Relationships Among Five Populations of European Black Pine (*Pinus nigra* ARN.) Using Morphometric and Isozyme Markers

By I. AGUINAGALDE[1]), F. LLORENTE[2]) and C. BENITO[2])

(Received 10th April 1996)

Abstract

Five European populations belonging to 3 subspecies of *Pinus nigra* ARN. (ssps. *nigra, salzmanii, laricio*) were analysed for 4 morphometric seeds characters (length, width, wing scar and weight/10 seeds) and for 23 isozyme loci. Significant differences were detected among the populations for the morphometric markers. Five isozyme systems (PGM, MDH, 6-PGD, SOD and EST) presented diagnostic loci that allowed the identification of Corsican population and others. Moreover, statistically significant differences among allele frequencies were found using aconitase (ACO), glutamate oxaloacetate transaminase (GOT) and esterase (EST) isozyme systems. The greatest amount of the total genetic diversity detected was localized within the populations. Genetic heterogeneity among populations indicated that only 30.9% of the total genetic diversity was interpopulations. The dendrogram obtained using genetic distances among populations indicated the existence of 3 groups, corresponding to the 3 subspecies. This data clearly pointed out that the Corsican population is the most distant, with relative lack of genetic variation, probably due to its geographic isolation.

Key words: Pinus nigra ARN., genetic relationships, morphometry, isozyme.

FDC: 165.3; 165.5; 174.7 *Pinus nigra.*

Introduction

Pinus nigra ARN. is a very variable species with a discontinuous circum-mediterranean distribution showing taxonomic problems. Following Flora Europea (TUTIN et al., 1964), its many geographical variants are often not clearly separable, because much of its variation shows a clinical pattern. Five subspecies are recognised, with no general consensus about it and some of them are sometimes regarded as distinct species. Subsp. *nigra* also often known as *austriaca*, corresponds with the forms growing from Austria to C. Italy. Greece and the former Jugoslavia. This subspecies has been introduced in N. Spain (Navarra) for repoblation because of its considerable tolerance to cold and humid habitats. Subsp. *salzmanii*, frequently known as *hispanica* inhabits the most occidental areas, the Pyrenees, C. and E. Spain and N. Africa. It grows over about 400,000 ha in Spain. Subsp. *laricio* grows in Corsica, Sardinia, and Calabria, occuping about 22,000 ha only on the Corsica Island. The other 2 subspecies: *dalmatica* and *pallasiana* growing in the NW. of the former Jugoslavia and in the Balkan peninsula respectively were not covered by this study. Populations belonging to subspecies *pallasiana*, together with *nigra* and *laricio* have been recently studied (SCALTSOYIANNES et al., 1994).

The difficulties in European Black Pine taxonomy explain the several studies on morphology, terpenoids composition and more recently on isozyme variation that have been conducted with this species (BONNET-MASIMBERT et al., 1978; NIKOLIC and TUCIC, 1983; SCALTSOYIANNES et al., 1994). AGUINAGALDE and BUENO (1994) have examined 2 very valuable forest of *P. nigra* subsp. *salzmanii* from the same forest area of the Iberian Range, looking for morphometric and/or isoenzymatic markers capable of differentiating between these very close populations. The present work should be considered as an extension of the former study, including populations from the same and from other subspecies of *Pinus nigra*. The high value in reforestation of the 4 selected Spanish populations belonging to 2 subspecies (*salzmanii* and *nigra*), explain our interest in finding morphologic and/or genetic markers, correlated with their provenance origins, which could be used for certifying the source of seed lots. The Corsican population was included in this work to study a third subspecies (*laricio*) and with the aim to clarify relationships among the 3 taxa. Distances between selected sites ranged from about 50 km (sample 2 to 3) to about 1,000 km (samples 2 and 3 to sample 5).

