# GENETIC VARIATION WITHIN AND AMONG *PINUS BRUTIA* TEN. SEED STANDS IN TURKEY IN THEIR ISOENZYMES[1]

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## SUMMARY

Thirty-two natural elite stands of *Pinus brutia* Ten. subsp. *brutia*, used for seed production, were analyzed by starch gel isoenzyme electrophoresis, to determine their genetic diversity and structure. The average number of alleles per locus was 2.16, the range from 1.95 to 2.40; average percentage of polymorphic loci, at the 95 %criterion was 68 % , the range from 65 to 75 % ; average effective number of alleles per locus was 1.49, the range from 1.41 to 1.60. The observed and expected heterozygosity were 0.191 (ranging from 0.144 to 0.267) and 0.271 (ranging from 0.233 to 0.312), respectively. Most of the genetic diversity (91 %) resides within populations. Inter-population gene flow according to Wright's formula was 2.55; the average gene flow between geographically grouped populations was 3.52, and that between latitudinally grouped populations was 2.81. There were significant linear regressions between allele frequencies in several loci among enzyme systems, and also between allele frequencies in several loci of different enzyme systems and the geographical variables of the stand (longitude, latitude, altitude, and slope aspect).

The results imply that the formerly designated seed distribution zones should be reconsidered, because each seed stand is unique in its set of alleles, therefore, its seeds can be distributed only in the adjacent forest areas that have similar ecological features.

Keywords: Allozymes, genetic diversity, genetic differentiation, gene flow, *Pinus brutia*

## INTRODUCTION

Much attention has been given lately to *Pinus brutia* Ten. subsp. *brutia* (NAHAL 1983), which grows naturally in the northern corner of the eastern Mediterranean, mainly in Turkey, the eastern Aegean islands, Crete, Cyprus, Lebanon and Iraq. This species was and is being introduced into countries around the Mediterranean, and in other regions in both the northern and southern hemispheres with homologous climates (e.g., PALMBERG 1975, SPENCER 1985). The species grows in a wide variety of sites, at altitudes from sea level up to 1500 m (MIROV 1967).

Availability in the 1970s of *Pinus brutia* seed material from 16 sites within its natural area of distribution (MORANDINI 1976) led to two different genetic approaches: (i) $F_1$-progeny tests (provenance trials) in many countries to elucidate and compare the success (survival, growth) of the various ecotypes (genotypes) in any given environment; (ii) analyses of morphological, anatomical and growth traits, and of genetic diversity, the results of which were reviewed by SCHILLER (1994).

Phenotypic variations in bole straightness, branching, crown shape and other traits were observed in natural populations. Populations growing at low altitudes have larger and thicker branches and less straight boles than those growing at higher sites (ISIK & KAYA 1993, ISIK *et al.* 1999). Because of the high ecological importance and economic value of *Pinus brutia,* much emphasis was placed on determining the amount of variation within several economically important traits such as crown shape, stem straightness, branching, etc. ISIK and KAYA (1993) wrote, "Within its range of distribution, *Pinus brutia* exhibits considerable variation in various form and growth characteristics", and these highlight the importance of the establishment of seed orchards, seed transfer zones, etc.

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Knowledge of the genetic diversity and structure of *Pinus brutia* elite populations is necessary for breeding programs. Therefore, the aims of the present study were: (i) to examine the variation of isoenzyme markers; (ii) to estimate the genetic differentiation of elite populations used as seed stands; and (iii) to try to find relationships between allozyme diversity and the geographic parameters of the seed stands that are scattered within the natural forest area of *Pinus brutia* in Turkey.

## MATERIALS AND METHODS

### Seed Materials

Figure 1 show the distribution of the seed orchards within the natural range of *Pinus brutia* in Turkey. Table 1 presents the geographic parameters of the selected natural seed stands, where collection of open pollinated seeds were made by the Turkish Forest Department, from which we received 32 bulked seed lots. They were the result of seed collection from many trees within each stand. Seeds of each of these stands should be distributed within a designated area of equal ecological features, i.e., seed zone distribution.

### Electrophoresis

The extraction of enzymes from the individual germinating seed megagametophyte tissue and horizontal starch gel electrophoresis were performed according to CONKLE *et al.* (1982) and WENDEL & PARKS (1982). A total of 15 enzyme systems encoded by 20 loci were analyzed with four different electrophoresis buffer systems as presented by KOROL & SCHILLER (1996). The enzyme systems were: alanine aminopeptidase (AAP, EC 3.4.11.2), aconitase (ACO, EC 4.2.1.3), acid phosphotase (ACP, EC 3.1.3.2), alcohol dehydrogenase (ADH, EC 1.1.1.1), catalase (CAT, EC 1.11.1.6),

