ALLOZYME VARIATION OF THE EUROPEAN BLACK (*Pinus nigra* ARNOLD) AND SCOTS PINE (*Pinus sylvestris* L.) POPULATIONS AND THE IMPLICATIONS ON THEIR EVOLUTION: A COMPARATIVE STUDY

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Abstract. *A comparative study of the type, magnitude and pattern of variation among thirteen Black pine (Pinus nigra Arn) and fourteen Scots pine (Pinus sylvestris L.) European populations were conducted by using isozyme gene markers. The evaluation of genetic diversity parameters (A/L, Ae, P%, He) for the two species indicated that both Black and Scots pine are characterized by high levels of variation. Between the two species, the genetic differentiation coefficient (Gst) and genetic distances were higher among the Black pine populations. For Scots pine populations, apart that of the Iberian Peninsula which seems to retain a Tertiary gene pool, genetic distances were low, even among populations of great geographical distance (Sweden-Balkan countries). The Principal Coordinate analysis and cluster analysis applied, also confirm the different genetic variation pattern of the two species. The above results can be well interpreted by the different evolutionary course of the two pine species, especially during the post-glacial period.*

Keywords: *variation pattern, genetic distances, evolution, Pinus nigra, Pinus sylvestris.*

Rezumat. Variaţia alozimelor de la populaţiile de pin negru european (*Pinus nigra* ARNOLD) şi pin scoţian (*Pinus sylvestris* L.) şi implicaţiile lor asupra evoluţiei: un studiu comparativ. *Un studiu comparativ al tipului, magnitudinii şi modul variaţiei între treisprezece populaţii de pin negru european (Pinus nigra ARN.) şi paisprezece populaţii de pin scoţian (Pinus sylvestris L.), a fost realizat prin utilizarea izozimelor genelor marker. Evaluarea parametrilor diversităţii genetice (A/L, Ae, P%, He) pentru cele două specii, au indicat că atât pinul negru cât şi pinul scoţian sunt caracterizate prin înalte nivele de variaţie. Între cele două specii, coeficientul de diferenţiere genetică (Gst) şi distanţele genetice au fost mai mari între populaţiile de pin negru. În cazul populaţiilor de pin scoţian, se pare că Peninsula Iberică, unde s-ar fi păstrat genofondul existent în Terţiar, distanţele genetice au fost mai mici, chiar între populaţiile situate la mari distanţe geografice (ţările din Peninsula Scandinavia şi din Balcani). Utilizarea analizei Coordinative Principale şi analiza clusterelor, a confirmat de asemenea modelul de variaţie genetică diferită a celor două specii. Rezultatele de mai sus, pot interpreta în mod corect diferite scheme ale evoluţiei celor două specii, în special în timpul perioadei post-glaciare.*

Cuvinte cheie: *modele de variaţie, distanţe genetice, evoluţie, Pinus nigra, Pinus sylvestris*.

INTRODUCTION

Pinus is considered one of the oldest genera of the plant kingdom. Today, approximately 100 species belong in genus *Pinus*, the largest number of species than any other gymnosperm mostly expanding to the Northern hemisphere (MIROV 1967, VIDAKOVIC 1991). In Europe, there are 11 pine species (LITTLE and CRITCHFIELD, 1969). Black (*Pinus nigra* ARN.) and Scots pine (*Pinus sylvestris* L.) first appeared during the Jurassic period (135-190 million years ago) (KLAUS 1989, MILLAR 1993). From a taxonomic point of view, both Scots and Black pine belong to the same subsection: *Sylvestres* (MIROV 1967, VIDAKOVIC 1991).

European Black pine was defined as a species in 1957 when Rohrig suggested the name *Pinus nigra*, previously given by Arnold, in 1785. The species covers a discontinuous area of 2.300.000 ha in 13 countries, ranging in 48 longitudinal (from the 6º west of Spain to the 42º east of Turkey) and 13 latitudinal degrees (from the 35º to the 48º to the North). It is considered a widely expanding species and can be found from the altitude of 1800 m to the sea level. It occurs in Southern Europe, Cyprus, Minor Asia, Crimea and Northwest Africa (CRITCHFIELD and LITTLE 1966). In Greece, Black pine grows mainly on the mountains of the continental country and in the islands of Thasos, Samos, Lesvos and Evia.