Material and Methods

Populations sampled

Four Spanish and 1 Corsican populations, representing 3 subspecies of Black Pine were chosen. Seeds were collected from natural populations as a gene pool. Bulk provenance collections were analysed to characterize the populations (see *Table 1*). Seed samples 2 and 3 were represented by other seed lots than in the previous paper, but from the same provenance. The origins of the populations studied are shown in *Table 1*.

Table 1. – Geographical origin of *Pinus nigra* ARN. populations used for this study.

Sample number	Subspecies	Origin
1	*P. nigra* subsp. *salzmanii* (Dunal) Franco	Los Palancares, Cuenca (Spain)
2	*P. nigra* subsp. *salzmanii* (Dunal) Franco	Cerro Gordo, Cuenca (Spain)
3	*P. nigra* subsp. *salzmanii* (Dunal) Franco	La Losilla, Cuenca (Spain)
4	*P. nigra* Arn. subsp. *nigra*	Aoiz Mountain, Navarra (Spain)
5	*P. nigra* subsp. *laricio* (Poiret) Maire	Bergeries de Ditalza, Corsica Island (France)

Morphometric studies

Morphometric measurements were carried out on 50 seed from each population using computerized image analysis with a "Summagraphics" MM1103 image scanner, programme VIDS III. The selected parameters for the characterization by image analysis were length, width and scar of wing insertion. The weight per 10 seeds was also carried out on 50 seeds.

Isozyme analysis

Extracts for isozyme analysis were obtained individually from 40 megagametophytes per population in order to obtain reliable estimates of allelic frequencies (EL-KASSABY, 1991). The extraction method and the techniques for horizontal starch

[1]) Dpto. Biología Vegetal, E.T.S.I. Agrónomos, Universidad Politécnica, Madrid, Spain

[2]) Dpto. Genética, Facultad de Biologia, Universidad Complutense, Madrid, Spain

gel electrophoresis in conifer seeds described by CONKLE et al. (1982) and MURPHY et al. (1990) were followed. The leucine aminopeptidase (LAP - E.C. 3.4.11.1), phosphoglucomutase (PGM – E.C. 2.7.5.1), aconitase (ACO – E.C. 4.2.1.3), malate dehydrogenase (MDH – E.C. 1.1.1.37) and 6-phosphogluconate dehydrogenase (6-PGD – E.C. 1.1.1.44) isozyme systems were resolved using tris-citric acid (0.043M, pH = 7.0) as the electrode buffer and histidine-HCl (0.006M, pH = 7.0) as the gel buffer. Glutamate oxaloacetate transaminase (GOT – E.C. 2.6.1.1), esterase (EST – E.C. 3.1.1.2) and superoxide dismutase (SOD – E.C. 1.15.1.1) were evaluated using tris-citric acid (0.15M, pH = 7.75) as the gel buffer and sodium borate (0.3M, pH = 8.6) as the electrode buffer.

The genetic control of the different studied isozyme loci has been previously described in the same or similar species (EL-KASSABY, 1981; NIKOLIC and TUCIC, 1983). Thus, in the genetic analysis of the MDH isozymes, the most complex to interpret, 4 loci were observed (as in other conifer species), 2 of which (*Mdh-2* and *Mdh-3*) regularly formed heterodimeric products (AGUINAGALDE and BUENO, 1994).

The isozymes were numbered in order of decreasing mobility from the anode. The locus that specifies the isozyme with the least anodic migration was labelled as 1, the next as 2, etc. At each locus alleles of different isozymes were also designated in order of decreasing mobility from the anode.

