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Genetic Variation of *Pinus brutia* from Islands of the Northeastern Aegean Sea

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Abstract

The present study concentrated on *Pinus brutia* TEN. one of the main forest species of the Aegean islands and one of the most important low-elevation Mediterranean conifers. In this report the amount and structure of genetic diversity of four *Pinus brutia* populations which forms an integral prerequisite for breeding efforts and for the protection of the species genetic resources, is presented. One population per island was sampled from four islands of the north-eastern Aegean namely Lesvos, Chios, Samos and Thasos. Seven isoenzymic loci (*Dia-1*, *Idh-1*, *Lap-1*, *Mdh-1*, *Mdh-4*, *Pgd-1* and *Pgi-2*) were identified and used for the evaluation of genetic variability in the above populations. Five of the loci studied were polymorphic, while a total of 17 alleles were detected. All populations presented significant amounts of genetic diversity and heterozygosity. The levels of genetic diversity parameters were higher than those of earlier reports regarding this species, but in agreement with values reported for conifer trees in general. Genotypic frequencies of the population samples were in agreement with those expected from HARDY-WEINBERG expectations. Results also point towards the absence of inbreeding and random genetic drift. Most of the genetic diversity of *Pinus brutia* (97.9%) was found within populations and only 2.1% among populations. Most of the alleles studied were common for all populations. Some differences among populations were detected for rare alleles. Populations present low values of NEI's genetic distance and CAVALLI-SFORZA and EDWARDS' chord distance. Two groups were revealed in the respective dendrograms: the first group formed by the populations of Lesvos, Chios and Samos, and the second by the Thasos population. The significance of these results in breeding and forest management practice is briefly discussed.

Key words: *Pinus brutia*, isoenzymes, Aegean, population genetics.

FDC: 165.3; 174.7 *Pinus brutia*; (495).

1. Introduction

Pinus brutia TENORE subsp. *brutia* is one of the main forest tree species of the Aegean islands, Greece. The species attains a tree form with a height of 15 m to 20 m, sometimes even 30 m with a usually straight main stem (ATHANASIADIS, 1986; PANETSOS, 1981). *P. brutia* populations present considerable phenotypic variation and plasticity (ISIK, 1986; PANETSOS, 1981). The distribution of *P. brutia* in the eastern Mediterranean basin and its ability to grow in adverse climatic and soil conditions make this species very important for multiple purpose forestry. Moreover in favorable sites *P. brutia* exhibits a significant growth potential, while the presence of mechanisms for regeneration after fire makes the species irreplaceable in the delicate Mediterranean ecosystem (PANETSOS, 1986).

Factors such as island geographic isolation, long term negative selection due to needs in wood and resin, soil mosaic, climatic variability due to differences in altitude, as well as forest fires, are expected to have contributed to the species present genetic structure. The destruction of *Pinus brutia* forests in the Aegean islands calls for the frequent employment of artificial reforestations. Protection of the local genetic resources and selection of the suitable planting material aquires a high importance in the frame of possible genetic divergence due to the island population subdivision and differentiation. One potential means to face the increasing demands for quality wood production (construction wood, ship-yard wood etc.) in the Aegean islands is the use of genetically improved material. The basic prerequisite for genetic improvement and for the protection of genetic resources is the study of genetic variability. In this paper an analysis of the isoenzymic genetic variability in natural populations of *Pinus brutia* from the islands of north-eastern Aegean is presented.

2. Materials and Methods

2.1. Sampling

A systematic approach was employed in sampling individuals from all populations. In the islands of Chios, Samos and Thasos samples were taken from the total distribution of *Pinus brutia*. Sampling was conducted in July 1995 and experiments were carried out in 1996. In these islands the distribution of the species is continuous. Individuals were sampled some 1 km to 2 km apart from each other. The sampling procedure capitalized on the mountainous terrain; care was taken to ensure that only one individual was sampled from a hill slope or valley, in order to secure both representative sampling and no filial relation of sampled individuals. Care was also taken to ensure that all typical stands in terms of altitude and orientation were visited and sampled. In Lesvos island were the species presents a discontinuous distribution with a number of large stands, samples were taken from the Tsamliki forest, the main *P. brutia* forest of the island. Individuals were sampled at equal distances on an east-west transect through the forest.

