Doyle, J.: Proembryogeny in *Pinus* in relation to that in other conifers — a survey. Proc. Royal Irish Acad. 13: 181—216 (1963). — Georgescu, C. C. and Ionescu-Barlad: Asupra statiunilor de *Pinus cembra* din Carpatii Romaniei. Rev. Pad. 8—9: 531—543 (1932). — Hagman, M. and Mikkola, L.: Observation on cross- self- and interspecific pollinations in *Pinus peuce*. Silvae Genetica 12: 73—79 (1963). — Halauer, A. R. and Miranda, F. O.: Quantitative genetics in maize breeding. Iowa State University, Ames, 468 p. (1981). — Heimburger,C.: Breeding of white pine for resistance to blister rust at the interspecific level. In: Bingham, R. T. et al. (Eds): Biology of rust resistance in forest trees. USDA, For. Serv., Misc. Publ. 1221, pp. 541—549 (1972). — Hoff, R. J., Bingham, R. T. and McDonald, G. I.: Relative blister rust resistance of white pines. Eur. Jour. Forest Path. 10 (5): 307—316 (1980). — Hoff, R. J. and McDonald, G. I.: Stem rusts of conifers and balance of nature. In: Bingham, R. T. et al. (Eds): Biology of rust resistance in forest trees. USDA, For. Serv., Misc. Publ. 1221, pp. 526—536 (1972). — Holzer, K.: Intrinsic qualities and growth-potential of *Pinus cembra* and *P. peuce* in Europe. In: Bingham, R. T. et al. (Eds): Biologi of rust resistance in forest trees. USDA, For. Serv., Misc. Publ. 1221, pp. 99—110 (1972). — Holzer, K.: Genetics of *Pinus cembra*. Anales Forestales, Zagreb, 6/5, pp 139—158 (1975). — Kriebel, H. B.: The timing of the incompatibility reaction in interspecific crosses of *Pinus strobus*. Proc. 14th IUFRO Congress, Munich, 3:77.87 (1967). — Kriebel, H. B.: Embryogeny and inviability in hybrids of the white pines. Proc. XII Int. Cong. Genetics. 1: 265 (1968). — Kriebel, H. B.: Embryo development and hybridity barriers in the white pines. Silvae Genetica 21 (1—2): 39—44 (1972). — Kriebel, H. B.: Methods for germinating seed of five needle pines. IUFRO Instructions, 2 p. (1973). — Leandru, L.: Hibrizi interspecifici la pin. ICAS, Seria II, Bucuresti, 78 p. (1982). Patton, R. F.: Interspecific hybridization in breeding for white pine blister rust resistance. In: Gerhold, H. D. et al. (Eds): Breeding pest resistant trees. Perg. Press, Oxford, pp. 367—376 (1964). — Righter, F. I. and Duffield, J. W.: Interspecies hybrids in pines. Jour. Hered. 42: 75—80 (1951). — Snedecor, G. W.: Statistical methods. Iowa State University Press. 534 p. (1956). — Ueda, K., Yoshikawa, K. and Inamori, Y.: On the fertilization of interspecies crossing in pine. Bull. Kyoto Univ. Forests 33: 137—155 (1961). — Wright, J. W.: Species hybridization in the white pines. Forest Sci. 5: 210—222 (1959). — Zobel, B. and Talbert, J.: Applied forest tree improvement. John Wiley and Sons, New York. 505 p. (1984).

Allozyme Frequency Distributions in Five European Populations of Black Pine (Pinus nigra Arnold)[1])[2])

I) Estimation of Genetic Variation Within and Among Populations
II) Contribution of Isozyme Analysis to the Taxonomic Status of the Species

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Summary

European black pine (*Pinus nigra* Arnold) is generally considered a collective species with many taxonomic problems.

Genetic variation in 5 natural populations of *Pinus nigra* (Austria, Bulgaria, Greece, Corsica, Calabria) was investigated, at the enzyme level, by using the technique of starch gel electrophoresis.

Isozyme patterns of 10 enzyme systems (MDH, 6PGD, MR, IDH, PGM, DIA, AAT, LAP, PGI, GDH) were studied. Analysis, using haploid megagametophyte tissue, demonstrated that the allozyme variants in the above enzyme systems were coded by a total of 42 structural genes in the 16 readable loci. In one of these loci (IDH), no variants were found.

The results of this study showed the following:

a) The loci (isozymes) of DIA appeared in MR enzyme system as well.
b) On the average, 70.0 % of the analyzed loci were polymorphic, while the number of alleles detected per locus (A/L) ranged from 1.87 (Calabria, Greece) to 2.31 (Bulgaria) with a mean value for all studied populations of 2.025 allleles per locus.
c) Heterozygosity ranged from 0.180 (Corsica) to 0.257 (Bulgaria).
d) 94% of the total variation of the species was due to intrapopulation gene diversity.
e) Isozymes contributed differently in the total variation of the species.
f) Isozyme variation is a useful tool for the taxonomy of *Pinus nigra*, since it can discriminate either different subspecies or different populations within a given subspecies.
g) The most appropriate enzyme systems for classification of the subspecies [ssp. *laricio, nigra (austriaca), pallasiana*] were MDH, DIA, 6PGD and AAT.
h) There was a clear distinction between *laricio* group (Calabria, Corsica) and *austriaca* group (Austria, Bulgaria).
i) The Greek provenance was closer to the *austriaca* group.

Key words: Pinus nigra, isozyme variation, taxonomy.

Introduction

European black pine (*Pinus nigra* Arnold) is one of the most valuable species in the Mediterranean region and it is distributed in a discontinuous area which includes Southern Europe, Minor Asia, Cyprus and North-Western Africa (Critchfield and Little, 1966). According to Stojanoff and Stefanoff (1929) and Mirov (1967), *P. nigra* is a

[1]) Part of this research was conducted by Dr. A. Scaltsoyiannes in his work under the title "Genetic variation of malate dehydrogenase isozymes in subspecies of European black pine (*Pinus nigra* Arnold)" granted by the Commision of the European Communities (Division for the Coordination of Agricultural Research) in the framework of the "Competitiveness of Agriculture and Management of Agricultural Resources" Programme. The work was conducted in the Lab. Interaction Plantes-Champignons et Micropropagation, Lyon, France.

[2]) Another part of the present research was submitted as a partial fullfilment of the "Diplome d'Associe aux Recherches — Diplome d'Etudes Superieures" of Ms M. Tsaktsira in the Lab. Interaction Plantes-Champignons et Micropropagation, University Claude Bernard, Lyon I, Lyon, France.

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typical Mediterranean species and is regarded as a relict species of the Tertiary period. Because of the pattern of its natural distribution, black pine is considered to be a very variable species in morphological, anatomical and physiological characteristics. This is also indicated by the large number of taxa (subspecies, varieties and forms) (RÖHRIG, 1957, 1966; LEE, 1968; ARBEZ and MILLIER, 1971; DEBAZAC, 1964, 1971; VIDAKOVIC, 1974; BASSIOTIS, 1967; ZOLLER et al., 1977; PANETSOS and MITSOPOULOS, 1978). However, the genetic variation of European black pine is not followed by geographic patterning (LEE, 1968; NIKOLIC and TUCIC, 1983).

According to many authors, black pine possesses a lot of taxonomic problems and there is no general consensus on its taxonomy. In the Balkan peninsula, for example, the variability and taxonomy of *P. nigra* are not yet completely clarified (VIDAKOVIC, 1974). In Yugoslavia only, VIDAKOVIC (1955, 1957) and FUKAREK (1971) classified European black pine into 5 subspecies, while in Greece, LEE (1968) and WHEELER et al. (1976), found such a high variation, in most of the traits examined, as the one found in the whole species.

The taxonomic problem of the species becomes more complicated by the existence of transitional forms between subspecies (VIDAKOVIC, 1974). The above seems to be a good explanation of the fact that so many researchers resulted in different classifications. First, RÖHRIG (1957) considered all forms and varieties of the European black pine as belonging to one species. According to VIDAKOVIC (1974), the most acceptable classification, with some modifications, is that of Flora Europaea (TUTIN et al., 1964), in which the European black pine is divided into the following 5 subspecies: ssp. *salzmanii* (Spain, South-West France, North Africa), ssp. *laricio* (Corsica, Calabria, Sicily), ssp. *nigra* (Austria, North-Eastern and Central Italy, Yugoslavia), ssp. *pallasiana* (Greece, Bulgaria, Romania, Turkey, Cyprus, the Crimea peninsula) and ssp. *dalmatica* (Coastal region and islands of North-West Yugoslavia). A considerable amount of information on European black pine classifications is given by VIDAKOVIC (1974) in his monograph.