Statistical analysis

Variance analysis of the morphometric results were conducted for each parameter studied (*Table 2*). Heterogeneity of allele frequencies among populations for each locus was tested with the χ^2 procedure (*Table 3*). Gene-diversity analysis were conducted using NEI's methods (NEI, 1973; NEI and CHAKRAVARTY, 1977). Total gene diversity (H_T), partitioned into gene diversity within populations (H_S) and among populations D_{ST}) and the relative amount of genetic differentiation among populations (G_{ST}) were studied (*Table 4*). The allelic frequencies were also used to obtain the genetic distances (GD) between populations (*Table 5*), following the method of NEI (NEI, 1972). Cluster analysis of populations based on genetic distances was conducted using the unweighted pair-group method, UPGMA, (SNEATH and SOKAL, 1973). The Numerical Taxonomy and Multivariate Analysis System (NTSYS) program by ROHLF (1990) was used to get phenetic clusters (*Fig. 2*).

[3]) As diploid genotypes were not determined in this study, observed heterozygosities could not be compared with expected values.

Results and Discussion

The average values obtained for the parameters used in the morphometric characterization of the seeds showed statistically significant differences. Seed length was the parameter that allowed to distinguish more populations. The average values of this character showed 3 statistically different groups: one of them with samples 1 and 5, another with 2 and 3 and the last one with sample 4. The data obtained showed significant differences between populations from Cerro Gordo and La Losilla (samples 2 and 3) only for wing scar character. However, in previous work, it was possible to distinguish these 2 populations using all 3 seed morphometric characters (AGUINAGALDE and BUENO, 1994). This different behavior has a possible explanation in that different seed lots were used for the present and for the former work. It would also indicate again that morphological features are very sensitive to selective pressures from the local environment.

The gametophytic tissue of conifer seeds in an ideal material to evaluate genotypes of these trees; the fact that it is haploid makes very easy to identify different isoenzymatic forms and the allele frequencies in those loci. The 8 enzymatic systems analysed in this study provided a total of 23 loci, all of them anodic axcept the *Got-1* locus which showed mobility towards the cathode. The allele frequencies obtained are indicated in *table 3*. Three genes were monomorphic (*Lap-1, Lap-2* and *Pgm-2*). In sample 5, *Pgm-1, Mdh-2, 6-Pgd-1* and *6-Pgd-2* could be consistently considered as "diagnostic loci". Each of them showed only a fixed and unique allele (*Pgm-1-4; Mdh-2-1; 6-Pgd-1-1* and *6-Pgd-2-3* respectively) that allowed to identify the Corsican population. Significant χ^2 values indicating heterogeneity ($p < 0.001$) in allele frequencies among populations were detected at the *Aco-1, Got-2, Est-3, Est-4, Est-5* and *Est-6* loci. The ACO and GOT isozyme systems also showed statistically significant differences between the Cerro Gordo and La Losilla populations in the previous paper (AGUINAGALDE and BUENO, 1994), where EST was not studied.

The percentage of polymorphic loci at 99 % (P) and values of expected heterozygosity[3]) (He) indicated much genetic diversity except in the population from Corsica (*Table 3*). Populations from Aoiz, Los Palancares and Cerro Gordo showed the same percentage of polymorphic loci (65.22 %); population from La Losilla had 69.57 %. The mean expected heterozygosity in populations 1, 2, 3, and 4 ranged from 0.28 to 0.24. These values are higher than those reported in conifers (average value He = 0.17) (GIBSON and HAMRICK, 1991; GIANNINI et al., 1991). The Corsica population (sample 5) has a very low P

Table 2. – Mean values of the morphometric analysis of 4 seed parameters in 5 *Pinus nigra* populations.

Sample number	Population origin	Parameters*			
		Length (mm)	Width (mm)	Wing scar	Weight/10 seed (g)
1	Los Palancares	7.301^{a}	$3.699^{a,b}$	4.041^{a}	$0.228^{a,b}$
2	Cerro Gordo	6.837^{b}	3.574^{b}	3.605^{b}	0.194^{b}
3	La Losilla	6.850^{b}	3.552^{b}	4.122^{a}	0.197^{b}
4	Aoiz	6.080^{c}	3.577^{b}	3.510^{b}	0.181^{b}
5	Bergeries de Ditanza	7.412^{a}	3.869^{a}	4.036^{a}	0.309^{a}

*) For each parameter, mean values with the same superscript letter are not significantly different at 1% level.