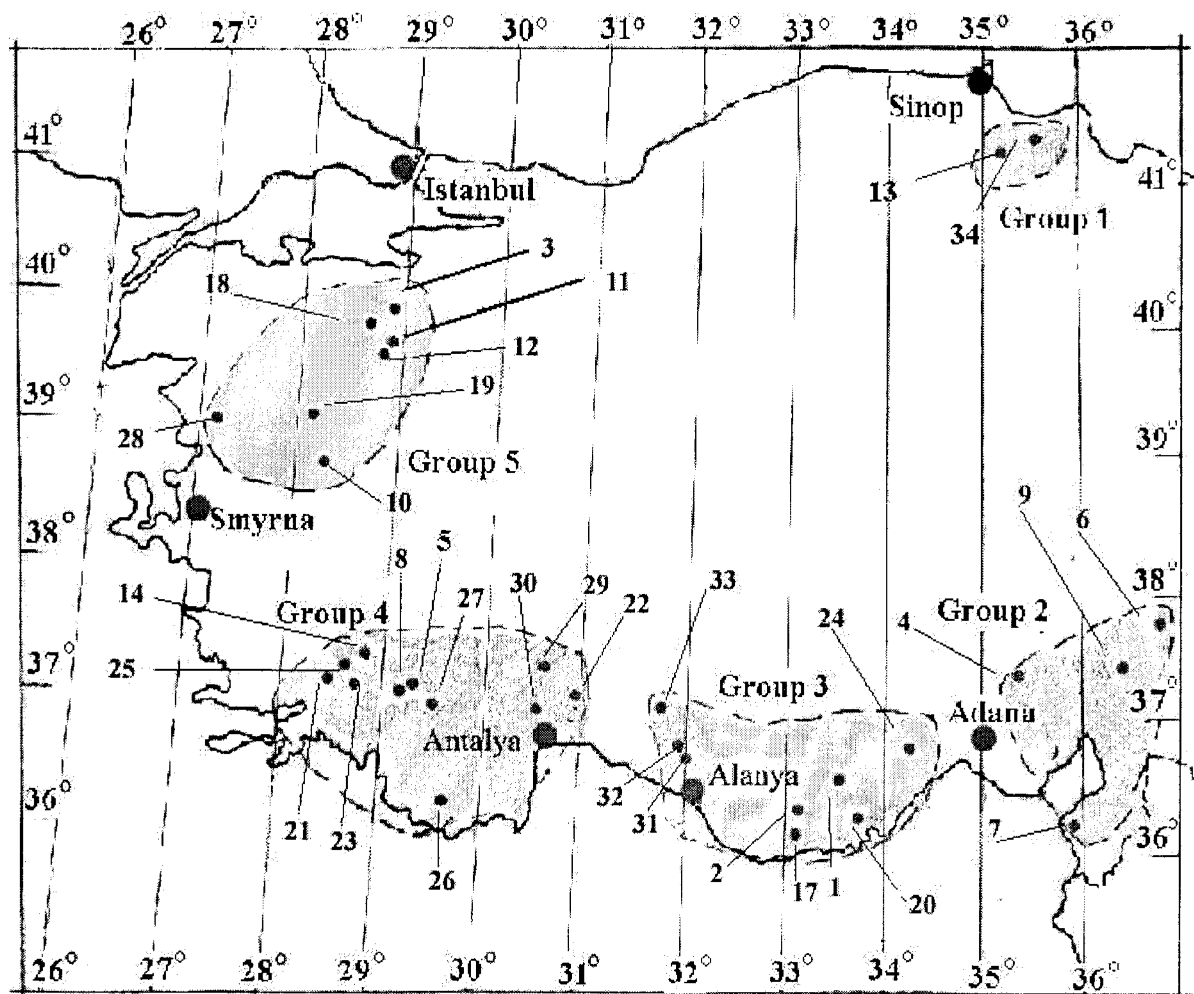


**Figure 1.** Locations of sampled *Pinus brutia* seed stands in Turkey.

**Table 1. Geographic location of seed stands of *Pinus brutia* analyzed in Turkey.**

| | Population | Latitude N | Longitude E | Regional Forest | Breeding zone | Altitude (m) | Aspect |
|---|---|---|---|---|---|---|---|
| 1 | Gulnar-Pembecik | 36° 14′ 30″ | 33° 15′ 20″ | Mersin | 1.2 | 650 | N |
| 2 | Anamur-Tokdag | 36° 09′ 27″ | 33° 05′ 55″ | Mersin | 1.1 | 250 | SE |
| 3 | Orhaneli-Goktepe | 40° 00′ 30″ | 28° 55′ 30″ | Bursa | 3.1 | 650 | S |
| 4 | Pos-Karsanti | 37° 34′ 30″ | 35° 24′ 00″ | Adana | 1.2 | 735 | W–N |
| 5 | Acipayam-Bozdag | 37° 16′ 50″ | 29° 15′ 20″ | Denzili | 1.3 | 870 | S |
| 6 | K. Maras-Sucati | 37° 46′ 30″ | 36° 42′ 00″ | K. Maras | 4.2 | 800 | S–SW |
| 7 | Antakya-Ulucinar | 36° 21′ 20″ | 35° 57′ 30″ | K. Maras | 1.1 | 385 | N |
| 8 | Cameli-Goldag | 37° 06′ 20″ | 29° 07′ 30″ | Denzili | 1.2 | 800 | N |
| 9 | Pos-Baspinar | 37° 35′ 30″ | 35° 21′ 10″ | Adana | 1.2 | 735 | S |
| 10 | Gordes-Sahinkaya | 38° 50′ 11″ | 28° 04′ 32″ | Izmir | 2.1 | 350 | N |
| 11 | Bayramic-Karakoy | 39° 50′ 00″ | 28° 55′ 30″ | Canakkale | 3.1 | 450 | various |
| 12 | Bayramic-katrandag | 39° 50′ 00″ | 28° 55′ 30″ | Canakkale | 3.1 | 450 | various |
| 13 | Durgan-Adadagi | 41° 27′ 13″ | 35° 05′ 33″ | Sinop | 6.1 | 400 | NW |
| 14 | Tavas-Kale | 37° 27′ 18″ | 28° 46′ 43″ | Denzili | 2.2 | 730 | S |
| 17 | Anamur-Toldag | 36° 13′ 20″ | 33° 06′ 20″ | Mersin | 1.1 | 350 | N |
| 18 | M.K.Pasa-Camkonak | 39° 58′ 45″ | 28° 40′ 54″ | Bursa | 3.1 | 250 | NE |
| 19 | Sindirgi-Seydan | 39° 12′ 00″ | 28° 08′ 00″ | Balkesir | 3.1 | 250 | N |
| 20 | Silifke-Kizlidagi | 36° 13′ 25″ | 33° 42′ 55″ | Mersin | 1.1 | 100 | S–W |
| 21 | Yilanli-Boyali | 37° 17′ 00″ | 28° 34′ 00″ | Mugla | 2.2 | 750 | SE |
| 22 | Serik-Pinargozu | 37° 16′ 00″ | 30° 59′ 40″ | Antalya | 1.2 | 500 | W–S |
| 23 | Koycegiz-Agla | 37° 01′ 27″ | 28° 44′ 56″ | Mugla | 2.2 | 650 | W |
| 24 | Mersin-Findikpinari | 36° 56′ 40″ | 34° 26′ 20″ | Mersin | 1.3 | 825 | E |
| 25 | Marmaris-Cetibeli | 37° 02′ 30″ | 28° 16′ 20″ | Mugla | 2.1 | 60 | NW |
| 26 | Kas-Karcasy | 36° 24′ 30″ | 29° 32′ 50″ | Antalya | 1.3 | 975 | S |
| 27 | Golhisar-Kocas | 37° 04′ 30″ | 29° 32′ 40″ | Isparta | 1.3 | 100 | SW |
| 28 | Bergama-Kozak | 39° 14′ 08″ | 27° 06′ 12″ | Izmir | 2.2 | 650 | N |
| 29 | Bucak-Melli | 37° 24′ 45″ | 30° 37′ 20″ | Isparta | 1.2 | 800 | E |
| 30 | Duzlercami | 36° 59′ 45″ | 30° 33′ 10″ | Antalya | 1.1 | 275 | flat |
| 31 | Gundogmus-Eskibag | 36° 44′ 40″ | 32° 00′ 00″ | Antalya | 1.3 | 1000 | NE |
| 32 | Gundogmus-Guzelbag | 36° 45′ 00″ | 31° 58′ 00″ | Antalya | 1.2 | 650 | N |
| 33 | Cevili-Ulupelit | 37° 10′ 22″ | 31° 43′ 53″ | Antalya | 1.3 | 850 | S |
| 34 | Bafra-Alacam-Camgolu | 41° 38′ 38″ | 35° 26′ 10″ | Amasya | 6.1 | 100 | N |

glutamate dehydrogenase (GDH, EC 1.4.1.3), glutamic-oxaloacetic transaminase (GOT, EC 2.6.1.1), isocitric dehydrogenase (IDH, EC 1.1.1.42), leucine aminopeptidase (LAP, EC 3.4.11.1), malate dehydrogenase (MDH, EC 1.1.1.37), menadione reductase (MNR, EC 1.6.99.2), phosphoglucose isomerase (PGI, EC 5.3. 1.9), phosphoglucomutase (PGM, 2.7.5.1), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), and shikimate dehydrogenase (SKDH, EC 1.1.1.25).