The following restrictions were imposed on the selection of individual trees: (a) to bear mature cones of the current year, (b) to have at least one neighbor within 25 m in order to avoid sampling trees that may have come from self-pollination. In addition, stands proximal to urban centers, or other areas of present or past human activity, were avoided during sampling in order to evade the possibility of probing artificially planted trees. The location of all sampled individuals was recorded and placed on maps for future reference. Sample sizes were 94 trees for Lesvos, 94 for Chios, 65 for Samos and 59 for Thasos. About 10 mature cones were selected from every individual.

2.2. Electrophoresis

The endosperm (megagametophyte) was used for enzyme extraction. The respective buffer of CONKLE *et al.* (1982) was employed and after homogenization the paste, was centrifuged at 13700 rpm and 4 °C for 10 minutes. Horizontal starch gel electrophoresis (10.5 % SIGMA® starch) was used for the study of the following enzyme systems (enzyme abbreviations and Enzyme Commission Numbers are given in parentheses): diaphorase (DIA 1.6.4.3.), isocitrate dehydrogenase (IDH 1.1.1.42), leucine aminopeptidase (LAP 3.4.11.1), malate dehydrogenase (MDH 1.1.1.37.), 6-Phosphoglyconate dehydrogenase (6PGD 1.1.1.44.), and phosphoglycoze isomerase (PGI 5.3.1.9.). Gel and electrode buffers, as well as enzyme-specific histochemical stains, followed the protocols of CHELIAK and PITEL (1984) except the histochemical stain of leucine aminopeptidase which was prepared according to CONKLE *et. al.* (1982). Electrophoresis was conducted at 4 °C with a 60 mA constant amperage. In enzyme systems where multiple isoenzymes were detected, the fastest migrating anodal zone was designated as locus 1. The fastest migrating banding phenotype within the zone was designated as allele A. Numbering of additional loci and alleles progressed sequentially in the cathodal direction. The use of endosperm in the determination of the genotype of a conifer mother tree is described by MORRIS and SPIETH (1978). In this particular case it has been established that the isoenzymes studied exhibit MENDELian inheritance and codominant allelic expression (ARAVANOPOULOS, 1996, and unpublished data), and the probability of false determination of a material genotype was p = 0.016 since seven endosperms were analyzed per individual.

2.3. Data analysis

Analysis was performed at two levels: within population and among populations. For the determination of genetic diversity, the following parameters were employed: average number of alleles per locus (A), percent polymorphic loci (P; 0.95 criterion), mean observed heterozygosity (H_{obs}), gene diversity (expected heterozygosity based on HARDY-WEINBERG expectations; NEI, 1973), and inbreeding coefficient (F; WRIGHT, 1978). The hypothesis that populations are in HARDY-WEINBERG equilibrium was tested by employing the χ^2 criterion. Population structure was studied through hierarchical F-statistics according to WRIGHT (1978). The values of the inbreeding coefficient F within populations (F_{IS}), between populations (F_{ST} also refereed to as fixation index) and of the total population (F_{IT}) if it is considered that the island populations form a total population, were determined. The unbiased genetic distances according to NEI (1978) and the chord distances according to CAVALLI-SFORZA and EDWARDS (1967) between populations, were also determined. Population relationships were depicted in two dendrograms: the former based on NEI's (1978) genetic distances subjected to cluster analysis with the employment of the unweighted pair group method using arithmetic averages (UPGMA); the latter based on the CAVALLI-SFORZA and EDWARDS (1967) chord distances subjected to the distance WAGNER procedure (FARRIS, 1972). The BIOSYS-1 software (SWOFORD and SELANDER, 1989) was used.