Table 3. – Allelic frequencies observed at 23 isozyme loci in 5 populations of *Pinus nigra* ARN. (P(99%)) percentage of polymorphic loci at 99% level; (He) expected heterozygosity per population and significance level of χ^2 test for the heterogeneity of allele frequencies (*** $p < 0.001$).

Loci	Alleles	Los Palancares 1	Cerro Gordo 2	La Losilla 3	Aoiz 4	Bergeries de Ditalza 5	χ^2
Lap-1	*1*	1.00	1.00	1.00	1.00	1.00	
Lap-2	*1*	1.00	1.00	1.00	1.00	1.00	
Pgm-1 &	*1*	0.00	0.02	0.00	0.00	0.00	
	2	0.52	0.41	0.50	0.55	0.00	
	3	0.48	0.57	0.50	0.45	0.00	
	4	0.00	0.00	0.00	0.00	1.00	
Pgm-2	*1*	1.00	1.00	1.00	1.00	1.00	
Aco-1	*1*	0.29	0.21	0.24	0.12	0.34	***
	2	0.66	0.58	0.55	0.44	0.50	
	3	0.05	0.21	0.21	0.44	0.16	
Mdh-1	*1*	0.97	1.00	1.00	0.97	1.00	
	2	0.03	0.00	0.00	0.03	0.00	
Mdh-2 &	*1*	0.38	0.40	0.35	0.30	1.00	
	2	0.00	0.00	0.00	0.23	0.00	
	3	0.52	0.54	0.48	0.36	0.00	
	4	0.00	0.00	0.00	0.09	0.00	
	5	0.10	0.06	0.17	0.02	0.00	
Mdh-3	*1*	0.93	0.96	0.85	0.80	1.00	
	2	0.07	0.04	0.15	0.20	0.00	
Mdh-4	*1*	1.00	0.98	0.96	0.94	1.00	
	2	0.00	0.02	0.04	0.06	0.00	
6-Pgd-1 &	*1*	0.33	0.40	0.34	0.38	1.00	
	2	0.05	0.00	0.00	0.00	0.00	
	3	0.62	0.58	0.64	0.60	0.00	
	4	0.00	0.02	0.02	0.02	0.00	
6-Pgd-2 &	*1*	0.07	0.21	0.30	0.12	0.00	
	2	0.17	0.00	0.06	0.08	0.00	
	3	0.76	0.79	0.64	0.80	1.00	
Got-1	*1*	0.59	0.56	0.53	0.52	#	
	2	0.41	0.44	0.47	0.48	#	
Got-2	*1*	0.29	0.36	0.30	0.39	1.00	***
	2	0.71	0.64	0.70	0.61	0.00	
Got-3	*1*	0.95	1.00	0.89	0.98	1.00	
	2	0.05	0.00	0.11	0.02	0.00	
Sod-1 &	*1*	1.00	1.00	1.00	0.00	0.00	
	2	0.00	0.00	0.00	1.00	1.00	
Sod-2 &	*1*	1.00	1.00	1.00	1.00	0.00	
	2	0.00	0.00	0.00	0.00	1.00	
Sod-3 &	*1*	1.00	1.00	1.00	1.00	0.00	
	2	0.00	0.00	0.00	0.00	1.00	
Est-1	*1*	0.00	0.00	0.00	0.06	#	
	2	1.00	0.87	0.85	0.69	#	
	3	0.00	0.13	0.15	0.25	#	
Est-2	*0*	0.95	0.92	0.75	1.00	1.00	
	1	0.05	0.08	0.25	0.00	0.00	
Est-3	*0*	0.59	0.42	0.56	0.44	1.00	***
	1	0.41	0.58	0.44	0.56	0.00	
Est-4	*0*	0.52	0.56	0.69	0.25	0.00	***
	1	0.48	0.44	0.31	0.75	1.00	
Est-5	*0*	0.62	0.62	0.71	1.00	1.00	***
	1	0.38	0.38	0.29	0.00	0.00	
Est-6	*0*	0.48	0.48	0.27	0.66	0.00	***
	1	0.52	0.52	0.73	0.34	1.00	
P (99%)		65.22 %	65.22%	69.57%	65.22%	4.76%	
He		0.24	0.25	0.28	0.26	0.03	