Up to date, most of the taxonomic studies of black pine were based on morphological and anatomical characteristics (DEBAZAC, 1964; TUTIN et al., 1964; LEE, 1968; ARBEZ and MILLIER, 1971; FUKAREK, 1971), except of very few studies that were based on biochemical markers, namely: a) monoterpenes (ARBEZ et al., 1974), b) flavonoids (LAURANSON, 1989) and c) isozymes (BONNET-MASIMBERT and BIKAY-BIKAY, 1978; NIKOLIC and TUCIC, 1983; FINESCHI, 1984).

During the last decades, the development of the isozyme technique has been an important step towards a further elucidation of the genetic structure on gene level of forest tree populations (LUNDKVIST and RUDIN, 1977). According to ROTHE (1990), isozymes have many evident advantages over the other characteristics: a) they are considered the direct products of specific allelic genes, b) their phenotype is not affected by environmental variation and c) they are relatively easy to handle and to assess. Up to now, all isozyme studies on black pine variation and taxonomy, are restricted to only few enzyme systems.

The present work was designed to: a) determine the genetic variation pattern of *Pinus nigra* by using as many as possible enzyme systems for allozyme analysis, b) examine the extent of the genetic diversity between and within populations of *Pinus nigra* and c) to clarify the taxonomic status in this species. Our analysis is based on 5 populations of *Pinus nigra* belonging in 4 subspecies according to Flora Europaea classification (1964).

Materials and Methods

Electrophoresis was conducted on haploid endosperms of germinated black pine seeds. Germination of seeds normally occured within 6 to 9 days, after placing them on moist filter paper in petri dishes in a seed germinator (16 hr photoperiod, 26 °C day, 21 °C night).

The seed material was bulk and was provided by the Office National de Forêts, (Service Graines et Plants, Secherie de la Joux, SUPT, Jura, France) except of the Greek provenance that was provided by the Laboratory of Forest Genetics and Plant Breeding (Aristotelian University of Thessaloniki). Information on the classification (from Flora Europea, based on morphological characteristics) and the locations of the examined populations of *Pinus nigra* are given on *table 1*.

Endosperms (megagametophytes) were dissected from germinated seeds (lenght of the radicle 4 mm to 6 mm) and were homogenized individually with a pestle and mortar by adding 0.20 M phosphate buffer (pH 7.5) (CONKLE et al., 1982). The homogenates were analysed for: asparate aminotransferase (AAT, E.C.2.6.1.1), acid phos-

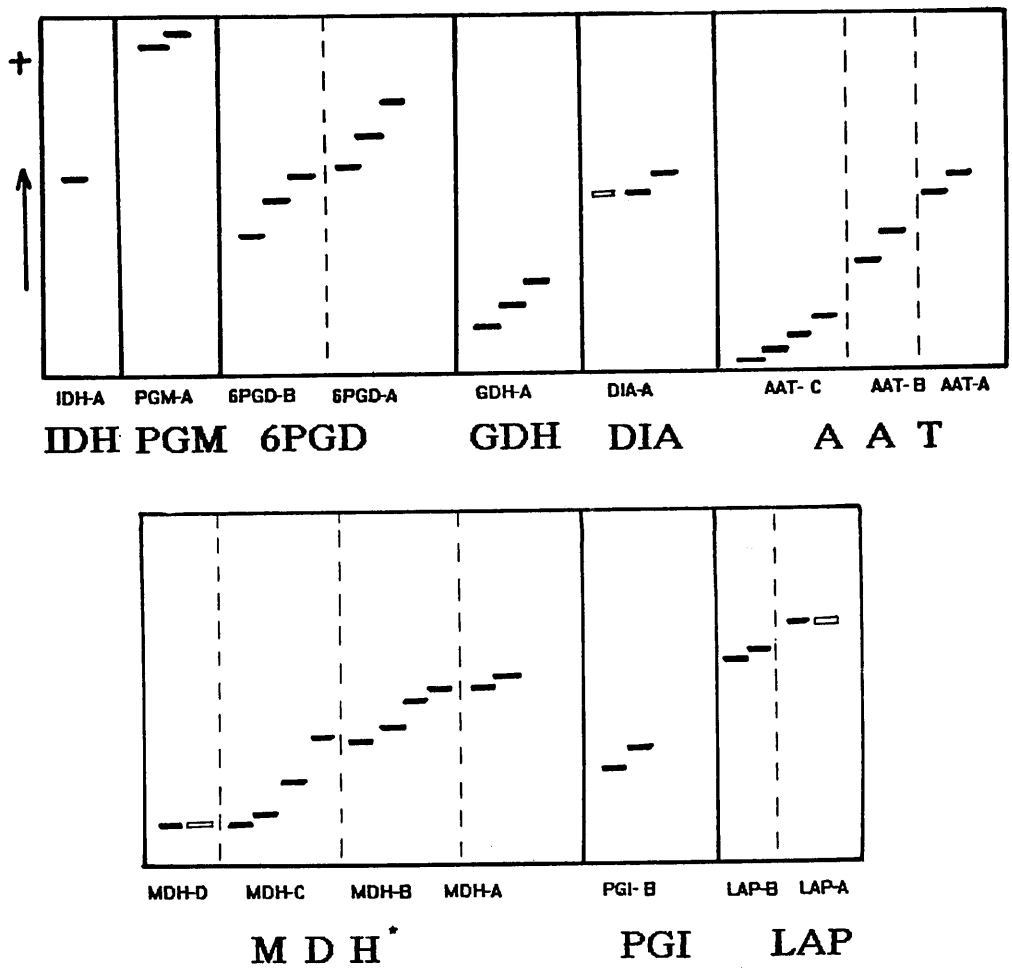
Table 1. — Locations of population samples of *Pinus nigra* ARNOLD.

Sample number	Country	Provenance	Subspecies (ssp.)
1	Austria	Austria	*nigra (austriaca)*
2	France	Corsica	*laricio*
3	Italie	Calabria (Verger de Bout)	*laricio*
4	Bulgaria	Kustendi	*nigra (austriaca)* (not identified)
5	Greece	Pieria	*pallasiana* (not identified)

Table 2. — Allele frequencies of 16 loci in 5 populations of *Pinus nigra* ARNOLD.