3. Results

Seven loci and a total of 17 alleles were revealed in the six enzyme systems that were studied (*Table 1*). Two additional loci (*Pgd-2* and *Pgi-1*) were not included in this work since

Table 1. – Allele frequencies of *Pinus brutia* TEN. in populations of north-eastern Aegean islands.

	Population			
Locus	Lesvos	Samos	Chios	Thasos
Dia-1				
(N)	93	64	93	59
A	0.925	0.906	0.909	0.915
B	0.075	0.094	0.091	0.085
Idh-1				
(N)	90	65	94	59
A	0.000	0.008	0.000	0.000
B	1.000	0.985	1.000	1.000
C	0.000	0.008	0.000	0.000
Lap-1				
(N)	94	65	94	59
A	1.000	1.000	1.000	1.000
Mdh-1				
(N)	90	65	94	59
A	0.328	0.385	0.293	0.229
B	0.672	0.615	0.707	0.771
Mdh-4				
(N)	90	65	94	59
A	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	1.000
Pgd-1				
(N)	90	65	94	59
A	0.639	0.577	0.484	0.678
B	0.361	0.423	0.516	0.229
C	0.000	0.000	0.000	0.093
Pgi-2				
(N)	94	65	94	59
A	0.027	0.000	0.000	0.000
B	0.330	0.331	0.383	0.203
C	0.644	0.669	0.617	0.780
D	0.000	0.000	0.000	0.017

Table 2. – Genetic variability parameters for seven loci in four *Pinus brutia* populations originating from islands of the Northeastern Aegean sea (standard errors in parantheses). N: average sample size, A: average number of alleles per locus, P: percentage of polymorphic loci (0.95 criterion), H_{obs}: average observed heterozygosity, H_{exp}: gene diversity (average expected heterozygosity (NEI, 1978), F: average inbreeding coefficient.

Population	N	A	P	H_{obs}	H_{exp}	F
Lesvos	91.6	1.70	57.1	0.213	0.218	0.023
	(0.8)	(0.3)		(0.086)	(0.088)	
Samos	64.9	1.90	57.1	0.220	0.231	0.048
	(0.1)	(0.3)		(0.083)	(0.088)	
Chios	93.9	1.60	57.1	0.230	0.223	-0.031
	(0.1)	(0.2)		(0.094)	(0.089)	
Thasos	59.0	1.90	57.1	0.160	0.193	0.171
	(0.0)	(0.3)		(0.070)	(0.077)	

inheritance analysis is pending. Five loci (*Dia-1*, *Mdh-1*, *Mdh-4*, *6Pgd-1*, and *Pgi-2*) were polymorphic. Allele frequencies among population did not differ significantly except in *Pgd-1B*. In the *Dia-1* locus one allele presented a low frequency (< 0.100) in all populations. In *Idh-1* two rare alleles (0.95 criterion) were present in the Samos population, while in the other populations this locus was monomorphic. In *Pgi-2* four alleles were detected: two common alleles in every population and two rare ones, one in the population of Lesvos island and the other in the population of Samos. Allele *Pgd-1C* was detected in the Thasos population only.

Genetic variability parameters are presented in *table 2*. Average number of alleles per locus varied between 1.70 and 1.90, and the percent polymorphic loci value was 57.1%. Gene diversity varied from 0.193 (Thasos) to 0.231 (Samos). Observed heterozygosity varied from 0.160 (Thasos) to 0.230 (Chios). In general variation and heterozygosity were higher in Samos, Lesvos, and Chios, but lower in Thasos. The average inbreeding coefficient was positive for Lesvos, Chios and Thasos (where the highest value was found) and negative for Chios.

The values of the inbreeding coefficient for the individual loci were generally low. A high value was detected for locus *Dia-1* in the Thasos population (*Table 3*). Significant deviations from the HARDY-WEINBERG expectations were not detected except for *Dia-1* in the Thasos population. The analysis of

Table 3. – Fixation indices (F_{IS}) and probability of deviating from HARDY-WEINBERG equilibrium in four *Pinus brutia* populations originating from islands of the Northeastern Aegean sea for all polymorphic loci.