& = Diagnostic loci for at least one population. Alleles designated by 0 are null alleles.
# = These loci could not be analysed.

value (4.76 %) and the He value (0.03) is also very low, showing variability only at 1 locus (*Aco-1*), together with numerous fixed alleles. The low genetic variability of the Corsican population can be observed in the zymograms showed in *figure 1* for malate dehydrogenase (MDH) and 6-phosphogluconate dehydrogenase (6-PGD).

Table 4 indicates that total genetic diversity (H_T) averaged 0.317. The greatest amount of the genetic diversity was localized within populations (H_S = 0.219), showing a low value for D_{ST} (0.098). Although conifer species differ in the manner by which they adapt to heterogeneous environments (REHFELDT, 1991), high values of genetic variation within Anatolian Black Pine

Table 4. – Genetic diversity parameters estimated for 23 loci in 5 *Pinus nigra* populations.

Loci	H_T	H_S	D_{ST}	G_{ST}
Lap-1	0	0	0	--
Lap-2	0	0	0	--
Pgm-1	0.642	0.400	0.241	0.377
Pgm-2	0	0	0	--
Aco-1	0.599	0.570	0.297	0.484
Mdh-1	0.024	0.024	0	--
Mdh-2	0.613	0.490	0.123	0.201
Mdh-3	0.494	0.156	0.338	0.684
Mdh-4	0.048	0.046	0.002	0.042
6-Pgd-1	0.522	0.392	0.130	0.249
6-Pgd-2	0.435	0.312	0.123	0.283
Got-1	0.495*	0.492*	0.003	0.006
Got-2	0.498	0.352	0.146	0.293
Got-3	0.070	0.064	0.006	0.086
Sod-1	0	0	0	--
Sod-2	0	0	0	--
Sod-3	0	0	0	--
Est-1	0.255*	0.235*	0.020	0.078
Est-2	0.140	0.122	0.018	0.128
Est-3	0.480	0.390	0.090	0.187
Est-4	0.482	0.358	0.124	0.257
Est-5	0.332	0.270	0.062	0.187
Est-6	0.470	0.368	0.102	0.217
Mean	0.287	0.219	0.068	0.237

H_T = Total genetic diversity for the species;
H_S = Gene diversity within populations;
D_{ST} = Gene diversity among populations;
$G_{ST} = D_{ST}$ expressed as a proportion of H_T.
*) Values obtained from only 4 populations.

Table 5. – NEI's genetic distances between the 5 analysed populations of *Pinus nigra*.

Sample number		Los Palancares 1	Cerro Gordo 2	La Losilla 3	Aoiz 4	Bergeries de Ditalza 5
1	Los Palancares	0.00000				
2	Cerro Gordo	0.00712	0.00000			
3	La Losilla	0.01317	0.00954	0.00000		
4	Aoiz	0.38594	0.38320	0.40393	0.00000	
5	Bergeries de Ditalza	0.09546	0.09036	1.04580	0.29797	0.00000

populations have been attributed to adaptation mechanisms to the microenvironments (KAYA and TEMERIT, 1994). Genetic diversity among populations relative to the total genetic diversity (G_{ST}) averaged 0.309, indicating that only 30.9% of the total genetic diversity was interpopulations. Following to SCALTSOYIANNES et al., (1994) *Pinus nigra* is characterized by a high total variability due to high intrapopulation gene differences, which also means that the genetic variation of Black Pine is high in local populations and the same alleles tend to be distributed throught the whole range of this species.