Because no segregation analysis to determine the observed variation as gene markers was done, wherever the term locus or allele is mentioned the authors refer to a putative locus or a putative allele (corresponding to putative homozygotes and putative heterozygotes).

## Statistical analysis

BIOSYS-1, release 2 (SWOFFORD & SELANDER 1989) was used to estimate the mean number of alleles per locus (*A/L*), the mean sample size per locus (*N/L*), the proportion of polymorphic loci (at $P$ = 0.95 or 0.99), and the observed and expected heterozygosity ($H_o$ and $H_e$). Estimations of diversity that were performed with the POPGEN version 1.32 software of YEH *et al.* (1997) included: the effective number of alleles per locus ($A_e$) according to CROW & KIMURA (1964); heterozygosity indices according to NEI (1973); and gene flow and F-statistics according to WRIGHT (1951, 1978) and NEI (1977). For each variable locus, fixation indices were calculated and then averaged across loci. Differentiation among *Pinus brutia* populations was calculated as a weighted average of $F_{st}$ for all alleles and all loci in each population.

The significance of excess and/or deficiency in heterozygotes was tested using Li's formula,

$$\chi^2 = NF^2 (a-1),$$

with df = $a(a-1)/2$, where $N$ is the total sample size and $a$ is the number of alleles at $a$ locus (LI & HORO-

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VITZ 1953). To test the significant differences among populations in allele frequencies, $\chi^2$ analysis was used: $\chi^2 = 2\,N\,G_{st}\,(a-1)$, df = (a–1) ($n$–1), where $N$ is the total sample size, $a$ is the number of alleles at the locus and $n$ is the number of populations (WORKMAN & NISWANDER 1970).

Estimation of gene flow among populations based on the indirect method represents the level of gene flow in an island model (WRIGHT 1978). The number of migrants per generation, $Nm$ was calculated according to WRIGHT's formula, $Nm = 0.25*(1-F_{st}) / F_{st}$.

For multiple comparisons of differences among populations and groups the analysis of variance (ANOVA) algorithm with SIGMASTAT STATISTICAL software (SPSS Inc.) was used. Correlation between allele frequencies in the various loci and the environmental factors was calculated by using the JMP software (Ver.3.2.2, SAS Institute, Inc.). The relationship between latitude and genetic parameters was examined by means of Pearson's correlation analysis (SIGMASTAT STATISTICAL software).

Canonical correlation analysis was implemented according to WESTFALL & CONKLE (1992).

## RESULTS

### Diversity in populations

Twenty loci were resolved in the 15 enzyme systems, of

**Table 2. Allozyme variation at 20 loci within šľ populations of *Pinus brutia* in Turkey.**

| | Population | $N$ | $A/L$ | $Ae$ | $P_{95\%}$ | $P_{99\%}$ | $H_o$ | $H_e$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Gulnar-Pembecik | 53 | 2.35 | 1.59 | 70 | 75 | 0.199 | 0.312 |
| 2 | Anamur-Tokdag | 71 | 2.30 | 1.45 | 70 | 75 | 0.205 | 0.256 |
| 3 | Orhaneli-Goktepe | 59 | 2.25 | 1.56 | 70 | 75 | 0.188 | 0.292 |
| 4 | Pos-karsanti | 62 | 2.25 | 1.47 | 65 | 70 | 0.208 | 0.251 |
| 5 | Acipayam-Bozdag | 69 | 2.15 | 1.48 | 65 | 75 | 0.183 | 0.273 |
| 6 | K. Maras-Sucati | 54 | 2.40 | 1.46 | 60 | 75 | 0.179 | 0.258 |
| 7 | Antakya-Ulucinar | 58 | 2.30 | 1.53 | 70 | 75 | 0.204 | 0.288 |
| 8 | Cameli-Goldag | 56 | 2.20 | 1.48 | 75 | 75 | 0.186 | 0.279 |
| 9 | Pos-Baspinar | 50 | 2.25 | 1.49 | 70 | 70 | 0.199 | 0.276 |
| 10 | Gordes-Sahinkaya | 70 | 2.25 | 1.53 | 60 | 65 | 0.225 | 0.260 |
| 11 | Bayramic-Karakoy | 52 | 2.35 | 1.52 | 70 | 75 | 0.183 | 0.282 |
| 12 | Bayramic-Katrandag | 36 | 2.15 | 1.58 | 70 | 70 | 0.206 | 0.300 |
| 13 | Durgan-Adadagi | 36 | 2.05 | 1.47 | 65 | 80 | 0.186 | 0.267 |
| 14 | Tavas-Kale | 57 | 2.15 | 1.60 | 75 | 75 | 0.203 | 0.302 |
| 17 | Anamur-Toldag | 36 | 2.20 | 1.45 | 65 | 70 | 0.210 | 0.257 |
| 18 | Pasa-Camkonak | 31 | 2.05 | 1.47 | 65 | 75 | 0.144 | 0.271 |
| 19 | Sindirgi-Seydan | 71 | 2.15 | 1.45 | 70 | 80 | 0.165 | 0.255 |
| 20 | Silifke-Kizlidagi | 57 | 2.10 | 1.47 | 65 | 70 | 0.202 | 0.263 |
| 21 | Yilani-Boyali | 64 | 2.00 | 1.48 | 60 | 80 | 0.162 | 0.269 |
| 22 | Serik-Pinargozu | 49 | 2.35 | 1.54 | 75 | 75 | 0.204 | 0.297 |
| 23 | Koycegiz-Agla | 52 | 2.20 | 1.57 | 70 | 75 | 0.189 | 0.301 |
| 24 | Mersin-Findikpinari | 49 | 1.95 | 1.42 | 65 | 65 | 0.124 | 0.235 |
| 25 | Marmaris-Cetibeli | 71 | 1.95 | 1.43 | 65 | 80 | 0.170 | 0.250 |
| 26 | Kas-karcasy | 46 | 2.10 | 1.46 | 75 | 75 | 0.185 | 0.268 |
| 27 | Golhisar-Kocas | 54 | 2.00 | 1.43 | 65 | 65 | 0.211 | 0.241 |
| 28 | Bergama-Kozak | 76 | 2.05 | 1.45 | 60 | 70 | 0.204 | 0.246 |
| 29 | Bucak-Meli | 52 | 2.10 | 1.51 | 70 | 75 | 0.195 | 0.277 |
| 30 | Duzlercami | 85 | 2.25 | 1.52 | 75 | 80 | 0.188 | 0.284 |
| 31 | Gundogmus-Eskibag | 34 | 2.20 | 1.56 | 70 | 70 | 0.267 | 0.292 |
| 32 | Gundogmus-Guzelbag | 41 | 2.00 | 1.48 | 70 | 75 | 0.189 | 0.277 |
| 33 | Cevili-Ulupelit | 71 | 2.15 | 1.56 | 75 | 80 | 0.209 | 0.292 |
| 34 | Bafra-Alacam-Camgolu | 39 | 2.00 | 1.41 | 65 | 70 | 0.158 | 0.233 |
| Overal means | | | 2.16 | 1.49 | 68 | 74 | 0.191 | 0.271 |

