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Genetic Comparison of *Pinus brutia* Ten. Populations from Different Elevations by RAPD Markers

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Abstract

Turkish Red Pine (*Pinus brutia* Ten.) is an important forest tree species in Turkey for various economic and ecological reasons. In this study, nine RAPD (Randomly Amplified Polymorphic DNA) primers were used to determine genetic variation within and among populations of *P. brutia* located at the Duzlercami common-garden test site. This site was established in 1979 and includes six natural populations of *P. brutia* from two altitudinal transects extending from the coast to higher elevations in the Antalya region of Turkey. A total of 32 polymorphic RAPD loci were found in the analyzed six populations. The mean proportion of polymorphic loci among population samples equals 100%, mean number of alleles for each locus = 2.0, effective allele number = 1.71, Shannon's information index = 0.58, and mean Nei (1973)'s gene diversity value = 0.4. According to G_{ST} results, a high proportion of genetic diversity (95-99%) is found within populations. A relatively high genetic differentiation was found among altitudinal population pairs in both transect. Also, data on quantitative traits (total height and/or diameter) at different ages (13, 17, 30 years) were compared with molecular data. There are similarities between the results obtained from RAPD markers and those obtained from the quantitative traits. The differentiation in quantitative traits appears to be due to local adaptation of populations. Data suggest that priority should be given to the selection of material based on geographic origin along the altitudinal gradients of *P. brutia* populations to conserve the genetic resource of species.

Keywords: altitudinal variation, genetic distance, local adaptation, Turkish red pine

Introduction

Turkish red pine (*Pinus brutia* Ten.) is distributed naturally in the eastern Mediterranean basin, mainly in the Mediterranean and Aegean regions of Turkey, with small isolated populations along the Black Sea Coast in the north. It covers 5.4 million ha of forestland, which constitutes 24% of the total forested areas in Turkey (Anonymous, 2006). Turkish red pine is one of the most important forest tree species in the region, from both an economical and ecological point of view. It provides timber resources, and is used widely in afforestation and reforestation programs (Boydak, 2004; Isik, 1986). The species normally forms pure stands from sea level up to 1200 m, and thrives sporadically up to 1500 m on the Taurus Mountains along the Mediterranean Sea in the South, and up to 600 m in the North. Natural stands are found on an extremely wide range of soil types and under a variety of climatic conditions, with significant amount of genetic variation in various form and growth characteristics (Arbez, 1974; Boydak, 2004; Isik and Isik, 1999; Kandemir *et al.,* 2004).

Turkish red pine is a relatively complex species with regional adaptations along its large distribution range in Turkey. Isik (1986) and Isik and Kara (1997), using the same populations and test sites as used in this study, have indicated that most of the morphological variation found in the region seems to be a function of altitude and associated climatic factors. In addition, they suggested that some portion of this variation is of adaptive importance and is under genetic control. Altitudinal differentiation on growth characters could indeed be related with climatic variation patterns in the region. As elevation increases, temperature and length of the growing season decrease in the study area (Isik, 1986; Isik *et al.*, 1987), which could promote phenological differentiation and a certain degree of isolation. Against this hypothetical role of altitude on morphological traits, different allozyme and chloroplast SSR studies in various pine species have reported low (F_{ST} < 0.015) or negligible levels of genetic differentiation among altitudinal gradients (Kara *et al.*, 1997; Navascues *et al.*, 2008; Saenz-Romero and Tapia-Olivares, 2003).

Random amplified polymorphic DNA (RAPD) markers are based on standard polymerase chain reaction (PCR) methodology and widely used in genetic studies of forest tree species (Neale *et al.*, 1992; Wang and Szmidt, 2001). The application of the RAPD method is relatively easy due to: use of arbitrary sequence primers without prior DNA sequence information, Mendelian inheritance of loci, requirement for a small amount of template DNA, relatively high speed of analysis, and easy separation of amplification products on an agarose gel. On the other hand, the main disadvantage of RAPD markers is that they are dominant genetic markers (Wang and Szmidt, 2001; Williams *et al.,* 1990). The utility of RAPD markers in Turkish red pine has been shown for linkage mapping (Kaya and Neale,

1995), and several genetic variation studies (Icgen *et al.*, 2006; Lise *et al.*, 2007; Kandemir *et al.*, 2004; Kaya and Neale, 1993).