provenance / alleles	AUSTRIA	CORSICA	CALABRIA	BULGARIA	GREECE
MDH-A					
MDH-A1	0.02	0.02	0.00	0.02	0.00
MDH-A2	0.98	0.98	1.00	0.98	1.00
MDH-B					
MDH-B1	0.62	0.04	0.25	0.48	0.16
MDH-B2	0.35	0.94	0.68	0.40	0.70
MDH-B3	0.00	0.00	0.07	0.12	0.14
MDH-B4	0.03	0.02	0.00	0.00	0.00
MDH-C					
MDH-C1	0.00	0.02	0.00	0.00	0.00
MDH-C2	0.35	0.79	0.62	0.40	0.38
MDH-C3	0.15	0.00	0.05	0.02	0.00
MDH-C4	0.50	0.19	0.33	0.58	0.62
MDH-D					
MDH-D1	0.95	1.00	0.98	0.98	1.00
MDH-Dn	0.05	0.00	0.02	0.02	0.00
DIA-A					
DIA-A1	0.50	0.00	0.08	0.38	0.46
DIA-A2	0.46	1.00	0.92	0.58	0.52
DIA-An	0.04	0.00	0.00	0.04	0.02
6PGD-A					
6PGD-A1	0.02	0.00	0.00	0.16	0.00
6PGD-A2	0.95	0.61	0.77	0.66	0.80
6PGD-A3	0.03	0.39	0.23	0.18	0.20
6PGD-B					
6PGD-B1	0.76	0.63	0.67	0.58	0.32
6PGD-B2	0.19	0.37	0.33	0.40	0.68
6PGD-B3	0.05	0.00	0.00	0.02	0.00
GDH-A					
GDH-A1	0.00	0.02	0.03	0.00	0.03
GDH-A2	1.00	0.98	0.97	0.98	0.97
GDH-A3	0.00	0.00	0.00	0.02	0.00
PGI-B					
PGI-B1	0.85	0.71	0.83	0.74	0.84
PGI-B2	0.15	0.29	0.17	0.26	0.16
LAP-A					
LAP-A1	0.94	0.98	1.00	0.98	1.00
LAP-An	0.06	0.02	0.00	0.02	0.00
LAP-B					
LAP-B1	0.00	0.00	0.00	0.00	0.04
LAP-B2	1.00	1.00	1.00	1.00	0.96
AAT-A					
AAT-A1	1.00	1.00	1.00	0.98	1.00
AAT-A2	0.00	0.00	0.00	0.02	0.00
AAT-B					
AAT-B1	0.04	0.02	0.02	0.04	0.02
AAT-B2	0.83	0.60	0.55	0.58	0.66
AAT-B3	0.13	0.38	0.43	0.38	0.32
AAT-C					
AAT-C1	0.11	0.20	0.03	0.12	0.24
AAT-C2	0.00	0.02	0.02	0.02	0.00
AAT-C3	0.87	0.74	0.95	0.86	0.76
AAT-Cn	0.02	0.04	0.00	0.00	0.00
PGM-A					
PGM-A1	0.00	0.00	0.00	0.06	0.08
PGM-A2	1.00	1.00	1.00	0.94	0.92
IDH					
IDH-A1	1.00	1.00	1.00	1.00	1.00

phatase (ACP, E.C.3.1.3.2), diaphorase (DIA, E.C.1.1.1.40), alpha esterase (a-EST, E.C.3.1.1.1), glutamate dehydrogenase (GDH, E.C.1.4.1.3), glucose-6-phosphate dehydrogenase (G6PD, E.C.1.1.1.49), isocitrate dehydrogenase (IDH, E.C.1.1.1.42), leucine aminopeptidase (LAP, E.C.3.4.11.1), malate dehydrogenase (MDH, E.C.1.1.1.37), menadione



*) Heterodimer bands are produced between MDH-B and MDH-C.

Figure 1. — Representative illustrations of allozyme band patterns for 9 enzyme systems observed in megagametophyte tissues of 5 populations of *Pinus nigra.*

reductase (MR, E.C.1.6.99.2), peroxydase (PER, E.C.1.11.1.7), 6-phosphogluconate dehydrogenase (6PGD, E.C.1.1.1.44), phosphoglucose isomerase (PGI, E.C.5.3.1.9) and phosphoglucomutase (PGM, E.C.2.7.5.1), in an horizontal electrophoresis system with 10.5% (w/v) Connaught starch (Connaught Laboratories, Willowdale, Ontario, Canada). Buffer systems, gel composition, electrode buffers and staining recipes were prepared according to CHELIAK and PITEL (1985), except for the staining recipes of LAP, a-EST and PER which were prepared according to CONKLE et al. (1982). Gels were run under refrigeration (4 °C) and constant amperages (60 mA) with the exception that they were not allowed to exceed 320 V. The loci (isozymes) and the alleles within each locus (allozymes) were numbered in decreasing order of anodal mobility. "Null" isozymes, that lacked staining activity under the conditions of our assays, were specified by the letter "n".

Estimations of heterozygosities: h (expected proportion of heterozygotes per locus and population), Ht (a measure of the total genetic variation), Hs (genetic variation within the population) and Dst (genetic variation among the populations) were conducted according to HARDY-WEINBERG expectations, by following the method of NEI (1973, 1978) and NEI and CHESSER (1983). The relative magnitude of gene differentiation for interpopulation gene diversity (coefficient of gene differentiation) was obtained by the formula Gst = Dst / Ht which can be used as an index of allele frequency heterogeneity (NEI, 1973; NICOLIC and TUCIC, 1983). Genetic relationships between populations were quantified by using NEI's (1978) unbiased genetic distance measure. Cluster analysis, based on the unweighted pair group method was performed on the matrix of NEI's genetic distance.

Isozyme patterns of all enzyme systems were examined on random samples of 80 to 100 endosperms of each population.

Results

Ten enzyme systems out of the 14 i.e. LAP, MDH, 6PGD, IDH, PGM, GDH, AAT, DIA, MR, PGI, representing 17 loci were resolved with sufficient consistency and clarity which permited us to state if one locus is monomorphic or polymorphic and when polymorphic, to score for allelic variants (*Table 2*) in the 5 European black pine populations. Zymogram phenotypes of the 9 enzyme systems are illustrated in *figure 1.* The loci (isozymes) of DIA appeared in the MR enzyme system as well. A total of 42 scorable alleles (allozymes) was detected in 16 readable loci. The allele frequencies at each locus and population are shown in *table 2.* Most of them were stable and easy to interpret, except of the isozymes of MDH enzyme system which could be interpreted only by a quite trained eye. In our case, the MDH system was coded by 4 loci and hetero-

dimers were formed between the 2 loci MDH-B and MDH-C. Very often the interpretation of MDH enzyme system zymogram was complicated because:

a) The 2nd locus (MDH-B) exhibited a very light staining band, in contrast to MDH-A that formed a dark staining band.

b) The 2 loci, MDH-A and MDH-B, often appeared as one broad and because of the similar migration rates of their most common alleles (MDH-A_2 and MDH-B_1).

c) One of the most common alleles of the 3rd locus (MDH-C_4) was "covered" by a dark staining band of the 4th locus.

Considering the above and also that: a) variation in MDH-B and/or MDH-C results in a change of position of the heterodimer band so that it is always midway between MDH-B and MDH-C and b) variation in MDH-A (e.g. MDH-A_1) and the presence of a null (silent) allele in the 4th locus revealed the existence of the alleles MDH-B_1 and MDH-C_4 respectively, 4 loci were recorded comprising 2, 4, 4, 2 alleles respectively. The presence of the heterodimer band between MDH-B and MDH-C was also noticed by EL-KASSABY (1981) in his study on genetic interpretation of MDH isozymes in conifer species.

With the exception of IDH enzyme system that was monomorphic in all populations, we have found variant alleles at every locus, although not present in every seed lot. From *table 2*, it is also indicated that 7 loci (MDH-B, MDH-C, 6PGD-A, 6PGD-B, PGI-B, AAT-B, AAT-C) were polymorphic in all populations and that most of these loci seemed to be fixed for their most common allele in all populations. *Table 3*, shows that the proportion of polymorphic loci (P) ranged from 0.625 (Corsica and Calabria) to 0.875 (Bulgaria). On the average, 70.0% of the analyzed loci were polymorphic, while the number of alleles detected per locus (A/L) ranged from 1.87 (Calabria, Greece) to 2.31 (Bulgaria) with a mean value for all studied populations, of 2.025 alleles per locus. Patterns of allele frequency distribution of polymorphic loci (*Table 2*) indicated considerable genetic heterogeneity among the 5 populations of *P. nigra*. The average amount of genetic variability (expected heterozygosity) was high (0.208) but varied considerably among populations. Populations from Corsica and Calabria were characterized by the lowest variability (0.180 and 0.183 respectively) whereas the Bulgarian population by the highest one (0.257) (*Table 3*).

Table 4, presents the allelic variation or the expected proportion of heterozygotes per locus and population (h) and suggests that the polymorphic loci contribute differently to the mean value of the genetic variability of the species. Only 8 (MDH-B, MDH-C, DIA-A, 6PGD-A, 6PGD-B, PGI-B, AAT-B, AAT-C) out of the 16 loci exhibited a high level of heterozygosity. Two loci of this category, namely MDH-C and 6PGD-A, possessed the unique alleles MDH-C_3, 6PGD-A_1, that were specific for the populations from Austria and Bulgaria respectively.

The great differentiation of allele frequencies, found at the isozyme loci among the 5 populations of *P. nigra* (*Table 2*), provides alleles with a diagnostic value adequate to discriminate subspecies or to identify each population.