$N$ = sample size; $P_{95\%}$ = percentage of polymorphic loci; $A_e$ = effective number of alleles (CROW & KIMURA 1964); $H_o$ = observed heterozygosity; $A/L$ = number allele per locus; $P_{99\%}$ = percentage of polymorphic loci; $H_e$ = expected unbiased heterozygosity (NEI 1978).

**Table 3. Correlation coefficients of linear regressions between allele frequencies among different enzyme loci.**

| Loci and alleles | *Aap-1*$_{.3}$ | *Aco-1*$_{.1}$ | *Acp-1*$_{.1}$ | *Adh-2*$_{.1}$ | *Gdh-1*$_{.1}$ | *Got-2*$_{.2}$ | *Mdh-1*$_{.1}$ | *Pgi-2*$_{.2}$ |
|---|---|---|---|---|---|---|---|---|
| *Aap-1*$_{.2}$ | | | 0.442* | 0.416* | 0.442* | | | |
| *Aco-1*$_{.1}$ | | | | | | | 0.427* | |
| *6Pgd-2*$_{.1}$ | | | | | | 0.578*** | | |
| *6Pgd-3*$_{.2}$ | | –0.426* | | | | | | 0.448* |
| *6Pgd-3*$_{.3}$ | –0.435* | 0.530** | | | | | | |
| *Skdh-1*$_{.3}$ | | | | | | | | |
| *Skdh-2*$_{.2}$ | | –0.423* | 0.527*** | | | –0.441* | | |

* – significant at 0.05; ** – significant at 0.01; *** – significant at 0.001; Notation of loci and alleles: *Aap-1*$_{.2}$ = the *Aap* locus 1, allele 2; *6Pgd-2*$_{.1}$ = the *6 Pgd* locus, 2, allele 1.

which 18 loci (90 % ) were polymorphic in at least one population; the *Lap* and *Pgm-1* loci were monomorphic in all analyzed populations.

Contingency analyses of heterogeneity showed that, among the 18 polymorphic loci, the heterogeneity in allele frequencies was not significant only in the *Idh* and *Mdh-1* loci ($\chi^2 = 67.2$, $P = 0.43$ and $\chi^2 = 21.3$, $P = 0.06$, respectively). Gene diversity parameters computed for the 32 populations are presented in Table 2. The number of alleles per locus (*A/L*) ranged from 1.95 to 2.40 and the overall mean was 2.16; effective number of alleles ($A_e$) ranged from 1.41 to 1.60 and the overall mean was 1.49. High genetic diversity was observed for the percentage of polymorphic loci and heterozygosity. The proportion of polymorphic loci (*P%* ) ranged from 65 to 75 % and the overall mean was 68 %, at the 0.95 criterion; at the 0.99 criterion, the corresponding figures were 65 to 80 % , and 74 %, respectively. Seven putative alleles were detected that were probably unique to individual populations, but not necessarily so, because of the low probability of detecting such an allele. Allele 4 in the *Aco* locus was detected in population No. 11; Allele 5 in the *Got-1* locus in population No. 6; Allele 2 in the *Idh locus* in population No. 32; Alleles 3 and 4 in the *Mdh-1* locus in population No. 6; Allele 2 in the *Pgm-2* locus in population No. 33; and allele 5 in the *Skdh-1* locus in population No. 20.

The observed heterozygosity ($H_o$) ranged from 0.124 to 0.267 with an overall mean of 0.191; and the expected heterozygosity ($H_e$) ranged from 0.233 to 0.312 with an overall mean of 0.271. The frequency of private alleles[2] varied from 0.019 to 0.383 in populations Nos. 29 and 22, respectively. The mean frequency of private alleles over all populations was 0.105. Thus, variability within the *Pinus brutia* populations analyzed appears to be rather high.

Interpopulation allozyme differentiation was estimated by means of the F-statistics (WRIGHT 1978). Genotype frequency deviations from the HARDY-WEINBERG equilibrium were inferred from the mean $F_{is}$ index of 0.281. Within species, there was high variability in the $F_{is}$ values, among the putative polymorphic loci. The $F_{is}$ values ranged from –0.441, representing excess in heterozygotes, in the locus *Acp*, to 0.833, indicating heterozygote deficiency, in the *Mnr-1*. In four loci, namely *Acp*, *Idh*, *Mdh-1* and *Mnr-2* excess in heterozygotes was found, but this excess was considerable only in the *Acp* locus. The coefficient of differentiation among subpopulations ($F_{st}$) was 0.089, i.e., 9 % of the total variation in the species, indicating that most of the diversity occurred within populations. The gene flow (*Nm*) calculated from the $F_{st}$ value was 2.55.

The ANOVA procedure, showed that there were significant correlation coefficients ($P < 0.05$) in 12 out of the 136 possible linear regressions between allele frequencies in the various enzyme systems (Table 3); these significant relationships might point to linkages among those loci.

**Variation with geography, topography and climate.**

Several attempts were made to find relationships between the putative allele frequencies in the 18 polymorphic loci in each of the populations, and their geographic and ecological parameters (the seed distribution zones), as presented in Table 1. Only 10 out the 68 possible linear regressions had significant correlation coefficients ($P < 0.05$) (Table 4). Most of the significant correlations were between allele frequencies and the latitude or longitude but not with the altitude; there was only one significant correlation with the aspect (expressed as degrees). No relationships were

[2] The table of the allele frequencies in 15 enzyme systems and in all 32 populations analyzed is not presented in this paper because of the large volume of data. The table will be sent by the authors to anybody upon request.