	Lesvos		Samos		Chios		Thasos	
Locus	F_{IS}	p	F_{IS}	p	F_{IS}	p	F_{IS}	p
Dia-1	-0.081	0.451	-0.103	0.430	0.029	0.735	0.563	<0.001
Idh-1	–	–	-0.012	1.000	–	–	–	–
Mdh-1	-0.084	0.455	-0.105	0.433	-0.002	0.977	-0.009	1.000
Pgd-1	-0.035	0.777	0.212	0.076	-0.171	0.107	0.116	0.553
Pgi-2	0.196	0.060	0.062	0.574	0.055	0.561	0.226	0.090

Table 4. – Genetic structure of *Pinus brutia* populations originating from islands of the Northeastern Aegean sea according to hierarchical F-statistics.

Locus	Within populations (F_{IS})	Among Populations(F_{IT})	Total(F_{ST})
Dia-1	0.100	0.101	0.001
Idh-1	-0.012	-0.003	0.009
Mdh-1	-0.054	-0.038	0.015
Pgd-1	0.030	0.064	0.036
Pgi-2	0.130	0.146	0.019
Mean	0.042	0.063	0.021

Table 5. – Genetic distance coefficients of *Pinus brutia* populations originating from islands of the Northeastern Aegean sea. Above the diagonal NEI's (1978) unbiased genetic distance; below the diagonal: CAVALLI-SFORZA and EDWARDS (1967) chord distance.

Population	Lesvos	Samos	Chios	Thasos
Lesvos	************ *	0.000	0.004	0.006
Samos	0.054	**************	0.002	0.010
Chios	0.057	0.046	**********	0.016
Thasos	0.104	0.110	0.115	**********

hierarchical F-statistics is presented in *table 4*. The values of the inbreeding coefficient F among populations (F_{IS}), between populations (F_{ST}) and of the total population (F_{IT}) were close to zero regardless of their sign. This result was generally found for all loci. The respective average values were very low and positive (*Table 4*).

Genetic distance values according to NEI (1978), and CAVALLI-SFORZA and EDWARDS (1967) are presented in *table 5*. The Lesvos and Samos populations presented negligible NEI's (1978) genetic distance. The Thasos population appears to be more distant from the other populations. The two-dimension population dendrogram of NEI's (1978) distances subjected to cluster UPGMA analysis is depicted in *figure 1*. Chord distances according to CAVALLI-SFORZA and EDWARDS (1967) also inferred that the Thasos population to appear more distant. In the respective dendrogram (*Fig. 2*) the Samos population appeared more related to the Chios population.

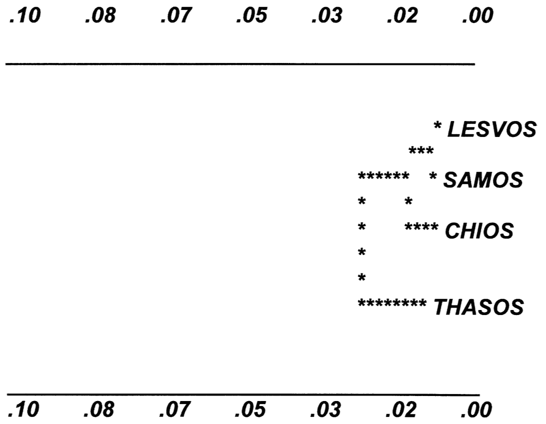

Figure 1. – Dendrogram of *Pinus brutia* populations originating from islands of the Northeastern Aegean sea according to NEI's (1978) genetic distances after an UPGMA cluster analysis.