Indeed, MDH-B_1 allele frequencies are higher for the Bulgarian and Austrian populations — spp. *nigra* — (0.48 and 0.62 respectively) than those for the rest populations or subspecies. The lowest frequency of MDH-B_1 allele (0.04) has been found in the Corsican population. MDH-B_2 allele is at high frequency (0.94) in the Corsican population. MDH-B_3 allele is absent from the Austrian and Corsican populations. The frequency of MDH-C_2 allele is higher for the populations from Corsica (0.79) and Calabria (0.62) — ssp. *laricio* — than from other subspecies or populations. The opposite occurs with MDH-C_4 allele frequency. DIA-A_2 allele frequencies are higher for the Corsican and Calabrian populations (1.00 and 0.92 respectively) than for the rest of the populations or subspecies. The alleles 6PGD-A_3, 6PGD-B_2 and AAT-B_3 are at their lowest frequencies (0.03, 0.19 and 0.13 respectively) in the Austrian population. Also, the lowest frequencies of 6PGD-B_1 and AAT-C_1 alleles have been found in the Greek (0.32) and the Calabrian (0.03) populations respectively. Finally, PGM-A_1 allele is absent from the Austrian, Corsican and Calabrian populations. The frequency distributions of the most useful isozymes for the discrimination of subspecies (ssp. *laricio, nigra, pallasiana*) of *P. nigra* are shown in *figure 3*.

Table 5 indicates that intrapopulation gene diversity was considerably high (0.208 or 94% of the total gene diversity of the species). The above is also indicated by the low value (0.06) of the coefficient of gene differentiation (Gst) which suggests either that heterogeneity within

Table 3. — Genetic variation of 16 loci in 5 populations of *Pinus nigra.*

Population	Mean number of alleles per locus (A/L)	Percentage of loci polymorphic (P)	Expected heterozygosity (He)	Standard error (SE)
AUSTRIA	2.125	68.7	0.197	0.053
CORSICA	1.937	62.5	0.180	0.053
CALABRIA	1.875	62.5	0.183	0.052
BULGARIA	2.312	87.5	0.257	0.059
GREECE	1.875	68.7	0.225	0.052
Mean	2.025	70.0	0.208	0.054

Table 4. — Estimation of expected heterozygosity per locus and population (he) in 5 populations of *Pinus nigra* Arnold.

locus \ provenance	AUSTRIA	CORSICA	CALABRIA	BULGARIA	GREECE	Mean
1. MDH-A	0.039	0.039	0.000	0.039	0.000	0.024
2. MDH-B	0.494	0.115	0.473	0.599	0.467	0.430
3. MDH-C	0.608	0.341	0.507	0.506	0.474	0.487
4. MDH-D	0.095	0.000	0.039	0.039	0.000	0.035
5. DIA-A	0.539	0.000	0.148	0.521	0.521	0.346
6. 6PGD-A	0.097	0.478	0.356	0.509	0.322	0.353
7. 6PGD-B	0.386	0.469	0.445	0.506	0.438	0.449
8. GDH-A	0.000	0.039	0.058	0.039	0.058	0.039
9. PGI-B	0.256	0.414	0.284	0.387	0.270	0.322
10. LAP-A	0.113	0.039	0.000	0.039	0.000	0.038
11. LAP-B	0.000	0.000	0.000	0.000	0.077	0.015
12. GOT-A	0.000	0.000	0.000	0.039	0.000	0.008
13. GOT-B	0.294	0.498	0.515	0.521	0.464	0.458
14. GOT-C	0.231	0.452	0.097	0.247	0.367	0.279
15. PGM-A	0.000	0.000	0.000	0.113	0.148	0.052
16. IDH-A	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.197	0.180	0.183	0.257	0.225	0.208

Table 5. — Gene diversity parameters in *Pinus nigra* subspecies.

Subspecies	Heterozygosity intrapopulation (Hs)	Total heterozygosity (Ht)	Heterozygosity interpopulation (Dst)	Coefficient of diversity (Gst)
ssp. nigra	0.226	0.233	0.007	0.020
ssp. laricio	0.185	0.186	0.001	0.005
P.nigra (five populations)	0.208	0.231	0.022	0.060

populations was higher than heterogeneity among populations or that interpopulation gene diversity is not strong. Gst value for the 2 subspecies (ssp. *laricio*, ssp. *nigra*) was found to be lower (0.005 and 0.020 respectively) than that of the whole species.

Unbiased genetic distances for all possible pairs of the 5 populations are presented in *table 6*. The average genetic distance is 0.035. The highest genetic distance is noticed between the populations from Corsica and Austria while the lowest one is noticed between populations from Corsica and Calabria. Generally, lower genetic distance values are found between populations belonging in the same subspecies.

The dendrogram of *P. nigra* populations, based on UPGMA clustering, was constructed by using the genetic distances of Nei (1978) and provides a graphic presentation of relationships among the 5 populations (*Figure 2*). In particular, the 5 populations of *P. nigra* are arranged into 2 distinct clusters: i) populations from Corsica and Calabria (ssp. *laricio)* and ii) populations from Austria and Bulgaria (ssp. *nigra*) plus the Greek population.

Discussion and Conclusions

Five European black pine populations have been analysed for allozyme variation in 16 loci. The allozyme analysis provided important information about the geographic pat-

Table 6. — Estimates of unbiased genetic distance*) (D) based on data from 16 loci between 5 populations of *Pinus nigra*.

	1 Austria	2 Corsica	3 Calabria	4 Bulgaria	5 Greece
1. Austria	0	0.080	0.040	0.016	0.039
2. Corsica		0	0.012	0.048	0.047
3. Calabria			0	0.020	0.031
4. Bulgaria				0	0.017
5. Greece					0

Note: Mean genetic distance 0.035 ± 0.006
*) According to Nei (1978)

tern and genetic variation in *P. nigra*. The data of gene frequencies obtained from our analysis imply that considerable genetic differentiation exists among the populations of *P. nigra* and this differentiation is not followed by a specific geographic pattern. Each one of the examined populations demonstrates significantly different allozyme distribution in the majority of the loci.

Moreover, the presence of alleles with diagnostic value

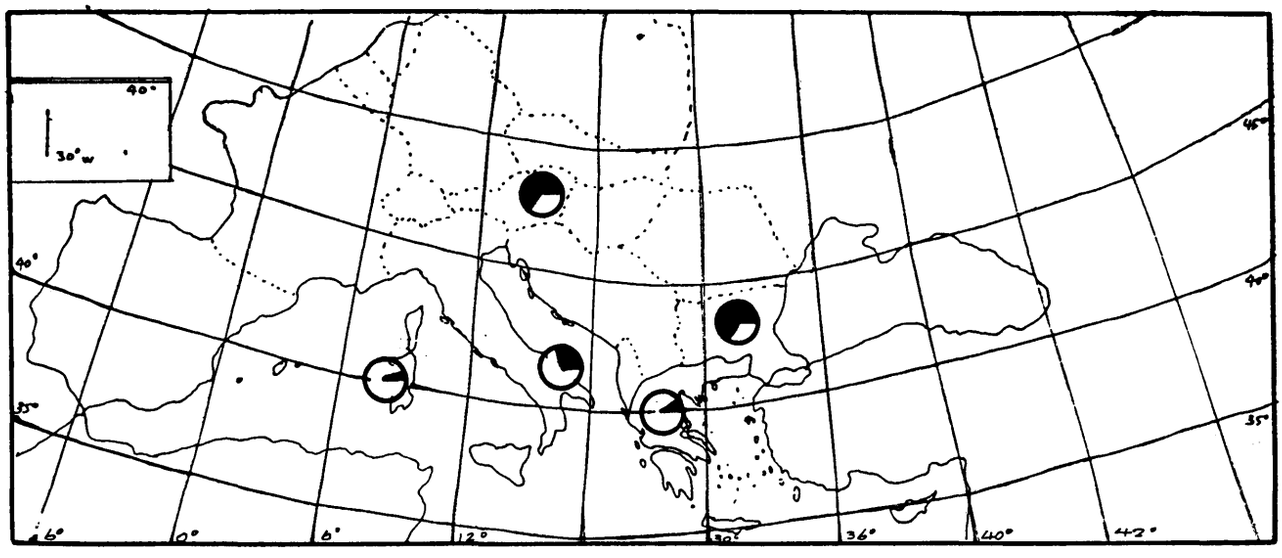


Figure 2a. — Frequency distribution of MDH-B_1 allele.