**Table 4. Correlation coefficients of linear regressions between allele frequencies and geographic variables.**

| Loci and alleles | Geographic Variables | | | |
|---|---|---|---|---|
| | Altitude | Aspect | Latitude | Longitude |
| $Aap\text{-}1_{.2}$ | | | 0.415* | |
| $Aco\text{-}1_{.1}$ | | | –0.533** | |
| $Acp\text{-}1_{.4}$ | | | | 0.424* |
| $Got\text{-}2_{.2}$ | | | | –0.466* |
| $Mdh\text{-}1_{.1}$ | | | –0.403* | |
| $Pgi\text{-}2_{.2}$ | | | | –0.449* |
| $6Pgd\text{-}2_{.2}$ | | –0.417* | | –0.563*** |
| $6Pgd\text{-}3_{.2}$ | | | | –0.448** |
| $Skdh\text{-}2_{.2}$ | | | 0.417* | |

* – significant at 0.05; ** – significant at 0.01; *** – significant at 0.001; Notation of loci and alleles: $Aap\text{-}1_{.2}$ = the *Aap* locus 1, allele 2; $6Pgd\text{-}2_{.1}$ = the *6 Pgd* locus 2, allele 1.

found between allele frequencies and the designated seed distribution zones. It is of interest that the few loci and alleles whose frequencies showed significant relations among themselves (Table 3) were also those that showed significant relations with the given geographic parameters.

To reveal eventual genetic relationships among the selected natural seed stands, genetic distances or identities were calculated according to NEI (1973). The calculations produced clusters that cannot be explained because: (i) the clusters aggregate populations growing very far apart in different geographical regions, or desegregated populations that grow in very close geographical proximity; (ii) the clusters aggregate the natural elite seed stands designated to very different ecological (seed distribution) zones (Table 1). Furthermore, the clusters produced by means of the distance-WAGNER procedure, or by the chord distance according to CAVALLI-SFORZA & EDWARDS (1967), also could not be explained by us because of the nature of the aggregations.

Because the linear relations shown in Table 4, between allele frequencies in several loci and the geographic parameters of the natural seed stands, have relatively low correlation coefficients ($r$ does not exceed 0.5 in most cases), and because the results of the various cluster analyses could not be explained, we attempted a different way to detect any relationships between the allele frequencies in the natural seed stands and the geographic and the seed distribution zone parameters. We aggregated the populations into: (i) five groups that correspond with clearly distinguishable geographic regions in Turkey, distributed from east to west; and (ii) zones according to one degree steps in latitude (Figure 1).

**Regional groups**

*Group one*; the mountainous region south of the cities of Sinop and Samsun in north Turkey, on the shores of the Black Sea, contained populations number 13 and 34. *Group two*; the region east of Adana and extending to Mt. Tahtali to the northeast and Mt. Nur to the southeast contained four populations numbered 4, 6, 7 and 9. *Group three*; the Taurus Mountains along the Mediterranean shore, between Alanya to the west and Adana to the east, included eight populations numbered 1, 2, 17, 20, 24, 31, 32 and 33. *Group four*; the various mountain chains between Antalya to the east and Mugla to the west, which contains 11 populations numbered 5, 8, 14, 21, 22, 23, 25, 26, 27, 29 and 30. *Group five*; the area between the larger towns of Manisia to the southwest, Bura to the north and Usak to the southeast contains seven populations numbered 3, 10, 11, 12, 18, 19 and 28. Allozyme variations within these five regional groups are presented in Table 5a. The differences among the five geographic groups, in the number of polymorphic loci (*PL*) and the effective number of alleles ($A_e$) were not significant. Total numbers of alleles ($a_T$) varied among the five groups, ranging from 48 in group one, to 62 in group three. The mean $F_{is}$ values ranged from 0.250 to 0.307, which indicated deviations from random mating within populations. Significant deviations from Hardy-Weinberg expectations occurred at eight polymorphic loci in group one, at nine polymorphic loci in group two, at 16 polymorphic loci in group three, and at 15 polymorphic loci in groups four and five. Genetic differentiation ($F_{st}$) differed considerably between group 1, on the one hand, and the other four groups, on the other hand. The gene flow within groups, as calculated from the $F_{st}$ values ranged between 1.6 and 5.7 with an average of 3.52.

*Latitudinal zones* (Figure 1) The 32 populations were grouped according to the latitudes of their respective sites: zone-A, between 40 and 41° N included two populations, zone-B, between 39 and 40° N included seven populations, zone-C, between 37 and 38° N included 13 populations and zone-D, between 36 and 37° N included 10 populations.

Table 5b shows that populations included in zone A differed from those in the other zones, in their allele frequencies at several loci, and that this zone had the highest degree of allele differentiation ($F_{st}$) among all the natural seed stands studied. Table 5b also shows that there was little variation among zones, in the mean values of the fixation index of individuals relative to the total population ($F_{it}$), in the percentage of polymorphic loci at the 95 % criterion, and in the direct-count heterozygosity that also correlated with latitude. $F_{it}$

**Table 5a. Estimates of allozyme variation at group level.**

| Name | Sample size | $PL$ | $a_T$ | $A_e$ | $P_{95\%}$ | $H_o$ | $H_e$ | $F_{is}$ | $F_{it}$ | $F_{st}$ | $Nm$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group1 | 75 | 16 | 48 | 1.548 | 80 | 0.176 | 0.284 | 0.290 | 0.384 | 0.133 | 1.635 |
| Group 2 | 222 | 15 | 53 | 1.512 | 75 | 0.198 | 0.277 | 0.253 | 0.284 | 0.042 | 5.739 |
| Group 3 | 412 | 17 | 62 | 1.526 | 85 | 0.199 | 0.284 | 0.250 | 0.292 | 0.056 | 4.250 |
| Group 4 | 655 | 17 | 60 | 1.540 | 85 | 0.188 | 0.295 | 0.307 | 0.363 | 0.081 | 2.846 |
| Group 5 | 395 | 16 | 58 | 1.543 | 80 | 0.190 | 0.287 | 0.296 | 0.348 | 0.074 | 3.126 |