The main objective of this study is to investigate genetic variation within and among six natural populations of *P. brutia* from two altitudinal transects planted in the Duzlercami common garden in Antalya region. Common garden material can provide valuable information to explain genetic differentiation and processes of adaptation of trees to different environments which can then be compared to molecular genetic information (Gonzalez-Martinez *et al.,* 2002; Whitlock, 2008). Specific study objectives are (i) to use highly polymorphic RAPD loci to determine levels of genetic variation and population genetic structure along altitudinal gradients; (ii) to compare molecular and quantitative data related to genetic diversity and differentiation estimates on the same populations and families; and finally, (iii) to interpret the results in view of forest management practices, conservation and genetic improvement of the species.

Materials and methods

Common garden experiments

The Duzlercami common-garden test site was established in 1979 and includes six natural populations of *Pinus brutia* from two altitudinal transects extending from the coast to higher elevations in the Antalya region of Turkey (Tab. 1 and Fig. 1). Three altitudinal groups were represented in each transect (coast, mid and high elevations). Each population was represented by (1) ten randomly-selected open pollinated families, and (2) 10 half-sib trees within each population. Detailed information about the populations, families and the common garden test sites are provided in Isık (1986), Isık *et al.* (1999), Isık and Isık (1999), and Kurt (2011).

Tab. 1. Geographic information on the location of *P. brutia* populations and common garden test site (see also Fig. 1.)

Transect*	Code	Name	Altitude (m)	Latitude N	Longitude E
A: Sampled populations					
1	S	Sarilar	92	36° 48'	31° 26'
	M	Murtbeli	490	37° 01'	31° 24'
	K	Kapan	933	37° 06'	31° 24'
2	D	Doyran	61	36° 52'	30° 32'
	B	Buk	480	36° 58'	30° 26'
	H	Hacibekar	1033	37° 19'	30° 11'
B: Common garden test site					
2	1	Duzlercami	350	36° 58'	30° 32'

*Transect: Altitudinal transects (1: Eastern and 2: Western regions of Antalya basin)

DNA extraction and RAPD primers

Needle samples for molecular analysis were collected from 360 trees (60 half-sib families × six trees per family) in the Duzlercami common garden test site. Total genomic DNA was extracted from the needles using the slightly modified protocol of Dellaporta *et al.* (1983). DNA quantity and quality were measured with a Nanodrop 1000 Spectrophotometer V3.7. At the beginning of the study 34 RAPD primers (10 oligonucleotide lengths) were selected from previous studies on *P. brutia* Ten. (Icgen *et al.*, 2006; Lise, 2000) and *P. halepensis* Mill. (Gomez *et al*., 2001). All 34 primers were screened on a randomly selected group of 10 samples to check the reproducibility and polymorphisms of the RAPD fragments they yielded using more than three runs for each primer. Prescreening of 34 random decamer primers revealed that nine primers (OPN-05, OPK-09, UBC-103, UBC-144, UBC-178, OPA-07, OPA-18, OPA-19 and OPP-10) could be useful for further study and data collection.