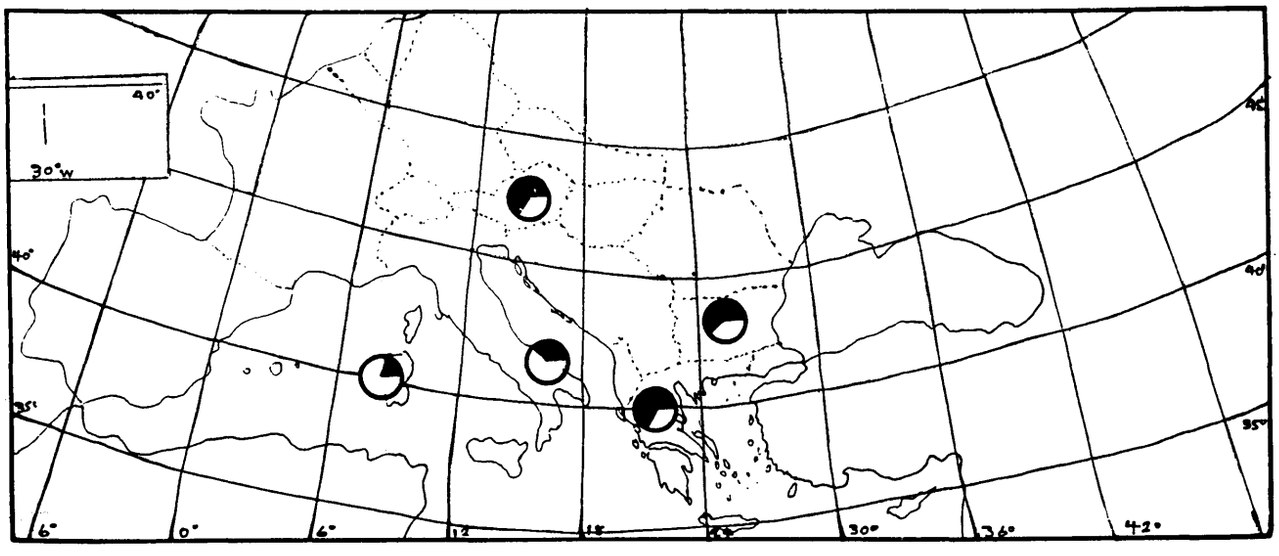


Figure 2b. — Frequency distribution of MDH-C_4 allele.

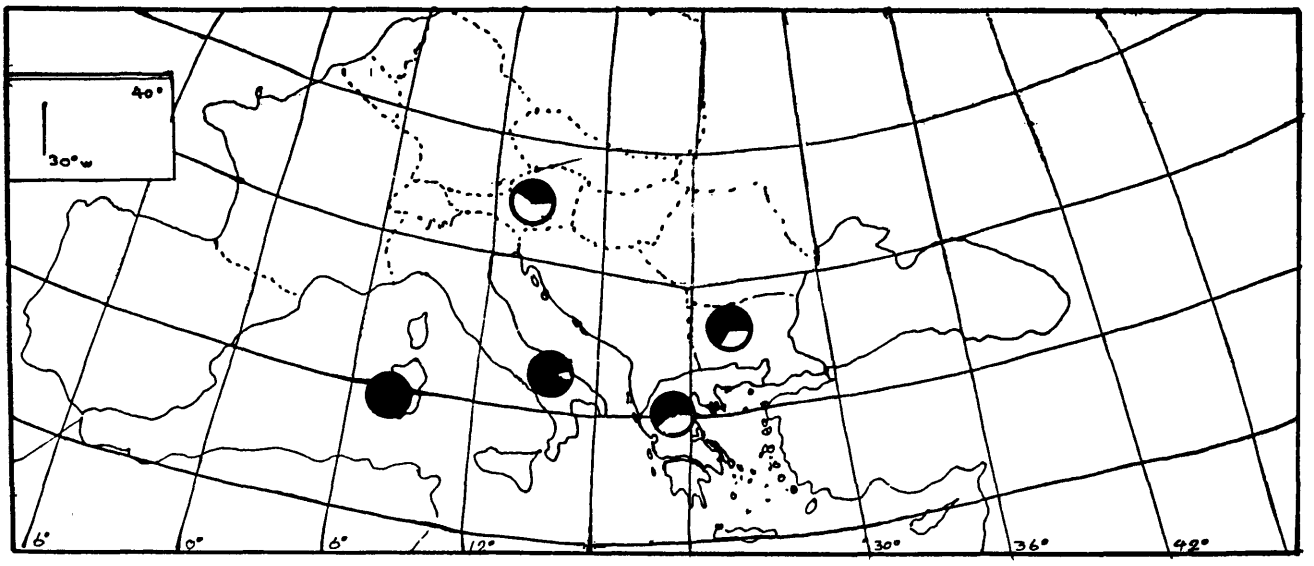


Figure 2c. — Frequency distribution of DIA-A_2 allele.

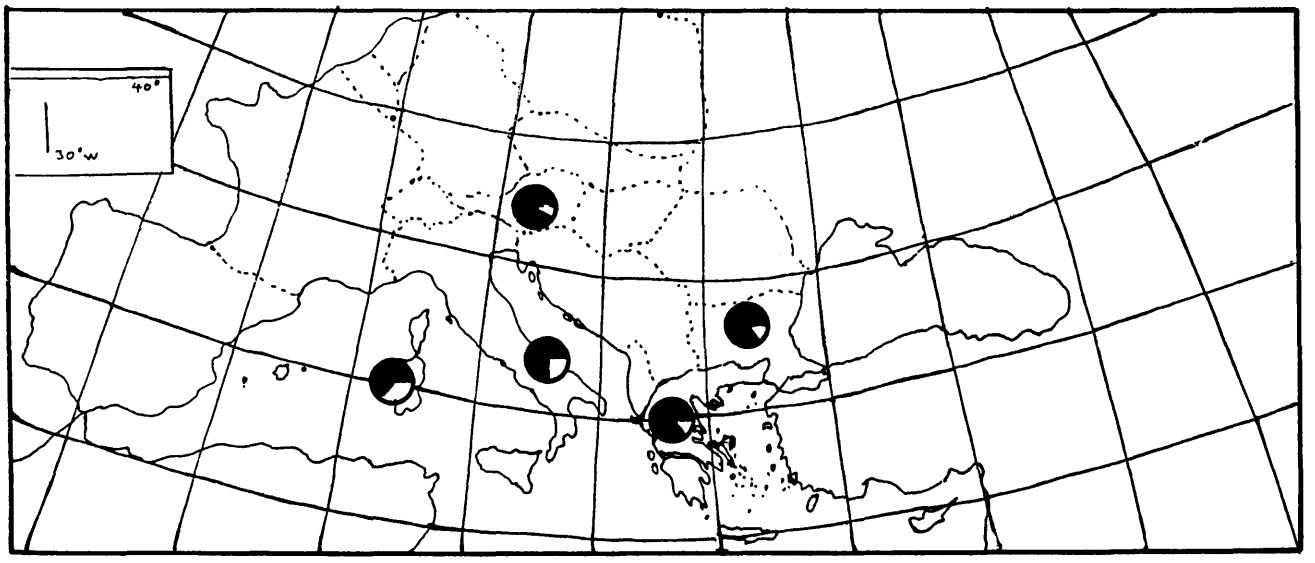


Figure 2d. — Frequency distribution of 6PGD-A_2 allele.