Black pine is a relic of the Tertiary period and during that geological period had a greater expansion. It is considered that the species originated from South-east Asia. In the Pliocene epoch, black pine was found at the Balkan Peninsula at the very same sites where is currently located (MIROV 1967). Even today, there is a controversy concerning the taxonomy of the species because of its wide and discontinuous expansion, long isolation of the populations and locations (e.g. island populations, North Africa, Austria, Crimea). Another reason is the existence of intermediate forms between the subspecies, a fact that renders difficult their discrimination.

Scots pine is one of the conifers with the largest geographic expansion in Northern Asia, Central and Northern Europe, situated within an area of 135 longitudinal and 30 latitudinal degrees. In Balkan Peninsula, the southernmost expansion point of the species is North Greece (VIDAKOVIC 1991). According to many researchers, in Central and Eastern Europe the gene pools of *Pinus sylvestris* were formed in post-glacial times during the migration of the species from the glacial refugia and intensive gene exchange between populations. On the contrary, Scots pine from the Iberian Peninsula probably did not take part in the colonization of Europe after the last glaciation and represents original

ancient Tertiary gene pool (MIROV 1967, HUNTLEY and BIRKS 1983, BENNETT et al. 1991, PRUS-GLOWACKI et al. 2003).

In Europe both species were studied extensively, regarding the magnitude and type of their variation by means of morphological, anatomical, growth and biochemical characteristics (KINLOCH et al. 1986, PRUS-GLOWACKI and STEPHAN 1994, SCALTSOYIANNES et al. 1994, GERBER et al. 1995, RAFII et al. 1996, AGUINAGALDE et al. 1997).

Although Black and Scots pine belong taxonomically to the same subdivision, complete comparative studies at the level of population genetic analysis are limited and are, usually, based on morphological and anatomical characteristics, particularly of needles and cones. Also, phylogenetic relations of the two species, resulting from crossing experiments were conducted (VIDAKOVIC 1974, MOULALIS and MITSOPOULOS 1975, MOULALIS et al. 1976, VIDAKOVIC 1991).

A recent comparative study (PASAGIANNIS et al. 2000) referring to the allozyme analysis on Balkan populations, indicated that Black and Scots pine had different evolutionary courses, especially during the post-glacial period on Balkan peninsula.

The aim of the present work was to perform a comparative analysis of allozyme variation of Black and Scots pine natural European populations, in order to reveal some trends of the post-glacial evolution of the two species in Europe.

MATERIALS AND METHODS

The plant material used for analyses, consisted of endosperms of germinated seeds derived from 14 Scots pine and 13 Black pine natural populations from the entire expansion area of the two species in Europe (Tables 1a, 1b). The seeds were sampled from at least 30 trees per population and used as bulk samples.

Table 1a. Origin of 14 analyzed Scots pine populations.
Tabel 1a. Originea celor 14 populații analizate de pin scoțian.

	Abbreviation	Provenance	Country
1	**XAN**	Xanthi (North Greece)	Greece
2	**TRA**	Trachoniou Dipotamou (Drama) (North Gre)	Greece
3	**LEY**	Leukogeia (Drama) (North Greece)	Greece
4	**FLO**	Florina (North Greece)	Greece
5	**ALM**	Almopia (North Greece)	Greece
6	**ELA**	Elatia (Drama) (North Greece)	Greece
7	**LAI**	Lailias (Serres) (North Greece)	Greece
8	**PER**	Kato Neurokopi (Drama) (North Greece)	Greece
9	**JUN**	Jundola	Bulgaria
10	**MAC**	Mala Krusa	FYROM
11	**BER**	Berovo	FYROM
12	**DEV**	Devin	Bulgaria
13	**SWE**	North Sweden	Sweden
14	**SPA**	Spain	Spain

Table 1b. Origin of 14 analyzed Black pine populations.
Tabel 1b. Originea celor 14 populații analizate de pin negru.