The NEI's genetic distances (GD) obtained (*Table 5*) are also an estimation of interpopulation genetic variability. Samples 1, 2 and 3, (*Pinus nigra* subsp. *salzmanii*) showed a very low genetic distance, indicating proximity of these 3 Mediterranean populations that could be connected by gene flow. The genetic distance obtained between the 2 populations from La Losilla and Cerro Gordo (samples 2 and 3) can not be compared with the value obtained in the former study (AGUINAGALDE and BUENO, 1994) because more loci have been now investigated. The geographic separation between Los Palancares and Cerro Gordo is about 50 km (GD = 0.007), between Los Palancares and La Losilla 40 km (GD = 0.013) and between Cerro Gordo and La Losilla is about 45 km (GD = 0.009). These small geographic distances would explain the low values, although some degree of local differentiation at isozyme levels could be observed (*Aco-1* and *Est-2*). Local differentiation at 3 loci (*Aco-1, Est-4* and *Est-6*) was found between *Pinus nigra* subsp. *nigra* and the remaining populations. A correlation with geography seems to exist. A previous paper (NICOLIC and TUCIC, 1983) reported that population differentiation exists in Black Pine, but also that differences in allelic frequencies do not follow geographic pattern.

The phenetic dendrogram obtained with the NEI's genetic distance matrix (*Fig. 2*) confirmed the already accepted genetic relationships among the taxa. Two clearly separated branches are showed: one of them with the strongly distant (GD = 0.375) Corsican population and a second one which showed inmediatly a dichotomy (GD = 0.100) between populations from E. Spain (Cerro Gordo, La Losilla and Los Palancares) and the population from N. Spain (Aoíz). These 3 groups clearly correspond to the 3 subspecies studied: *laricio, salzmanii* and *nigra*.

The high level of polymorphic loci (P) detected in the Spanish populations of *Pinus nigra* agrees with previous work on other *Pinus nigra* populations (SCALTSOYIANNES et al., 1994), showing that genetic variation resides within populations and only a small but significant component exists among populations. On the other hand, the data obtained for the Corsican population were different, showing a very uniform population with lack of genetic variation, probably due to its geographic isolation in Corsica Island.

Summarizing, morphometric characterization using image analysis seems to be a useful tool for population differentiation. Isozyme markers are genetic markers not always able to discriminate between very close populations. For the other populations, geographically further apart, the enzyme differentiation is so high that diagnostic loci could be detected and the genetic distance discriminates between them. The high value obtained for intra-population gene diversity is only limited due to the low heterogeneity of sample 5. Curiously enough, SCALTSOYIANNES et al., (1994) found the lowest coefficient of gene diversity between populations of subspecies *larico*, $G_{ST} = 0.005$, a value which is much lower than that obtained in the same study for the whole species ($G_{ST} = 0.060$).