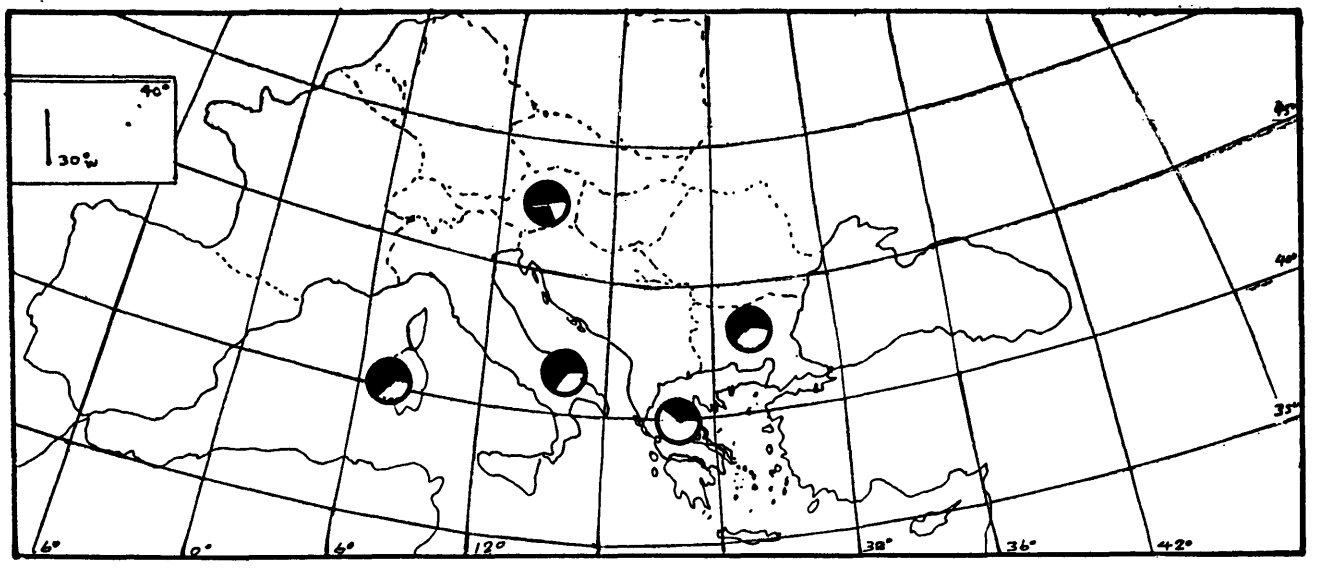


Figure 2e. — Frequency distribution of 6PGD-B_1 allele.

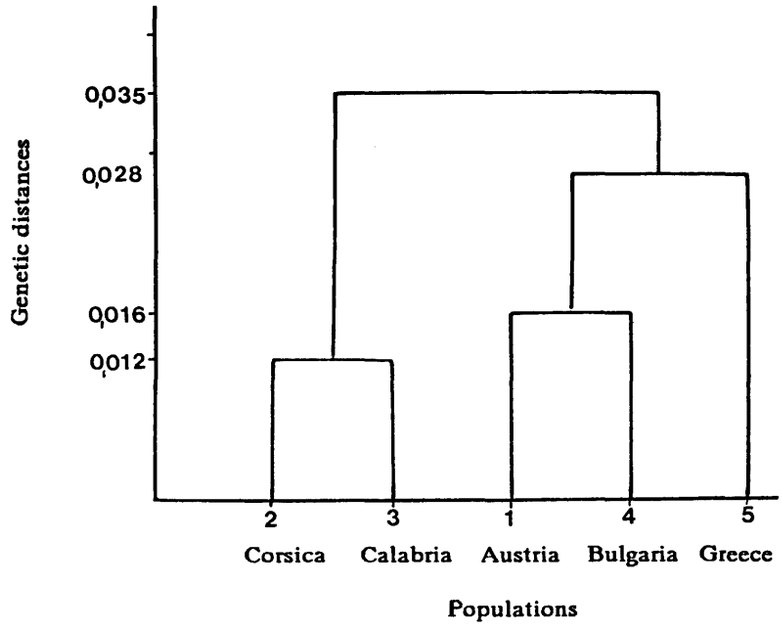


Figure 3. — UPGMA cluster analysis of Nei's unbiased genetic distances between 5 populations of *Pinus nigra*.

was adequate to provide strong evidence for the subdivision of black pine into subspecies or to discriminate and identify each population in every subspecies. In fact, the ssp. *laricio* (Corsica and Calabria) can be discriminated from the ssp. *nigra* and the Greek population by the very high frequency of DIA-A_2 allele or/and by the high frequency of MDH-C_2 allele. The ssp. *nigra* (Austria and Bulgaria) can be discriminated from the ssp. *laricio* and the Greek population by the higher frequency of MDH-B_1 allele. On the other hand, the Austrian population differs from the Bulgarian one by the significantly higher frequencies of MDH-C_3, 6PGD-A_2, 6PGD-B_1, PGI-B_1, AAT-B_2 alleles. The Corsican population differs from the Calabrian one either by the significantly higher frequency of MDH-B_2, MDH-C_2, 6PGD-A_3, AAT-C_1 or/and by the absence of DIA-A_1. Finally, the Greek population differs from the rest populations by the higher frequency of 6PGD-B_2 or/and by the high frequency of MDH-B_2 in combination with the high frequency of MDH-C_4. In the present work, the most useful isozymes, for classification of the subspecies (ssp. *laricio, nigra, pallasiana*) of *P. nigra* were MDH-B, MDH-C, DIA-A, 6PGD-A, 6PGD-B. The same isozymes were, also, proved to be useful for the discrimination of ssp. *salzmanii* from the rest of the subspecies (Rohr et al., 1993). The different contribution of loci (isozymes) to the genetic variation of conifer populations was also noticed by Nicolic and Tucic (1983) in *P. nigra*, by Steinhoff et al. (1983) in *P. monticola*, by Yeh and Layton (1979) in *P. contorta*, by Yeh and Arnott (1986) in *Picea sitchensis, P. glauca* and their hybrid.

Genetic differentiation among black pine populations was also noticed by several other studies on variation which involved provenance trials (Wilcox and Miller, 1975; Wheeler et al., 1976), analysis of morphological, anatomical and physiological characters (Bassiotis, 1967; Lee, 1968; Arbez and Millier, 1971; Debazac, 1971; Matziris, 1984) and analysis of biochemical markers, i.e. monoterpenes (Arbez et al., 1974), flavonoids (Lauranson, 1989) and isozymes (Bonnet-Masimbert and Bikay-Bikay, 1978; Nikolic and Tucic, 1983; Fineschi, 1984). In particular, in isozyme studies up to now, only 5 enzyme systems (ACP, EST, LAP, AAT, SKDH) have been used for the taxonomic classification and genetic analysis of the *P. nigra* populations. The absence of any geographical variation pattern was also observed by Lee (1968) and Wheeler et al. (1976) on morphological, anatomical and chemical data and by Bonnet-Masimbert and Bikay-Bikay (1978) and Nikolic and Tucic (1983) on allozyme analysis.

The wide range of the genetic variability values (P, A/L, h, H) was also noticed by Nikolic and Tucic (1983) on *P. nigra* and according to Yeh and El-Kassaby (1980) this is typical for several conifer species.

The estimation of heterozygosity (*Table 3*) indicates that *P. nigra* exhibits high levels of genetic variation and that there are considerable differences of the genetic variants among the populations. The above is in agreement with the findings of Nikolic and Tucic (1983) in *P. nigra* populations or with the findings of other workers who studied different conifer species (Steinhoff et al., 1983; Yeh and Arnott, 1986).

Generally, conifers are characterized by very high levels of genetic variation and their group is one of the most variable groups of species (Hamrick, 1979). Up to now, notable exceptions to this rule were *Pinus resinosa* (Fowler and Morris, 1977), *Pinus torreyana* (Ledig and Conkle, 1983), *Thuja plicata* (Copes, 1981) and *Cedrus deodara* (Panetsos et al., 1992). According to Hamrick et al. (1979), Mitton (1983), Ledig (1986), the possible factors accounting for the high variation of conifers are: a) the life-history of conifers (i.e. long-lived, outcrossed trees with high fecundity) and b) the divergent selection for macro-micro geographical adaptation.

The analysis of the genetic variation shows (*Table 5*) that *P. nigra* is characterized by a high total variability (Ht = 0.23) and that a large portion of this is due to high intrapopulation gene differences (94 %). This means that the genetic variation of black pine is high in local populations, and the same alleles tend to be distributed

throughout the whole range of the species. Nevertheless, the observed decrease in the coefficient of genetic differentiation (Gst) in the 2 subspecies of *P. nigra* suggests that, in spite of the low degree of interpopulation gene diversity (Dst = 0.022), there are significant differences among the populations and the proposed subdivision of *P. nigra* into these subspecies according to Flora Europaea (1964), is confirmed.