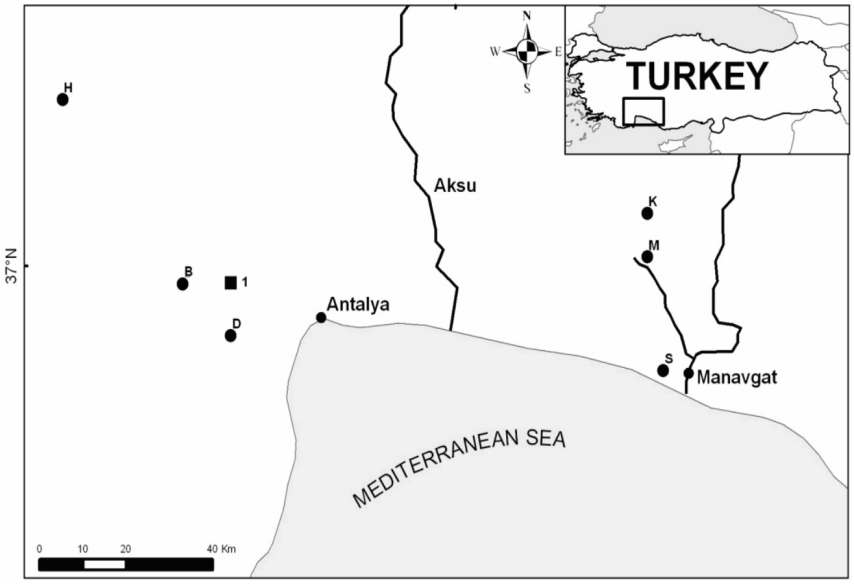

Fig. 1. Location of *Pinus brutia* populations (S, M, K, D, B and H) and common garden test site (1) included in the study (see also Tab. 1)

PCR conditions and interpretation of gels

Different DNA template quantities (3ng, 6ng and 12 ng), primer concentrations (3 pmol, 6 pmol and 9 pmol), $MgCl_2$ concentrations (1.5 mM, 2.5 mM and 3 mM), and Taq polymerase quantities (1 U and 1.5 U) were assayed in order to determine optimum reaction conditions for RAPD analysis and its reproducibility. The final PCR reactions were performed in volumes of 12 µl containing 1x reaction buffer, 6 ng DNA, 6 pmol primer, 2.5 mM $MgCl_2$, 0.3 mM dNTP, and 1 U Taq DNA polymerase (MBI Fermentas, Lithuania). The previously optimized PCR amplification parameters for Turkish red pine were used (Icgen *et al.*, 2006). Amplification products were visualized on 1.7% agarose gels using a 100 base DNA ladder plus (Generuler™ MBI Fermentas) to determine the size of RAPD bands during data collection. All the samples were analyzed twice for all nine primers to secure the reproducibility of the PCR product.

Data analysis

The sizes of amplification products on gels for each primer were determined by using Gel Quant software ver 2.7 (DNR Bio-Imaging Systems Ltd, Israel). Amplification products were scored as discrete, binary states (present/absent). The bands with very low frequencies or poor staining were dropped out of the final data set. Genetic diversity parameters such as polymorphic loci (P), allele frequencies, allelic richness, effective number of alleles, observed and expected heterozygosities, and Shannon's information index were calculated using the software POPGENE version 1.31 (Yeh *et al.*, 1997). Also, Nei's (1987) gene diversity and G_{ST} values were estimated. Nei's (1987) genetic distance and identity were used to construct neighbor-joining trees between populations.

Results

Nine RAPD primers initially revealed 63 polymorphic bands among 360 samples. Some bands with poor staining and/or very low frequencies (<10%) were discarded from the data set. Thus, in the final interpretation, 32 polymorphic bands (loci) with 2 alleles were used in the statistical analysis. The loci numbers ranged from five (OPK-09) to ten (OPP-10) in the primers used. The estimated mean number of observed alleles (N_a) was 2.00. The highest number of effective alleles (N_e) was observed in the Murtbeli and Kapan populations (1.72), whereas, the lowest N_e was found in Hacibekar (1.53). When all populations were considered, overall mean N_e was 1.71 (Tab. 2).