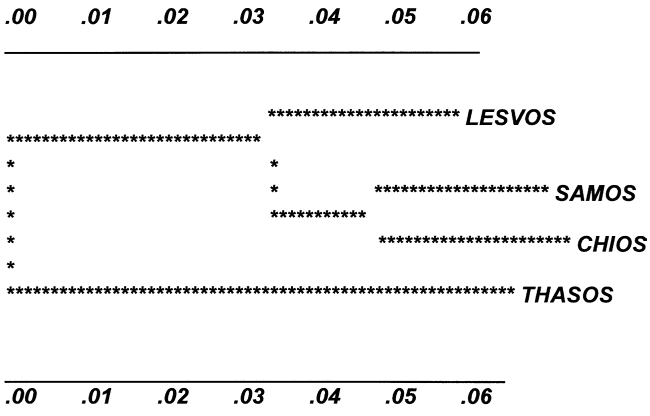

Figure 2. – Dendrogram of *Pinus brutia* populations originating from islands of the Northeastern Aegean sea according to CAVALLI-SFORZA and EDWARDS (1967) chord distances.

4. Discussion

4.1. *Within population diversity*

Generally, elevated amounts of genetic diversity, were detected in all populations. Values for average number of alleles per locus, percent polymorphic loci, observed heterozygosity and gene diversity, were within the range reported for *P. brutia* (CONKLE *et al.*, 1988; PANETSOS *et al.*, 1995). The Thasos population presented relatively lower values for heterozygosity and gene diversity. In general, conifers present high levels of genetic variability and heterozygosity (MITTON, 1983) and *Pinus brutia* seems to follow this trend. The values of the fixation index indicate a slight deficiency of heterozygotes in Lesvos and Samos and of homozygotes in Chios. The value of the average inbreeding coefficient indicates that the Thasos populations presented a rather higher deficiency in heterozygotes.

In all populations and for every locus there was agreement between the detected allele frequencies and those expected under HARDY-WEINBERG equilibrium. The *Dia-1* locus in the Thasos population was an exception. When the inbreeding coefficient was considered, it became evident that there was a notable deficiency in heterozygotes at the above locus. This phenomenon has elevated the value of the average inbreeding coefficient in the Thasos population. The reasons for observing this heterozygote deficiency are not known. Nevertheless, inbreeding should not be regarded as a potential cause since it would have affected other loci as well. In addition, any potential causes probably will not include epigenetic or post-translational modifications, since the inheritance analysis at this isoenzyme locus showed typical MENDELian behavior (ARAVANOPOULOS, 1996). In general, the agreement of the observed allele frequencies with the HARDY-WEINBERG expectations does not permit us to reject the null hypotheses of random mating and of the absence of significant amounts of genetic drift in the *Pinus brutia* populations of the northeastern Aegean.

According to the results of the hierarchical F-statistics the levels of inbreeding are generally low. The low values of the fixation indices (F_{ST}) in every locus indicate that the existence of relatively isolated populations in every island, did not lead to genetic drift phenomena within populations. The existence of geographic (and possibly genetic) isolation seems to be counterbalanced by other factors such as the large population sizes and the considerable levels of outcrossing. The largest part of the *P. brutia* genetic diversity (97.9%) existed between populations and only 2.1% was among populations. The inbreeding coefficient within populations (F_{IS}) indicates that the possibility of non-random mating within populations is minimal and that there is no detectable inbreeding. The total inbreeding coefficient (F_{IT}) which reflects the possible effect of the of the *P. brutia* total population partitioning in island subpopulations, as well as the effects of possible non-random mating within subpopulations, showed minimal inbreeding in *P. brutia* as a whole. Indirectly the above finding also provides