The organisation of gene diversity in black pine is quite comparable with that observed for other conifers. For example, Gst values reported for: a) lodgepole pine ranged between 1% (KNOWLES, 1984) and 6.1% (WHEELER and GURIES, 1982), b) scots pine varied widely from 2% (GULBERG et al., 1985) to 16% MEJNARTOWITCZ, 1979), c) norway spruce ranged between 2% (LUNDKVIST and RUDIN, 1977) and 6% (TIGERSTEDT, 1974) and d) douglas fir varied from 0.1% (MERKLE and ADAMS, 1987) to 7.1% (LI and ADAMS, 1989) for coastal populations and from 4.3% (YEH, 1981; LI and ADAMS, 1989) to 12.2% (LI and ADAMS, 1989) for interior populations. EL-KASSABY (1991) based on the data gathered from 55 studies conducted on 24 conifer species resulted in the same conclusion, that is: the majority of genetic variation resides within populations and a small but significant component exists among populations.

The above, according to YEH and LAYTON (1979) is probably due to the ecological amplitude, the breeding system and the lack of effective barriers to prevent gene flow among conifer populations.

Since the similarity of isozyme patterns is most likely to be a reflection of their close taxonomic relationship (ROTHE, 1990), our dendrogram, which was constructed from the estimated genetic distances, indicates that: a) there is a clear distinction between ssp. *laricio* (Corsica and Calabria) and ssp. *nigra* (Austria and Bulgaria) and b) the Greek population is closer to ssp. *nigra* than to ssp. *laricio*.

Despite the small number of examined populations, our classification seems to be in agreement with the taxonomic classification of *P. nigra* conducted either by isozyme analysis (BONNET-MASIMBERT and BIKAY-BIKAY, 1978) or by morphological traits (TUTIN et al., 1964; FUKAREK, 1958; DEBAZAC, 1964, 1971). On the contrary, our results are in disagreement with the findings of ARBEZ and MILLIER (1971), ARBEZ et al. (1974), LAURANSON (1989) and NIKOLIC and TUCIC (1983), who found that the populations from Corsica and Calabria belong in different subspecies.

The taxonomic status of *P. nigra*, will be further elucidated by the application of isozyme analysis to populations from the whole natural distribution of the species.

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References

ARBEZ, M., BERNARD-DAGAN, D. and FILLON, CH.: Variabilité intraspécifique des monoterpénes de *Pinus nigra* ARN. bilan des premiers resultats. Ann. Sci. Forest. **1**: 14 pp. (1974). —— ARBEZ, M. and MILLIER, C.: Contribution à l'étude de la variabilité géographique de *Pinus nigra* ARN. Ann. Sci. Forest **28**: 23—49 (1971). —— BASSIOTIS, C.: Variation in characteristics of black pine in Greece. Scientific Annals of Agriculture and Forestry Faculty, Thessaloniki **11**, 79—133 (1967). —— BONNET-MASIMBERT, M. and BIKAY-BIKAY, V.: Variabilité intraspécifique des isozymes de la glutamate-oxalacetate-transaminase chez *Pinus nigra* ARNOLD. Intérêt pour la taxonomie des sous espèces. Silvae Genet. **27**, 71—79 (1978). —— CHELIAK, W. M. and PITEL, J. A.: Techniques for starch gel electrophoresis of enzymes from forest tree species. Information report PI-X-42, Petawawa National Forestry Institute (1985). —— CONKLE, M. T., HODGSKISS, P. D., NUNNALLY, L. B. and HUNTER, S. C.: Starch gel electrophoresis of conifer seeds: a laboratory manual. In: General Technical Report PSW-64 (1982). —— COPES, D. L.: Isoenzyme uniformity in western red cedar seedlings from Oregon and Washington. Can. J. For. Res. **11**: 451—453 (1981). —— CRITCHFIELD, W. B. and LITTLE, L., jr.: Geographic distribution of the pines of the world. USDA Misc. pub. 991, 97 pp. (1966). —— DEBAZAC, E. F.: Le pin *laricio* de Corse dans son aire naturelle. Rev. for. franc. **16**: 188—215 (1964). —— DEBAZAC, E. F.: Contribution à la connaissance de la répartition et de l'écologie de *Pinus nigra* ARN. dans le sud-est de l'Europe. Ann. Sci. Forest. **28**: 91—139 (1971). —— EL-KASSABY, Y. A.: Genetic interpretation of malate dehydrogenase isozymes in some conifer species. The Journal of Heredity **72**: 451—452 (1981). —— EL-KASSABY, Y. A.: Genetic variation within and among conifer populations: review and evaluation of methods. In: Biochemical markers in the population genetics of forest trees. pp. 61—76. Edited by S. FINESCHI, M. E. MALVOLTI, F. CANNATA and H. H. HATTEMER. SPB Academic Publishing bv, The Hague (1991). —— FINESCHI, S.: Determination of the origin of an isolated group of trees of *Pinus nigra* through enzyme gene markers. Silvae Genet. **33**, 169—172 (1984). —— FOWLER, D. P. and MORRIS, R. W.: Genetic diversity in red pine: evidence for low genic heterozygosity. Can. J. For. Res. **7**: 343—347 (1977). —— FUKAREK, P.: Sume borova na jugoslavenskom krsu. Simp. ozastiti prirode u nasem krsu. Jug. Akademija znanosti i umjetnosti, Zagreb: 145—160 (1971). —— GULBERG, U., YAZDANI, R., RUDIN, R. and RYANNEN, N.: Allozyme variation in Scots pine (*Pinus sylvestris* L.) in Sweden. Silvae Genet. **34**: 193—201 (1985). —— HAMRICK, J. L.: Genetic variation and longevity. In: SOLBRIG, O. T., JAIN, S. and RAVEN, P. H. (eds): Plant Population Biology, pp. 84—113. Columbia University Press, N. Y. (1979). —— HAMRICK, J. L., LINHART, Y. B. and MITTON, J. B.: Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. Ann. Rev. Ecol. Sys. **10**: 173—200 (1979). —— KNOWLES, P.: Genetic variability among and within closely spaced populations of lodgepole pine. Can. J. Genet. Cytol. **26**: 177—184 (1984). —— LAURANSON, J.: Exploration de la diversité biochimique chez les conifers: contribution à l'étude de l'hybridation *Pinus uncinata* RAM. x *P. sylvestris* et à la connaissance du complexe spécifique *Pinus nigra* ARN. Thèse, Université Claude-Bernard, Lyon I. 270 pp. (1989). —— LEDIG, F. T.: Heterozygosity, heterosis and fittness in outbreeding plants. In: SOULE, E. (ed.): Conservation Biology: The science of Scarcity and Diversity. pp. 77—104. Sinauer Association, Sunderland, Massachusetts (1986). —— LEDIG, F. T. and CONKLE, M. T.: Gene diversity and genetic structure in a narrow endemic, Torrey pine (*Pinus torreyana* PARRY ex CARR.). Evolution **37**: 79—85 (1983). —— LEE, C. H.: Geographic variation in European black pine. Silvae Genet. **17**: 165—172 (1968). —— LI, P. and ADAMS, W. T.: Range-wide patterns of allozyme variation in Douglas-fir [*Pseudotsuga menziesii* (MIRB.) FRANCO]. Can. J. For. Res **19**: 149—161 (1989). —— LUNDKVIST, K. and RUDIN, D.: Genetic variation in eleven populations of *Picea abies* as determined by isozyme analysis. Hereditas **85**: 67—74 (1977). —— MATZIRIS, D. I.: Genetic variation in morphological and anatomical needle characteristics in the black pine of Peloponnesos. Silvae Genet. **33**: 164—169 (1984). —— MEJNARTOWITCZ, L.: Genetic variation in some isozyme loci in Scots pine (*Pinus sylvestris* L.) populations. Arboretum Kornickie **24**: 91—104 (1979). —— MERKLE, S. A. and ADAMS, W. T.: Patterns of allozyme variation within and among Douglas fir breeding zones in southwest Oregon. Can. J. For. Res. **17**: 402—407 (1987). —— MIROV, N. T.: The Genus Pinus. The Ronald Press Company, New York. 602 pp. (1967). —— MITTON, J. B.: Conifers. In: TANKSLEY, S. D. and ORTON, T. J. (eds.): Isozymes in Plant Genetics and Breeding. pp. 443—472. Elsevier Science Publishers B. V., Amsterdam (1983). —— NEI, M.: Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. U.S.A. **70**: 3321—3323 (1973). —— NEI, M.: Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics **89**: 583—590 (1978). —— NEI, M. and CHESSER, R. K.: Estimation of fixation indices and gene diversities. Ann. Hum. Genet. **47**: 253—259 (1983). —— NIKOLIC, D. and TUCIC, N.: Isoenzyme variation within and among populations of European black pine (*Pinus nigra* ARNOLD). Silvae Genet. **32**, 3—4 (1983). —— PANETSOS, K. P., CHRISTOU, A. and SCALTSOYIANNES, A.: First analysis on allozyme variation in Cedar species (*Cedrus* sp.). Silvae Genet. **41**(6): 339—342 (1992). —— PANETSOS, K. P. and MITSOPOULOS, D. J.: Variation in some characteristics of *Pinus nigra* ARN. in Greece. Scientific Annals of Agriculture and Forestry School, Vol. **21**: 41—63 (1978). —— ROHR, R., PANETSOS, K. P., SCALTSOYIANNES, A. and TSAKTSIRA, M.: Isozyme variation in six populations of *Pinus nigra*. In preparation (1993). —— RÖHRIG, E.: Über die Schwarzkiefer (*Pinus nigra* ARN.)