Mean Nei's (1987) gene diversity (or heterozygosity, h) and Shannon's Information index (I) equaled 0.40 and 0.58, respectively. Heterozygosity (h) was lowest in Hacibekar (0.31) and greatest in Murtbeli (0.41) (Tab. 2). Populations were compared using total average heterozygosity (H_t), average heterozygosity within populations (H_s), and genetic differentiation coefficient (G_{ST}) in three different categories (Tab. 3a, b, c). Total average heterozygosity ranged from 0.37 to 0.41, while average heterozygosity within the populations was between 0.36 and 0.40. Genetic differentiation coefficient was found to be between 0.01and 0.05 in three categories (Tab. 3a, b, c). Thus, a very high proportion (ranging from 95% to 99%) of genetic variation was due to differences within populations. H_t and H_s values in the east transect were slightly higher than in the west transect (Tab. 3a). Middle elevation populations were shown to have higher H_t and H_s values than coastal and high elevation populations. High elevation populations had the greatest G_{ST} value (Tab. 3b).

Nei's (1987) genetic distance coefficient ranged from 0.020 (Sarilar-Buk) to 0.085 (Murtbeli-Hacibekar) among all possible population pairs. This indicates that differentiation among the studied populations is low. The phenogram, based on UPGMA (Unweighted Pair-Group Method using arithmetic Averages) clustering, was constructed using Nei's (1987) genetic distance values (Fig. 2). According to phenogram, Buk and Sarilar populations appears to be genetically more similar to each other than the other populations studied. Doyran, Murtbeli and Kapan populations were then added to branches of the phenogram in decreasing order of similarity. Hacibekar, which is the most geographically isolated population (Fig. 1), was found to be the most genetically differentiated (Fig. 2).

Discussion

Comparison of genetic diversity parameters

The level of genetic variation in *Pinus brutia* was found to be within the range previously reported from studies of

Tab. 2. Genetic diversity parameters for the studied populations. [Estimated mean number of observed alleles (N_a), mean number of effective alleles (N_e), Nei (1987)'s gene diversity (h), Shannon's information index (I), and proportion of polymorphic loci (P), ± Standard errors]

Population	N_a	N_e	h	I	%P
Doyran	1.94 ± 0.03	1.65 ± 0.04	0.37 ± 0.02	0.54 ± 0.03	93.75
Sarilar	2.00	1.63 ± 0.04	0.36 ± 0.02	0.54 ± 0.02	100
Murtbeli	2.00	1.72 ± 0.03	0.41 ± 0.01	0.59 ± 0.01	100
Buk	2.00	1.71 ± 0.03	0.40 ± 0.01	0.58 ± 0.02	100
Kapan	2.00	1.72 ± 0.03	0.40 ± 0.02	0.59 ± 0.02	100
Hacibekar	1.81 ± 0.05	1.53 ± 0.05	0.31 ± 0.02	0.46 ± 0.03	81.25
Overall mean	2.00	1.71 ± 0.01	0.40 ± 0.006	0.58 ± 0.007	100

Tab. 3. Total average heterozygosity (H_t), average heterozygosity in populations (H_s), genetic differentiation coefficient (G_{ST}). Standard deviations in parentheses

	No. of analyzed sample	H_t	H_s	G_{ST}
a) Based on two transects (geographical distance)				
Eastern transect	180	0.41 (0.01)	0.39 (0.01)	0.05
Western transect	179	0.38 (0.02)	0.36 (0.02)	0.05
b) Based on three altitudinal population pairs				
Coastal elevation	120	0.37 (0.02)	0.37 (0.02)	0.02
Middle elevation	120	0.41 (0.01)	0.40 (0.01)	0.02
Higher elevation	119	0.37 (0.02)	0.36 (0.02)	0.04
c) At the population level (regardless of transect and altitude)				
Six populations	359	0.40 (0.01)	0.40 (0.01)	0.01