**Table 5b. Estimates of allozyme variation at zone level.**

| Name | Sample Size | $A/L$ | $A_e$ | $P_{95\%}$ | $H_o$ | $H_e$ | $F_{is}$ | $F_{it}$ | $F_{st}$ | $Nm$ | Latitude North |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Zone A | 75 | 2.03 | 1.44 | 65 | 0.172 | 0.250 | 0.290 | 0.384 | 0.133 | 1.635 | 40° – 41° |
| Zone B | 395 | 2.18 | 1.51 | 66 | 0.188 | 0.274 | 0.296 | 0.348 | 0.074 | 3.126 | 39° – 40° |
| Zone C | 761 | 2.17 | 1.50 | 68 | 0.192 | 0.274 | 0.288 | 0.338 | 0.070 | 3.323 | 37° – 38° |
| Zone D | 530 | 2.18 | 1.49 | 70 | 0.197 | 0.273 | 0.268 | 0.321 | 0.073 | 3.170 | 36° – 37° |
| $r$ (Pearson) | | –0.792 | –0.623 | *–0.969* | *–0.964* | –0.850 | 0.708 | *0.960* | 0.818 | 0.240 | |
| $P$ | | 0.208 | 0.277 | *0.031* | *0.035* | 0.150 | 0.292 | *0.040* | 0.182 | 0.760 | |

$PL$ = the number of polymorphic loci; $P_{95}\%$= the percentage of polymorphic loci; $A_e$ = effective number of alleles; $H_e$ = expected heterozygosity; $F_{st}$ = coefficient differentiation of population; $A/L$ = number of alleles per locus; $a_T$ = total number of alleles; $H_o$ = observed (direct count) heterozygosity; $F_{is}$ = Wright's fixation index within population; $F_{it}$ = Wright's fixation index in the total population.

correlated with the regional distribution ($r$ = 0.960; $P$ < 0.05). Strong correlations were found between the observed heterozygosity ($H_o$) and the latitude, and the percentage of polymorphic loci ($P$ % ). The Pearson correlation analysis revealed a correlation between gene differentiation and the latitude in only two loci, namely *Aap* and *Adh-2*; differences between the zones in allele frequency were significant. The correlation coefficient for the locus *Aap* was $r$ = 0.990 and $P$ = 0.009; and for the *Adh*-2 locus $r$ = –0.9637 and $P$ = 0.036. These loci also had significant relationships among themselves.

Canonical correlation analysis was used to improve our understanding of the relationships between allele frequencies and geographic parameters. The canonical variables were created by using geographical data and the mean allele frequencies at each locus at the species level (over all the populations). The best canonical variable of the geographic parameters presented in Table 1 was given by the equation:

$$Geo = + 0.00069 \cdot Alt. - 0.338 \cdot Asp. + 0.528 \cdot Lat. - 0.177\ Long.$$

The best canonical variable of the allele frequency information was given by the equation:

$$Gen = - 2.243 \cdot Adh\text{-}2 + 5.825 \cdot Got\text{-}2_{-1} - 0.406 \cdot 6Pgd\text{-}2_{-1} + 6.08 \cdot 6Pgd\text{-}3_{-2} - 1.906 \cdot Skdh\text{-}1_{-2} + 4.617 \cdot Skdh\text{-}2_{-2}.$$

The *Gen* equation includes loci that do not have simple relationships with geographic parameters (Table 4), but they do have significant relationships with other enzyme systems. The squared correlation coefficient between the two canonical variables (*Geo, Gen*) was $R^2$ = 0.72, Pro > $F$ = 0.0001. These results point to the genetic and geographic uniqueness of each seed stand.

## DISCUSSION

In comparison with previous studies on genetic diversity in *Pinus brutia* (e.g., CONKLE *et al.* 1988; KARA *et al.* 1997, PANETSOS *et al* 1998, GANI-GULBABA & OZKURT 2000) there are similarities and differences that are summarized in Table 6. The differences are probably a result of the number of populations analyzed and their geographic distribution, the number of enzyme systems and of surveyed loci, and the number of alleles scored in each locus.

Most of the conifers have similar life histories: they are long-lived, grow in heterogeneous environments, are wind pollinated, and have high capacity for adaptation, therefore, they may show similar levels of genetic variability. According to KRUTOVSKII *et al.* (1995) the mean number of alleles per locus ($A/L$) for 30 pine species was 2.08, and the expected heterozygosity value was 0.169. According to HAMRICK *et al.* (1992a) and

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**Table 6. Comparison between results of several studies of allozyme diversity in *Pinus brutia* Ten.**

| | Parameter Analyzed | 1* | 2* | 3* | 4* | 5* |
|---|---|---|---|---|---|---|
| 1. | No. of enzyme systems analyzed | 20 | 12 | 12 | 6 | 15 |
| 2. | No. of loci analyzed. | 30 | 17 | 20 | 7 | 20 |
| 3. | No. of populations analyzed | 10 | 9 | 8 | 4 | 32 |
| 4. | *A/L* | 1.53 | 2.10 | 1.70 | 1.80 | 2.16 |
| 6. | *P* (95 % ) | 43 | 69.6 | 61.9 | 57.1 | 68.0 |
| 7. | $H_o$ | | 0.219 | 0.149 | 0.205 | 0.191 |
| 8. | $H_e$ | 0.118 | 0.265 | 0.216 | 0.216 | 0.271 |
| 11. | $F_{st}$ | | 0.053 | 0.024 | 0.021 | 0.089 |
| 12. | $F_{is}$ | | 0.147 | 0.295 | 0.042 | 0.281 |
| 13. | $F_{it}$ | | 0.180 | 0.311 | 0.063 | 0.341 |

1* – CONKLE *at al*; 1988; 2* – KARA *et al*; 1997; 3* – PANETSOS *et al*; 1998; 4* – GANI-GULBALA & OZKURT 1998; 5*– According to the present study.

PARKER & HAMRICK (1996), the mean number of alleles per locus (*A/L*) in gymnosperms is 2.38 and the mean Hexp value is 0.169.

Inter-population allozyme differentiation was estimated by means of the F-statistics (WRIGHT 1978). Genotype frequency deviations from the Hardy-Weinberg equilibrium, inferred from the mean $F_{is}$ index of 0.281, showed heterozygote deficiency at the species level similar to that deduced from the $F_{is}$ value of 0.295 found by GANI-GULBABA & OZKURT (2000). Within species, there was high variability in the $F_{is}$ values, among the putative polymorphic loci: the $F_{is}$ values ranged from –0.441, representing an excess of heterozygotes, in the locus *Acp*, to 0.833, indicating heterozygote deficiency, in *Mnr-1*.