und ihre Formen. I. Teil: Die natürlichen Standorte. Silvae Genet. 6: 39—53 (1957). — RÖHRIG, E.: Die Schwarzkiefer (*Pinus nigra* ARN.) und ihre Formen. II. Erste Ergebnisse von Provenienzversuchen. Silvae Genet. 15: 21—26 (1966). — ROTHE, G. M.: Forest Genetic Resources. Informations no. 18. Food and Agriculture Organisation of the United Nations, 1—15 pp. (1990). — STEINHOFF, R. J., JOYCE, D. G. and FINS, L.: Isozyme variation in *Pinus monticola*. Can. J. For. Res. 13: 1122—1132 (1983). — STOJANOFF, N. and STEFANOFF, B.: Beitrag zur Kenntnis der Pliozanflora der Ebene von Sofia (Fossile Pflanzenreste aus den Ablagerungen bei Kurilo). Ztschr. Bulg. Geol. Ges. 1: 3—115 (1929). — TIGERSTEDT, P. M. A.: Genetic structure of *Picea abies* populations as determined by the isozyme approach. In: Proc. Joint IUFRO Meeting S-02.04.1-3, Stockholm, pp. 283—291 (1974). — TUTIN, T. G., HEYWOOD, V. H., BURGES, N. A., VALENTINE, D. H., WALTERS, S. M. and WEBB, D. A. (Eds.): Flora Europaea. Vol. 1. Cambridge University Press. 464 p. (1964). — VIDAKOVIC, M.: Znacenje anatomske grade iglica kod svojta crnog bora u Jugoslaviji. Sum. list 7—8: 244—253 (1955). — VIDAKOVIC, M.: Oblici crnog bora u Jugoslaviji na temelju anatomije iglica. (Forms of *Pinus nigra* in Yugoslavia on the basis of anatomical structure of the needles). Glas. Sum. Pokuse 13: 111—248, Zagreb (1957). — VIDAKOVIC, M.: Genetics of European black pine (*Pinus nigra* ARN.). Annales Forest. Acad. Sci. et Artium Slavorum Meridionalium 6/3. 86pp. (1974). — WHEELER, N. C. and GURIES, R. P.: Population structure, genic diversity and morphological variation in *Pinus contorta* DOUGL.. Can. J. For. Res. 12: 595—606 (1982). — WHEELER, N. C., KRIEBEL, H. B., LEE, C. H., READ, R. A. and WRIGHT, J. W.: 15-Year performance of European black pine in provenance tests in North Central United States. Silvae Genetica 25, 1—7 (1976). — WILCOX, M. D. and MILLER, J. T.: *Pinus nigra* provenance variation and selection New Zealand. Silvae Genet. 24 (5—6) (1975). — YEH, F. C.: Analyses of gene diversity in some species of conifers. In: CONKLE, M. T. (tech. coord.). Proc. Symp. Isozymes of North Am. For. Trees and For. Insects. pp. 48—52. USDA For. Serv. Gen. Tech. Rep. PSW-48 (1981). — YEH, F. C. and ARNOTT, J. T.: Electrophoretic and morphological differentiation of *Picea sitchensis, Picea glauca* and their hybrids. Can. J. For. Res. 16 (4): 791—798 (1986). — YEH, F. C. and EL-KASSABY, Y. A.: Enzyme variation in natural populations of Sitka spruce (*Picea sitchensis*). I. Genetic variation patterns among trees from 10 IUFRO provenances. Can. J. For. Res. 10: 415—422 (1980). — YEH, F. C. and LAYTON, C.: The organization of genetic variability in central and marginal populations of lodgepole pine *Pinus contorta* spp. *latifolia*. Can. J. Genet. Cytol. 21: 487—503 (1979). — ZOLLER, H., GEISSLER, P. and ATHANASIADIS, N.: Beiträge zur Kenntnis der Wälder, Moos- und Flechtenassoziationen in den Gebirgen Nordgriechenlands. Bauhinia 6(1): 215—255 und 14 Tabellen und 5 Figuren (1977).

Pairwise Competition Among Progenies from Matings Within and Between Three Origins of Picea abies in a Nursery Trial

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Summary

Picea abies (L.) KARST. full-sib progenies from a factorial mating design were classified into 8 types according to the origin of the parents: northern Sweden, central Sweden, or continental Europe. Seedlings were planted in 36-tree plots with 40 cm × 40 cm spacing, either a single mating type, or 2 types mixed (checker-board), on sandy, unfertilized soil. Seven out of the 28 possible pairwise mixtures were included. Height and stem diameter were assessed annually from age 2 (height) or 5 (diameter) through 9 years. The effects of direct mating type and of associate mating type were quantified by a factorial analysis of variance. For height, the direct effect was large at ages 2 to 4, as a result of juvenile free growth in the southern mating types. However, the direct effect declined and was smaller than the associate effect at age 9. An average mixed-plot superiority compared to pure plots was significant from age 3 through 6 for height, from age 6 through 8 for diameter. At most, this superiority was about 5%, both for height and for diameter. Similar competition effects have been reported for other conifers, with other types of genetic entries, when growing on nutrient-poor soils.

Key words: Competition, Provenance matings, Contrast plots, Narrow spacing, Mixture effects, Progeny test, *Picea abies* (L.) KARST.

Introduction

Many effects of competition between adjacent trees in the forest are obvious, and are the motives for silvicultural strategies such as various thinning and selective cutting regimes. With increasing intensity of cultivation and increasing control of the genetic set-up of entries, prediction of growth performance in mixtures of particular entries, or more generally, in defined competitive environments, becomes increasingly attractive. This would be possible through the identification of ideotypes (DONALD, 1968; CANNELL, 1982). A forest tree ideotype could be defined by different kinds of traits, e.g. phenological, morphological, or physiological. A highly heritable, narrow crown type of *Picea abies* in Finland, has been proposed as a crop ideotype that would yield more merchantable wood per unit area (PULKKINEN, 1991; PULKKINEN and TIGERSTEDT, 1992). However, for the ideotype concept to be useful, more knowledge and experimental data are required in areas such as sink dynamics, ageing, and competition processes (DICKMAN, 1985). Causes and effects of competition may depend on the type of competing entries (species, provenances, half-sibs, clones, etc), site conditions (soil fertility, spacing, etc.), age, and other factors. JOHANSSON and KEDDY (1991), working on a large number of wetland annual plant species, experimentally confirmed two general hypotheses related to competition, that (i) intensity, i.e. average growth depression, increases with the degree of similarity of the competing species, and that (ii) asymmetry, i. e. lack of balance between growth alterations of the competing species, decreases with increasing similarity. They also found that "competitive similarity" between species was difficult to measure, although a large number of traits, considered relevant for competition, were observed. This emphasizes the need for a better understanding of competitive mecha-