations and the lowest gene flow value was between Muğla and eastern populations (Adana & K. Maraş). In other word, geographically close populations exchange their genes at relatively high level, whereas distant populations exchange their genes at relatively low level. This finding is also supported with the result of cluster analysis.

The result of cluster analysis after optimization is shown in Fig. 2. The five populations under consideration were clustered into two main branches, which then split into three subgroups mainly according to geographic proximity (see Fig. 1). The Adana and K.Maraş populations, which are the most eastern in the distribution range of the species and closer to each other geographically, are clustered on the same branch. Muğla & Isparta populations, which are also close to each other geographically, form a separate group. Mersin population, which is closer to Adana & Mersin, also forms a group together with them. The phylogenetic tree indicates that there is a close association between the geographic proximity and genetic similarity among the populations.

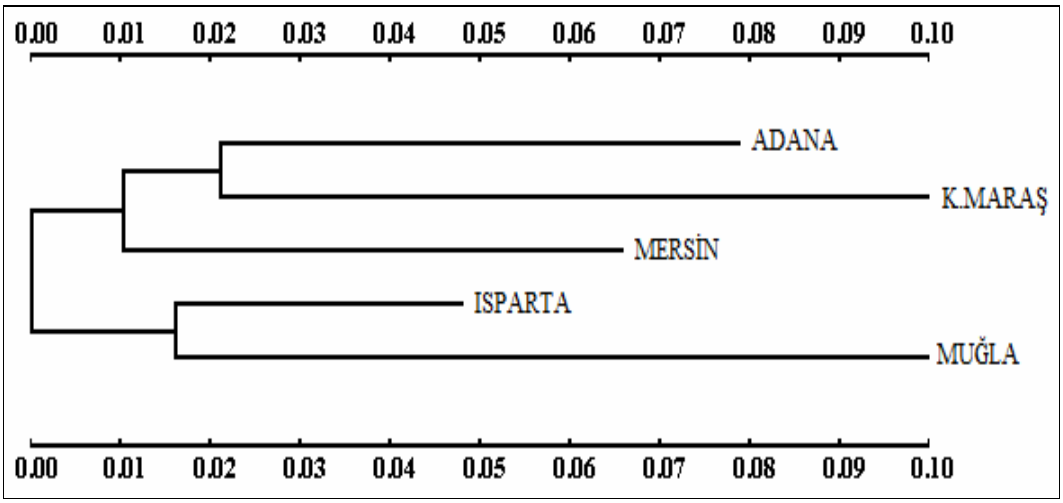

Fig. 2. Phylogenetic tree based on "E" distance, showing the relations among five *Pinus brutia* populations located in the Mediterranean region, Turkey.

Conclusions

Genetic diversity enables populations to exist in the future against changing environmental conditions. Therefore, the maintenance of genetic diversity in *P. brutia* populations and especially conservation of populations with high genetic diversity are the most important issues for sustainable *P. brutia* forests in the future. Based on 14 loci in *P. brutia*, the populations in the western and central parts (Muğla, Isparta and Mersin) of the Mediterranean region have relatively higher genetic variation than the populations in eastern part (Adana & K.Maraş). The longitudinal variation as illustrated by the dendrogram categorized the *P. brutia* populations into two groups: the western (Muğla, Isparta) and the eastern group (Mersin, Adana, K. Maraş). In accordance with several previous studies, our results also showed that genetic differentiation among the neighboring *P. brutia* populations is relatively small possibly because of high gene flow among the adjacent and contiguous populations.

Our findings show that each population has a specific genetic structure, especially western (Muğla, Isparta) and the eastern group (Mersin, Adana, K. Maraş). Probably these two groups might have differentiated in terms of their gene pool, gene combination and adaptation values due to longitudinal and other micro-climate differences, although all populations in the study are nearly at the same altitude. Studies of the geographical structure of genetic variation in forest trees often give valuable information at better defining seed transfer rules among provenance regions, and generate a background for conservation studies.

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