	Abbreviation	Provenance	Country
1	**FRA**	Gagneres	France
2	**SPA**	Algarbe	Spain
3	**CAL**	Calabria	Italy
4	**COR**	Pozzo di Najja	Corsica
5	**AUS**	Alpes Seches	Austria
6	**BUL**	Kustendil	Bulgaria
7	**YUG**	Yugoslavia	Yugoslavia
8	**ROD**	Petrota (North Greece)	Greece
9	**THAS**	Thasos (Island)	Greece
10	**MET**	Kalambaka (Central Greece)	Greece
11	**SAM**	Samos (Island)	Greece
12	**MIT**	Mitilini Island)	Greece
13	**KAL**	Kalamata (South Greece)	Greece

The isozyme analysis was conducted on the haploid endosperms of germinated seeds (at least 80 endosperms per population) by using the technique of horizontal starch gel electrophoresis, following the protocols of CONKLE et al. (1982) and CHELIAK and PITEL (1984). After seed germination (root length 3-4 mm), their endosperms were homogenized by using the extraction buffer of CONKLE et al. (1982). Both species were analyzed for the following 9 enzyme systems: Acid phosphatase (**ACP;** EC3.1.3.2), Glutamic dehydrogenase (**GDH;** EC1.4.1.2), Isocitric dehydrogenase (**IDH;** EC1.1.1.42), Leucine aminopeptidase (**LAP;**EC3.4.11.1), Malate dehydrogenese (**MDH;**

EC1.1.1.37), Menadione reductase (**MNR;** EC1.6.99.2), 6-Phosphogluconate dehydrogenase (**6PGD;** EC1.1.1.44), Phosphoglucose isomerase (**PGI;** EC5.3.1.9) and Phosphoglucomutase (**PGM;** EC5.4.2.2).

By the method of Nei (1973, 1978) the following population-genetic parameters of variation were estimated: mean number of alleles per locus (**A/L**), effective allele number (**Ae**), percentage of polymorphic loci (**P**), expected heterozygosity per population (**He**), the differentiation coefficient (**Gst**), and the genetic distances (**D**). The dendrogram based on the **UPGMA** method and the Principal Coordinate analysis (**PcoA**) were performed on the values of genetic distances.

RESULTS

Isozymes of nine enzyme systems were resolved with consistency and clarity and thus, fourteen loci were recorded, coded by 53 alleles for Scots pine and 61 for Black pine (Table 2a, 2b).

Table 2a. Allele frequencies at 14 gene loci in the 14 studied populations of Scots pine.
Tabel 2a. Frecvența alelelor locilor celor 14 gene, în cele 14 populații studiate de pin scoțian.