Large heterozygote deficiencies were also found in the *Aap*, *Gdh*, *Got-1*, *Skdh-1* and *Skdh-2* loci. Excess heterozygosity was found in four loci, namely *Acp*, *Idh*, *Mdh-1* and *Mnr-2*, but this excess was considerable only in *Acp*. Heterozygote deficiency ($F_{it}$) in the total population has been reported for many pines (e.g., DANCIK & YEH 1983, EDWARDS & HAMRICK 1995, PARKER & HAMRICK 1996, and SCHILLER *et al.* 1999), and in our present study of *Pinus brutia* the overall heterozygote deficiency ($F_{it}$) was 0.346, indicating heterozygote deficiencies within the populations analyzed. There are two possible explanations for this phenomenon. The first is that samples might have been collected from trees of different ages; younger trees might differ appreciably from older ones, in their allele frequencies, which would produce a temporary Wahlund effect within the site (PARKER & HAMRICK 1996). The second possibly explanation is that microscale genetic differentiation might have occurred within *Pinus brutia* populations, in response to heterogeneous selection, such as has been reported for other pines (DANCIK & YEH 1983).

Conifers often have low genetic differentiation among populations (EDWARDS & HAMRICK 1995). However, this differentiation is influenced by the effective population size and the geographic distribution (HAMRICK *et al.* 1992b). Pine species with a wide geographic range that is divided into isolated groups have higher inter-population genetic differentiation ($F_{st}$ values) probably as a result of restricted gene flow, whereas pine species with a continuous geographic distribution probably have higher gene flow and are therefore less genetically differentiated (e.g., DANCIK & YEH 1983, WHEELER & GURIES 1982, HAMRICK & GODT 1989, GURIES & LEDIG 1982, KRUTOVSKII *et. al.* 1995). The mean level of inter-population differentiation for the genus *Pinus*, is estimated to be ~0.06–0.07 on the basis of allozymes (PARKER & HAMRICK 1996), so that the $F_{st}$ value of 0.089 in *Pinus brutia* that is spread over large areas in Turkey is slightly higher than that in other widespread species. The higher level of inter-population differentiation observed in our present study may be attributed to barriers to gene flow formed by the highly dissected mountainous terrain, and/or selection of some alleles along clines as presented in Table 4. On the other hand, similarities among populations might be the result of high and effective inter-population gene flow (via pollen transport) that reduces the effects of selection and enhances homogeneity. WRIGHT (1951) showed that at drift-migration equilibrium, the average number of migrants per population and per generation (Nm) is inversely proportional to the levels of inter-population genetic differentiation, measured as the Fst. This relationship is based on the assumption that gene flow is uniform among populations. The value of Nm calculated from the Fst value indicates that the gene flow is large enough. In the absence of large gene flow the importance of the local environmental heterogeneity increases. The gene flow

analysis presented in Table 5a shows that the exchange of genes within groups is not even. Group one, which contains the two populations growing in the Kure Mountains near Sinop on the shores of the Black Sea show a low level of gene flow in comparison with the other geographic groups. This group has the highest $F_{st}$ value among the groups, and contrasting values of other genetic parameters. There are two possible explanations of these results: (i) the movement of pollen between group 1 and the other groups is probably restricted because its geographic location is far from the main area distribution of the species in Turkey; (ii) an earlier study by CONKLE *et al.* (1988) found *P. brutia* of this region to be more closely related to *P. eldarica* than to *P. brutia* of the other regions in Turkey.

In our previous research significant relationships were found between mean expected heterozygosity and altitude, and also between the frequencies of the alleles $Mdh\text{-}1_{-1}$, $Mdh\text{-}4_{-1}$, $Mdh\text{-}4_{-3}$, $Skdh\text{-}1_{-1}$, $Skdh\text{-}1_{-3}$, $Aco_{-1}$ and $Gdh_{-3}$ and altitude in the Antalya region (KARA *et al.* 1997). Also, SCHILLER and GRUNWALD (1987) and CONKLE *et al.* (1988) pointed to differentiation between populations that depended on altitude. In the present study, no significant relationships between allele frequencies or calculated genetic variables, on the one hand, and the altitude, on the other hand, could be established. However, several significant relationships between allele frequencies in several loci and a longitudinal or latitudinal cline were revealed. Of the 20 loci, *Aap* and *Aco* were the only ones in which the differentiation of the loci along a cline indicated gradual change in crossing latitudinal zones. A similar pattern of diversity, i.e., clear differentiation among large regions and small differentiation within them has also been found in other conifers such as *Abies alba or Abies grandis*; it may be the result of migration following the ice age (KONNERT & BERGMAN 1995; KONNERT & RUETZ 1997).

The various cluster analysis procedures failed to aggregate the 32 populations according to any of the geographical parameters of the sites or of the designated seed zones (ecological zones). This implies that the formerly designated seed distribution zones area should be reconsidered, their size should be reduced to fit the adjacent ecological area of each seed stand.

To conclude, the relationships between the allele frequencies in several loci and geographic parameters, and the very significant relationships between the assemblage of geographic parameters and that of allele frequencies point to the uniqueness of each seed stand. This knowledge should be used to designate seed distribution zones to each of the seed stands, according to its ecological parameters.

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## REFERENCES

CAVALLI-SFORZA, L. L. & EDWARDS, A. W. F. 1967: Phylogenetic analysis: Models and estimation procedures. *Evolution* **21**:550–570.

CONKLE, M. T., HODGSKISS, P. D., NUNNALLY, B. L. & HUNTER, S. C. 1982: Starch Gel Electrophoresis of Conifer Seeds: a Laboratory Manual. USDA Forest Service, Pacific Southwest Forest and Range Experiment Station, *General Technical Rapport PSW–64*.

CONKLE, M. T., SCHILLER, G. & GRUNWALD, C. 1988: Electrophoretic analysis of diversity and phylogeny of *Pinus brutia* and closely related taxa. *Systematic Botany* **13**:411–424.

CROW, J. F. & KIMURA, M. 1964: The number of alleles that can be maintained in a finite population. *Genetics* **49**: 725–738.

DANCIK, B. P. & YEH, F. C. 1983: Allozyme variability and evolution of lodgepole pine (*Pinus contorta* var. *latifolia*) and jack pine (*P. banksiana*) in Alberta. *Canadian Journal of Genetics & Cytology* **25**:57–64.

EDWARDS, M. A. & HAMRICK, J. L. 1995: Genetic variation in shortleaf pine, *Pinus echinata* Mill. (Pinaceae). *Forest Genetics* **2**:21–28.

GANI-GULBABA, A & OZKURT, N. 2000: [Isoenzyme diversity in Turkish red pine (*Pinus brutia* Ten.) populations sampled from the Bolkar Moutains]. Technical Bulletin No. 5 of the Eastern Mediterranean Forestry Research Institute, Tarsus, Turkey.