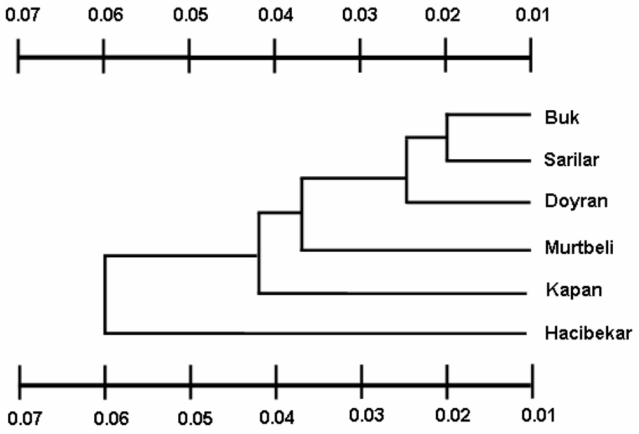

Fig. 2. Phenogram constructed using Nei's (1987) genetic distance values for six studied *Pinus brutia* populations

other *P. brutia* populations when measured by isozymes (Aravanopoulus *et al*., 2004; Conkle *et al*., 1988; Kara *et al*., 1997; Korol *et al*., 2002; Panetsos *et al*., 1998) or RAPD markers (Icgen *et al*., 2006; Lise *et al*., 2007; Kandemir *et al*., 2004). The genetic diversity parameters calculated in this study are similar in level to values obtained from isozyme and RAPD studies on *P. brutia*, and also to isozyme studies on other conifer species (Hamrick *et al*., 1992; Tolun *et al*. 2000). Hamrick *et al*. (1992) estimated the mean level of diversity for the genus *Pinus* using G_{ST} as 6.5%. Tolun *et al*. (2000) estimated total average heterozygosity (H_t) as 0.227 for *Pinus nigra* populations sampled from the Bolkar Mountains.

Nei's (1987) gene diversity, Shannon's information index and mean proportion of polymorphic loci were relatively higher than previously reported RAPD studies on *P. brutia* (Icgen *et al*., 2006; Lise *et al*., 2007; Kandemir *et al*., 2004; Velioglu *et al*., 2002). This high level of genetic diversity may be due to the broad altitudinal range of the samples included in the study. Additionally, the low level of genetic variation in previously reported RAPD studies could be due to using seed stands and/or seed orchards as sample populations, which generally have a relatively narrow genetic base (Icgen *et al*., 2006; Kandemir *et al*., 2004; Velioglu *et al*., 2002).

Overall heterozygosity (H_t = 0.40) and overall heterozygosity within populations (H_s = 0.40) is compatible with other studies on *P. brutia* (Icgen *et al*., 2006; Lise *et al*., 2007; Kandemir *et al*., 2004). However, genetic differentiation coefficient (Gst) values were between 1% and 5% in this study, while G_{ST} ranged from 7% to 35% in previously reported RAPD studies on *P. brutia* (Icgen *et al*., 2006; Lise *et al*., 2007; Kandemir *et al*., 2004; Velioglu *et al*., 2002). The high differentiation rate of those studies could be explained by various reasons; such as using seed stands, seed orchards and/or plantations which are geographically isolated and established far away from each other, low pollination mechanism between seed stands and/or seed orchards, mating system of species, and low seed distribution range and low level of gene flow between seed stands and seed orchards (Kang *et al*., 2001). In contrast, the relatively low polymorphism level within populations in the same studies could be attributed to certain characteristics of seed stands, seed orchards, and/or plantations. Compared to natural populations, these seed stands, seed orchards, and plantations were established in relatively small areas, used small numbers of selected genotypes for their establishment, and have limited seed production and harvesting (Icgen *et al*., 2006; Kandemir *et al*., 2004; Kang *et al*., 2001).