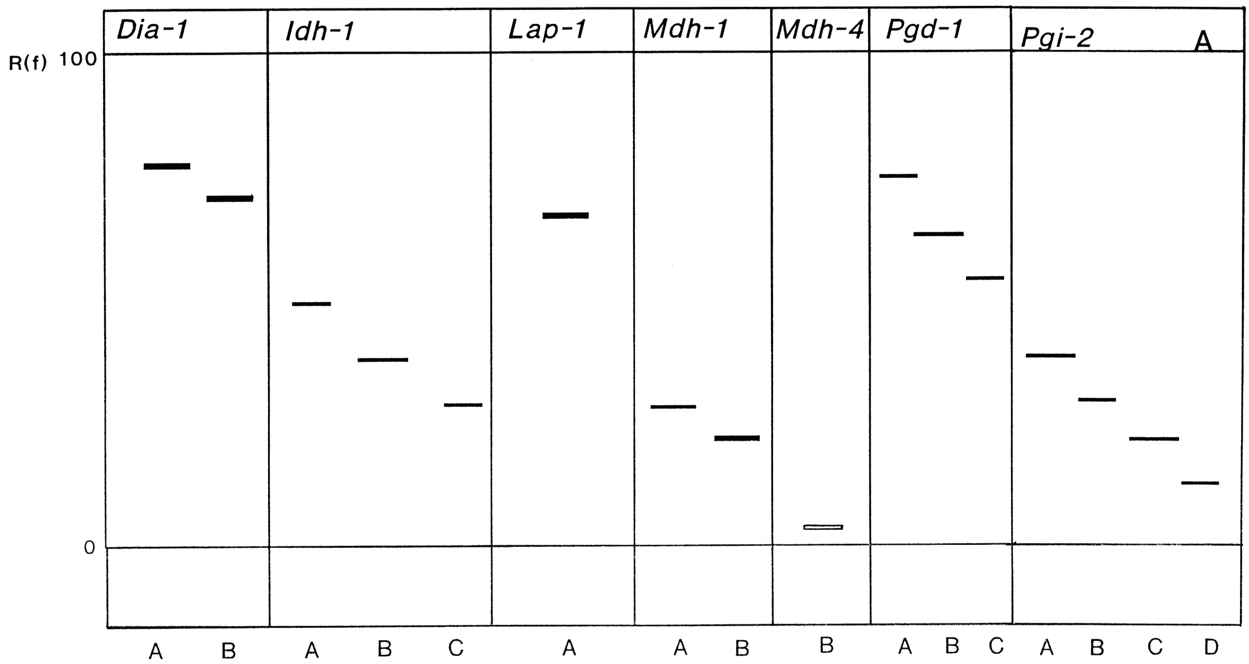

Figure 3. – Zymogram banding patterns and assignment of alleles in the enzyme systems studied in *Pinus brutia* individuals.

an indication for minute differentiation of the total *P. brutia* population, from the Hardy-Weinberg equilibrium.

4.2. Genetic diversity among populations

The distribution of allelic frequencies and the detection of rare alleles in specific island populations, lead to the notion that populations present a degree of genetic differentiation. Most of the alleles that were revealed in this study, were also found in earlier works (Panetsos *et al.*, 1995). In addition, it was noted that the frequencies of common alleles in the island populations that were studied, were comparable to the respective frequencies of continental *P. brutia* populations situated in Asia Minor and Iraq (Conkle *et al.*, 1988). The case of *Idh-1* warrants for some particular notion. Insofar one allele has been detected in this locus (*Idh-1B*) which was found fixed in *P. brutia* (Conkle *et al.*, 1988; Panetsos *et al.*, 1995). In the Samos population two rare alleles were detected (*Idh-1A* and *Idh-1C*). The former has been reported in earlier studies as fixed in *Pinus halepensis* (Conkle *et al.*, 1988; Tessiere *et al.*, 1995), and this is the first report of its presence in a *P. brutia* population. It is characteristic that the individual which possesses this allele, is within a typical *P. brutia* stand situated in the southern slopes of the Ambelos mountain in Samos island. Therefore, this individual is far away from any urban areas where *P. halepensis* could have been occasionally introduced in the past, in parks and gardens. The *Idh-1C* allele was found in the same population and it is the first time that this allele was detected in *P. brutia*. The existence of some *Pinus halepensis* pollen flow cannot be definitely excluded, nevertheless it can be regarded as highly unlikely.