GURIES, R. P. & LEDIG, F. T. 1982: Genetic diversity and population structure in pitch pine (*Pinus rigida.* Mill.). *Evolution* **36**:387–402.

HAMRICK, J. L. & GODT, M. J. 1989: Allozyme diversity in plant species. *In*: Plant Population Genetics Breeding and Genetic Resources (eds. A. H. D. Brown, M. T. Clegg, J. L. Kahler and Weir). pp. 43–63. Sinauer Associates, Sunderland, MA.

HAMRICK, J. L., GODT, M. & SHERMAN-BROYLES, S. L. 1992a. Factors influencing levels of genetic diversity in woody plant species. *In*: Population Genetics of Forest Trees. (eds W. T. Adams, S. H. Strauss, D. L. Copes & A. R. Griffin). pp. 95–125. Kluwer Academic Publishers, London.

HAMRICK, J. L., GODT, M. J. W. & WHERMAN-BROYLES, S.L. 1992b. Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**:95–124.

ISIK, F. & KAYA, Z. 1993: Genetic variation in *Pinus brutia* Ten. natural populations. *In*: Proceedings of the FAO – IUFRO and the Turkish Ministry of Forestry, International Symposium on *Pinus brutia* Ten., Marmaris, Turkey pp. 243–253.

ISIK, F., ISIK, K. & LEE, S. J. 1999: Genetic variation in *Pinus brutia* Ten. in Turkey: I. Growth, biomass and stem quality traits. *Forest Genetics* **6**:89–99.

KARA, N., KOROL, L., ISIK, K. & SCHILLER, G. 1997: Genetic diversity in *Pinus brutia* Ten: Altitudal variation. *Silvae Genetica* **46**:155–161.

KONNERT, M. & BERGMANN, F. 1995: The geographical distribution of genetic variation of silver fir in relation to its migration history. *Plant Systematic &. Evolution* **196**:19–30.

KONNERT, M. & RUETZ, W. 1997: Genetic variation among provenances of *Abies grandis* from the Pacific Northwest. *Forest Genetics* **4**:77–84.

KOROL, L. & SCHILLER, G. 1996: Relations between native Israeli and Jordanian Aleppo pine (*Pinus halepensis* Mill.) based on allozyme analysis: a note. *Forest Genetics* **3**:197–202.

KRUTOVSKII, K. V., POLITOV, D. V. & ALTUKHOV, Y. P. 1995: Isozyme study of population genetic structure, mating system and phylogenetic relationship of the five stone pine species (subsection *Cembrae*, section *Strobi*, subgenus *Strobus*). *In*: Population Genetics and Genetic Conservation of Forest Trees. (eds Ph. Baradat, W. T. Adams and G. Müller-Starck). pp. 279–304. SPB Academic Publishers, Amsterdam.

LI, C. C. & HOROVITZ, D. G. 1953: Some methods of estimating the inbreeding coefficient. *American Journal of Human Genetics* **5**:107–117.:

MIROV, N. T. 1967: The Genus *Pinus*. The Rohald Press Co., New York, 602 pp.

MORANDINI, R. 1976: Mediterranean conifers. *In*: Forest Genetic Resources. Information No: Forestry Occasional Paper 1976/1, FAO, Rome, pp. 12–18.

NAHAL, I. 1983: Le pin brutia (*Pinus brutia* Ten.) *Forêt Méditerranéenne* **2**:165–172.

NEI, M. 1973: Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science, USA* **70**:3321–3323.

NEI, M., 1977: F-statistic and analysis of gene diversity in subdivided populations. *Annals of Human Genetics* **41**:225–233.

NEI, M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**:283–290.

PALMBERG, C. 1975: Geographic variation and early growth in south-eastern semi-arid Australia of *Pinus halepensis* Mill. and the *P. brutia* Ten. species complex. *Silvae Genetica* **24**:150–160.

PANETSOS, K. P., ARAVANPOULOS, F. A. & SCALTSOYIANNES, A. 1998: Genetic variation of *Pinus brutia* from islands of the northeastern Aegean Sea. *Silvae Genetica* **47**:115 –120.

PARKER, K. C. & HAMRICK, J. L. 1996: Genetic variation in sand pine (*Pinus clausa*). *Canadian Journal of Forest Research* **25**:224–254.

SCHILLER, G. 1994: Diversity among *Pinus brutia* subsp. *brutia* and related taxa – A review. Orman Fakültesi Dergisi, Series A, **44**:133–147.

SCHILLER, G. & GRUNWALD, C. 1987: Cortex resin monoterpene in *Pinus brutia* provenances grown in Israel. *Bioch. Sys. and Ecology* **15**:389–394.

SCHILLER, G., KOROL, L., UNGAR, E. D., ZAHAVI, A., GIL, S. L. & CLIMENT, M. J. 1999: Canary Islands pine (*Pinus canariensis* Chr. Sm. ex Dc.) 1. Differentiation among native populations in their isoenzymes. *Forest Genetics* **6**:257–276.

SPENCER, D. J. 1985: Dry country pines: Provenance evaluation of *Pinus halepensis – P. brutia* complex in the semi-arid region of south-east Australia. *Australian Forestry Research* **15**:263–279.

SWOFFORD, L. D. & SELANDER, B. R. 1989: BIOSYS-1, A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Release 2.0. University of Illinois at Urbana-Champaign. Urbana, IL, USA.

WENDEL, J. F. & PARKS, C. R. 1982: Genetic control of isozyme variation in *Camellia japonica* L. *Journal of Heredity* **73**:197–204.

WHEELER, N. B. & GURIES, R. P. 1982: Population structure, genetic diversity and morphological variation in *Pinus contorta* Dougl. *Canadian Journal of Forest Research* **12**:595–606.

WESTFALL, R. D. & CONKLE, M. T. 1992: Allozyme markers in breeding zone designation. *In*: Population Genetics of Forest Trees. (eds W. T. Adams, S. H. Strauss, D. L. Coes & A. R. Griffin). pp. 279–310. Kluwer Academic Publishers, London,.

WRIGHT, S. 1951: The genetical structure of populations. *Ann. Eugen.* **15**:323–354.

WRIGHT, S. 1978: Evolution and the Genetics of Populations, Vol. 4. Variability within and among natural populations. University of Chicago Press, Chicago, IL, USA.

WORKMAN, P. L. & NISWANDER, J. D. 1970: Population studies on southwestern Indian tribes. II. Local genetic differentiation in the Papago. *American Journal of Human Genetics* **22**:24–29.

YEH, F. C., YANG, R-C, BOYLE, T. B. J., YE, Z-H. & MAO, J. X. 1997: POPGENE. The user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Center, University of Alberta, Canada.