Comparison of the populations

The results showed that all genetic diversity parameters were relatively greater for the middle and upper-middle elevation populations than those from the lower and higher elevation populations (Tab. 2 and 3). The previous results based on growth characters (Isik, 1986; Isik and Isik, 1999; Isik *et al*., 1999) and isozyme analysis (Isik and Kara, 1997; Kara *et al*., 1997) on the same populations and test sites as in this study also indicate that middle elevation and middle-upper elevation populations contain greater genetic variability than coastal and higher elevation populations. Similar results were reported for many plant species (Ohsawa and Ide, 2008). This situation is attributed to optimal habitat conditions for middle elevation populations. The results suggest that geographically "core" and/or "central" populations receive genes both from coastal and high elevation populations, causing them to have higher genetic diversity. In addition, the lower genetic variation and high differentiation level of coastal and high elevation populations could be explained by the effects of suboptimal conditions (early and late frosts in high elevation populations; drought stress, exploitation and/or negative selection by human in coastal populations), relatively limited gene flow, population size and possibly founder effects (Isik and Kara, 1997; Lise *et al*., 2007; Kara *et al*., 1997; Ohsawa and Ide, 2008).

According to the phenogram (Fig. 2), the Hacibekar population has been distinctly differentiated from the others. Hacibekar also showed slightly higher G_{ST} values

based on altitudinal population pairs (Tab. 3). Divergence of the Hacibekar population may be due to its unique features related to its geographic location (Fig. 1). Firstly, this population is located above 1000 m altitude, which is close to the altitudinal periphery of the species. Secondly, it is located on a transition region between Mediterranean and continental climates, where average annual precipitation is much lower than in the core distribution range of the species (30.9 mm vs. 100.1 mm). These two factors, either alone or in combination, could have contributed to accumulation of new mutations, and eventual isolation over time (Kurt, 2011). Buk population was genetically more similar to the Sarilar and Doyran populations than to any others (Figs. 1 and 2). The location of Buk is geographically close to Doyran and it has similar local climatic patterns as the low elevational populations. Therefore, it is likely that evolutionary forces, such as natural selection, might have operated in the same direction for the Buk and Sarilar populations (Isik *et al*., 1987).

Growth characteristic results (Isik, 1986; Isik and Isik, 1999; Isik *et al*., 1999; Kurt, 2011) obtained from the same populations and test sites, displayed significantly greater values for genetic differentiation among populations in terms of quantitative traits (Q_{ST}) than G_{ST} values as observed in this study (Tab. 3). This would provide evidence that Turkish red pine is a complex species with regional adaptations along its large distribution range in Turkey. Isik (1986), Isik and Kara (1997), and Kara *et al*., (1997) reported that most of these variations seem to be a function of altitude and associated climatic factors in the Taurus Mountains. Altitudinal and longitudinal variations have also been shown by Dangasuk and Panetsos (2004) on some morphological traits of the species on Crete Island, Greece. Gonzalez-Martinez *et al*., (2002) indicate that environmental heterogeneity and genotype-by-environment interactions seem to have a major role in quantitative differentiation when gene flow is high. Also, they suggested that some ecological factors (rainfall, temperature, altitude, soil types) are the main determinants of the interaction in maritime pine in Spain. This could also apply to Turkish red pine in this study. Also, some morphological traits of various plant species (especially those of forest trees) showed genetic differentiation along altitudinal gradients as a result of various factors (Ohsawa and Ide, 2008; Saenz-Romero *et al*., 2006; Vitasse *et al*., 2009; Viveros-Viveros *et al*., 2009). All the differentiation along altitudinal gradients can be explained by gene flow (phenological) isolation and selective pressure, eventually resulting in environmental (temperature, precipitation and others) clines (Ohsawa and Ide, 2008; Vitasse *et al*., 2009).

Conclusions

The present results indicate that Turkish red pine populations contain genetic variation associated with seed source elevation. Therefore, for forestry practices such as selection of seed sources, determination of seed transfer zones, and genetic resource conservation programs, the populations with higher genetic variation should first be defined with strong emphasis on elevation gradients. It should be noted that the populations in this study, although representing a wide elevational range of the species, come from a relatively small geographic part of the species distribution area. In view of patterns of variation found within and among populations, further comparative studies, including both molecular and quantitative information should systematically be carried out on a great number of stands (and families), covering various altitudinal and horizontal gradients within the entire geographic range of the species.

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