References

AGUINAGALDE, I. and BUENO, M. A.: Morphometric and Electrophoretic Analysis of Two Populations of European Black Pine (*Pinus nigra* ARN.). Silvae Genetica **43**: 195–199 (1994). — BONNET-MASIMBERT, M., BIKAY-BIKAY, V. and CAPELLI, P.: Variabilité intraspécifique des isozymes de la glutamate-oxalacetate-transaminase chez *Pinus nigra* ARNOLD. Intérét pour la taxonomie des sous-espéces. Silvae Genetica **27**: 71–79 (1978). — CONKLE, M. T., HODGSKISS, P. D., NUNNALLY, L. B. and HUNTER, S. C.: Starch gel electrophoresis of conifer seeds: a laboratory manual. USDA. For. Serv. Gen. Tech. Rep. PSW-64 (1982). — 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 genetic of forest trees". pp. 61–76. Eds.: FINESCHI, S., MALVOLTI, M. E., CANNATA, F. and HATTEMER, H. H. SPB Academic Publishing, The Hague, The Netherlands (1991). — GIANINI, R., MORGANTE, M. and VENDRAMIN, G. G.: Allozyme Variation in Italian Populations of *Picea abies* (L.) KARST. Silvae Genetica **40**: 160–166 (1991). — GIBSON, J. P. and HAMRICK, J. L.: Genetic diversity and structure in *Pinus pungens* (Table Mountain pine) populations. Can. J. For. Res. **21**: 635–642 (1991). — KAYA, Z. and TEMERIT, A.: Genetic Structure of Marginally Located *Pinus nigra* var *pallasiana* Populations in Central Turkey. Silvae Genetica **43**: 272–277 (1994). — MURPHY, R. W., SITES, J. W., BUTH, C. D. G. and HAUFLER, CH. H.: Proteins I: Isozyme Electrophoresis. In: "Molecular Systematics". pp. 45–126. Eds: D. M. HILLIS and C. MORITZ. C. Sinauer Associates, Sunderland, Mass., USA (1990). — NEI, M.: Genetic distance between populations. American Naturalist **106**:

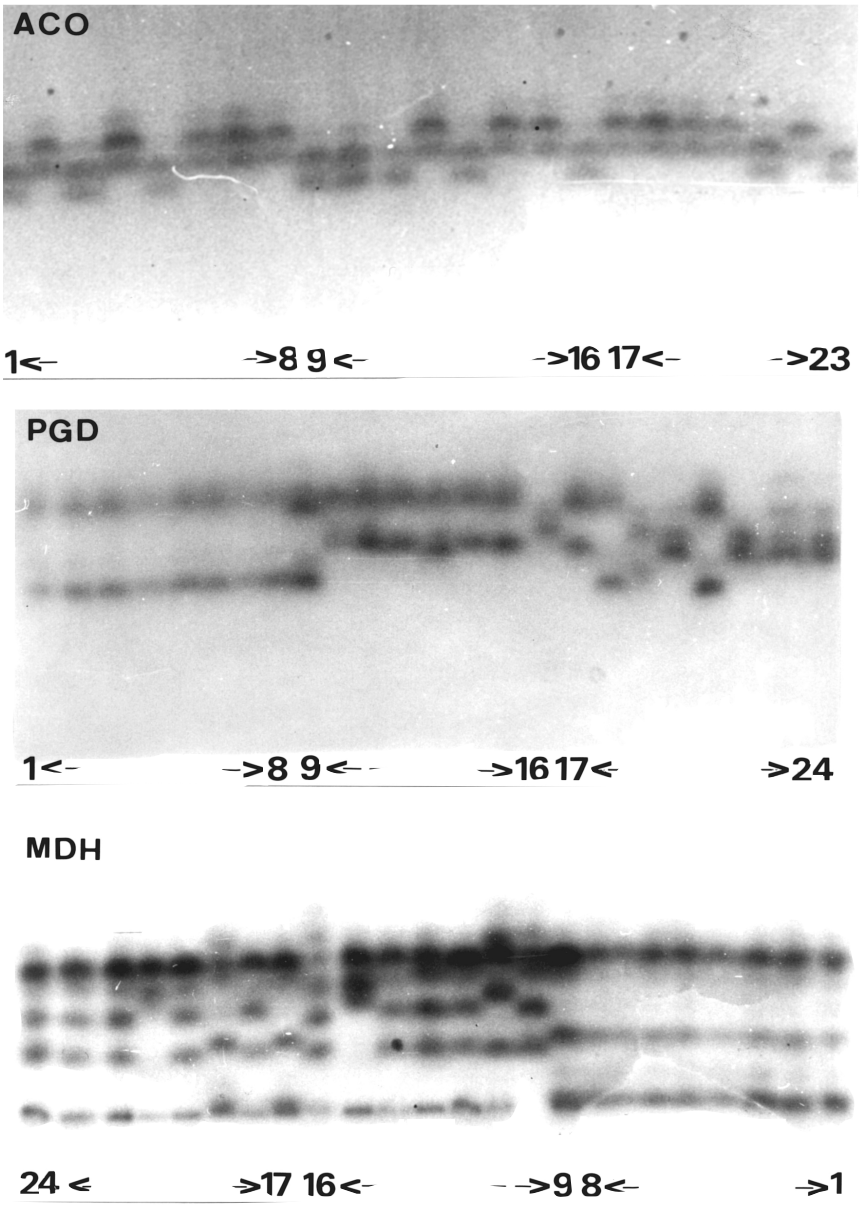

Figure 1. – Zymograms showing isozyme phenotypes of gametophytic tissue of seeds from 3 different populations. Lines 1 to 8: Bergeries de Ditalza (from Corsica Island), Lines 9 to 16: Aoiz (from Navarra, Spain) and Lines 17 to 23 or 17 to 24: Los Palancares (from Cuenca, Spain).
ACO: Aconitase, PGD: 6-phosphogluconate dehidrogenase and MDH: Malate dehidrogenase. It is possible to observe the uniformity of the 6-PGD and MDH isozyme phenotypes of Bergezies de Ditalza population from Corsica Island (Lines 1 to 8).

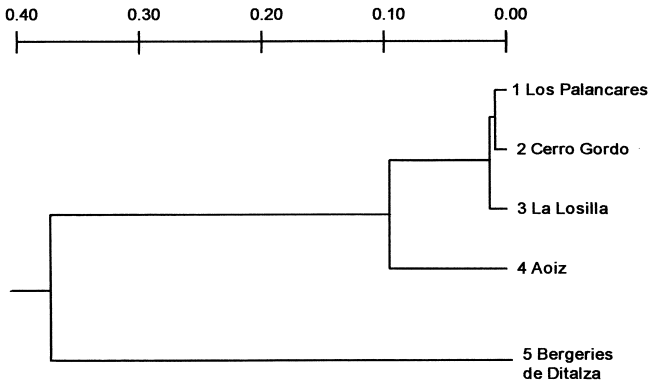

Figure 2. – Phenogram of the 5 Black Pine populations obtained using NEI's genetic distances and the UPGMA method.

283–292 (1972). — NEI, M.: Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. Wash. **70**: 3321–3323 (1973). — NEI, M. and CHAKRAVARTY, A.: Drift variances of F_{ST} and G_{ST} statistics obtained from a finite number of isolated populations. Theor. Pop. Biol. **11**: 307–325 (1977). — NIKOLIC, D. and TUCIC, N.: Isoenzyme Variation within and among Populations of European Black Pine (*Pinus nigra* ARNOLD). Silvae Genetica **32**:80–89 (1983). — REHFELD, G. E.: Gene Resource Managements: Using Models of Genetic Variation in Silviculture. USDA-Forest Service, Genetic/Silviculture Workshop, Wenatchee, WA, USA. pp. 31–44 (1991). — ROHLF, J.: NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Sept. Ecol. Evol., New York (1990). — SCALTSOYIANNES, A., ROHR, R., PANETSOS, K. P. and TSAKTSIRA, M.: Allozyme Frequency Distribution in Five European Populations of Black Pine (*Pinus nigra* ARNOLD). Silvae Genetica **43**: 20–30 (1994). — SNEATH, P. H. A. and SOKAL, R. R.: Numerical taxonomy. In: Freeman W. H. & Co., San Francisco (1973). — TUTIN, T. G., HEYWOOD, V. H., BURGES, N. A., VALENTINE, D. H., WALTERS, S. M. and WEBB, D. A.: Eds. Flora Europea. Volume I. pp. 33–34. Cambridge University Press (1964).