As it can be deduced from the Nei (1978), and the Cavalli-Sforza and Edwards (1967) distances, *Pinus brutia* island populations of the north-eastern Aegean, do not present a strong differentiation. Especially Nei's (1978) genetic distance values are small, and the population of Lesvos and Samos in particular, show a high genetic similarity. In general the corresponding dendrogram depicts two groups: (1) Lesvos, Chios, Samos where a minute genetic differentiation among populations was observed, and (2) Thasos. In contrast to Thasos, the other islands of the first group presented higher amounts of variation and heterozygosity. The islands of the first group present similarities in their geological history and emerged at about the same geological period some 1 million to 1.5 million of years ago (Fytikas, 1997, pers. comm.[1]). Among the two dendrograms based on the cluster analysis (Nei's distance) and on the distance Wagner procedure (Cavalli-Sforza and Edwards distance) there is only one difference in relation to population clustering within the first group as presented above. The methodologies of distance estimation and dendrogram construction differ both in the respective computational process as well as in their assumptions with regards to evolutionary rates and times of divergence. It is therefore not unusual to find some differences in the topologies of the particular dendrograms (Gootman and Weight, 1989). According to Westfall and Conkle (1992), the Cavalli-Sforza and Edwards (1967) chord distance coupled with the dendrogram construction based on the distance Wagner procedure (Farris, 1972) present some attributes which make this approach better suited for investigation of geographic patterns of genetic variation.

5. Conclusions

The *P. brutia* populations of the islands of the north-eastern Aegean present significant genetic variability and heterozygosity. Based on the loci that were studied, it can be asserted that aside from the fact that these populations have been exposed for a long time to forest fires and to various anthropogenic influences, no signs of genetic erosion were detected. Results indicate also the existence of Hardy-Weinberg equilibrium, random mating and absence of notable inbreeding. Therefore the populations in question present a level of genetic stability which implies no threat with regards to their genetic resources. The observed large within population variation and high outcrossing rate are encouraging for potential breeding applications. Despite the little genetic differentiation among populations, the presence of two groups in the dendro-

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grams constructed, could point towards a conservative approach regarding the origin of plant material for artificial regeneration. In light of the above discussion, and provided that the results presented are coupled with provenance studies, the use of plant material originating from Thasos island should be limited to this locale. Nevertheless, the opposite practice could be beneficial in a program of breeding through intraspecific hybridization.

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Analysis of Random Amplified Polymorphic DNA Markers in Three Conifer Species

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Abstract

From 40 arbitrary primers tested with one DNA from each species, two, three and four were chosen to generate RAPD profiles in Norway spruce, silver fir and Scots pine respectively. Sample sites were Northern Vosges, Hunsrück mountains and, between them, the forest around the University of Saarbrücken. The similarity indices of intra- as well as between-sampling sites averaged for all primers, were distinctly lower (0.48) in all spruce and fir stands than in Scots pines' (0.79). Almost all variability resided within samples. Mean genetic distances among sampling sites were generally small and seemed slightly elevated in Scots pine only. Some applications of RAPD data are discussed.

Key words: Abies alba MILL., *Picea abies* L. KARST., *Pinus sylvestris* L., RAPDs, polymorphism.

FDC: 165.5; 165.3; 174.7; *Abies alba;* 174.7 *Picea abies;* 174.7 *Pinus sylvestris.*

Introduction

Allozyme studies have shown that conifers are one of the genetically most variable groups of forest tree species, probably because they are long-lived, have large geographic distributions, exhibit wind pollination linked to high fecundities, shed winged seeds and are common in later successional stages (MITTON, 1983). Some species, however, such as red pine, Torrey pine and western red cedar harbour almost no genetic variation for soluble proteins. This may possibly be due to their restricted and isolated populations, where genetic drift is known to erode allozyme variability more quickly in smaller populations. Though conifers, in large and continuous stands, shed more than 90% outcrossed seed, small stands and isolated trees are increasingly forced to selfing. However, inbred progeny suffer severe inbreeding depression and it is thought that selection against deleterious alleles found in homozygotes arising from consanguineous matings results in an overall excess of heterozygotes in older stands. On account of these counteracting evolutionary strategies and contradictory find-

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