Provenances / Gene loci & Alleles		XAN	TRA	LEY	FLO	ALM	ELA	LAI	PER	JUN	MAC	BER	DEV	SWE	SPA
PGI-B	PGI-B1	0,04	0,00	0,00	0,00	0,00	0,06	0,02	0,12	0,04	0,00	0,04	0,08	0,06	0,04
	PGI-B2	0,95	1,00	0,88	1,00	0,98	0,91	0,98	0,85	0,91	1,00	0,96	0,86	0,94	0,96
	PGI-B3	0,01	0,00	0,12	0,00	0,02	0,03	0,00	0,03	0,05	0,00	0,00	0,06	0,00	0,00
IDH-A	IDH-A1	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00
PGM-A	PGM-A1	0,02	0,05	0,06	0,05	0,02	0,06	0,00	0,07	0,10	0,00	0,10	0,01	0,14	0,00
	PGM-A2	0,90	0,91	0,91	0,91	0,93	0,89	1,00	0,90	0,86	0,91	0,85	0,88	0,86	1,00
	PGM-A3	0,08	0,02	0,00	0,02	0,05	0,05	0,00	0,00	0,04	0,09	0,05	0,10	0,00	0,00
	PGM-A4	0,00	0,02	0,03	0,02	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,01	0,00	0,00
MNR-A	MNR-A1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	MNR-A2	0,53	0,81	0,65	0,81	0,65	0,64	0,57	0,65	0,60	0,71	0,68	0,45	0,70	0,82
	MNR-A3	0,04	0,00	0,00	0,00	0,02	0,01	0,03	0,05	0,04	0,03	0,07	0,04	0,05	0,10
	MNR-A4	0,18	0,00	0,10	0,00	0,18	0,04	0,14	0,00	0,10	0,00	0,06	0,08	0,05	0,00
	MNR-A5	0,24	0,19	0,25	0,19	0,15	0,31	0,26	0,30	0,26	0,26	0,19	0,41	0,20	0,04
	MNR-A6	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,04
ACP-A	ACP-A1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,04
	ACP-A2	0,89	0,75	0,85	0,75	0,83	0,89	0,93	0,83	0,81	0,96	0,71	0,87	0,88	0,92
	ACP-A3	0,03	0,25	0,03	0,25	0,00	0,03	0,00	0,02	0,05	0,00	0,11	0,08	0,02	0,04
	ACP-A4	0,08	0,00	0,12	0,00	0,17	0,08	0,07	0,15	0,14	0,04	0,18	0,05	0,10	0,00
LAP-A	LAP-A1	0,94	0,96	1,00	0,96	0,90	0,98	0,94	0,99	0,99	1,00	0,98	0,95	0,98	1,00
	LAP-A2	0,06	0,04	0,00	0,04	0,10	0,02	0,06	0,01	0,01	0,00	0,02	0,05	0,02	0,00
LAP-B	LAP-B1	0,05	0,03	0,00	0,04	0,00	0,02	0,07	0,00	0,01	0,00	0,05	0,00	0,00	0,00
	LAP-B2	0,95	0,94	1,00	0,95	0,95	0,98	0,92	1,00	0,99	0,95	0,91	0,99	0,99	1,00
	LAP-B3	0,00	0,03	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,05	0,01	0,01	0,01	0,00
	LAP-B4	0,00	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00
GDH-A	GDH-A1	0,18	0,19	0,09	0,13	0,10	0,29	0,33	0,15	0,24	0,32	0,25	0,27	0,36	0,15
	GDH-A2	0,82	0,81	0,91	0,87	0,90	0,71	0,63	0,85	0,75	0,68	0,75	0,73	0,54	0,85
	GDH-A3	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,00	0,01	0,00	0,00	0,00	0,10	0,00
MDH-A	MDH-A1	0,07	0,01	0,00	0,07	0,05	0,07	0,01	0,02	0,01	0,02	0,02	0,02	0,06	0,02
	MDH-A2	0,93	0,99	1,00	0,93	0,84	0,93	0,94	0,98	0,99	0,98	0,98	0,98	0,94	0,98
	MDH-A3	0,00	0,00	0,00	0,00	0,11	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	MDH-A4	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
MDH-B	MDH-B1	0,00	0,03	0,02	0,00	0,00	0,00	0,02	0,00	0,02	0,00	0,02	0,03	0,00	0,00
	MDH-B2	1,00	0,89	0,96	1,00	1,00	1,00	0,98	0,98	0,96	1,00	0,94	0,93	1,00	0,96
	MDH-B3	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,02	0,00	0,04
	MDH-B4	0,00	0,08	0,02	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,04	0,02	0,00	0,00
MDH-C	MDH-C1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00
	MDH-C2	0,00	0,02	0,02	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00
	MDH-C3	0,73	0,76	0,55	0,69	0,65	0,72	0,83	0,85	0,73	0,89	0,66	0,76	0,43	0,67
	MDH-C4	0,26	0,16	0,38	0,31	0,35	0,25	0,17	0,15	0,23	0,07	0,34	0,22	0,57	0,33
	MDH-C5	0,01	0,06	0,05	0,00	0,00	0,03	0,00	0,00	0,04	0,04	0,00	0,00	0,00	0,00
MDH-D	MDH-D1	0,26	0,08	0,17	0,11	0,12	0,20	0,08	0,17	0,09	0,04	0,17	0,07	0,08	0,10
	MDH-D2	0,28	0,33	0,37	0,32	0,27	0,19	0,30	0,40	0,26	0,43	0,34	0,51	0,10	0,34
	MDH-D3	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,16	0,00	0,00	0,00	0,00
	MDH-D4	0,01	0,11	0,00	0,00	0,09	0,05	0,05	0,00	0,00	0,00	0,00	0,07	0,09	0,00
	MDH-D5	0,45	0,48	0,46	0,57	0,52	0,56	0,57	0,43	0,65	0,37	0,49	0,35	0,73	0,56
PGD-A	6PGD-A1	0,04	0,04	0,01	0,02	0,00	0,00	0,05	0,00	0,00	0,01	0,05	0,03	0,02	0,02
	6PGD-A2	0,49	0,48	0,70	0,60	0,46	0,74	0,59	0,55	0,62	0,50	0,50	0,64	0,34	0,30
	6PGD-A3	0,11	0,00	0,00	0,02	0,00	0,04	0,09	0,00	0,04	0,05	0,02	0,00	0,02	0,00
	6PGD-A4	0,36	0,48	0,29	0,31	0,54	0,22	0,27	0,45	0,34	0,44	0,40	0,31	0,62	0,66
	6PGD-A5	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,02	0,00	0,02
PGD-B	6PGD-B1	0,85	0,82	0,90	0,72	0,84	0,98	0,89	0,80	0,95	0,89	0,72	0,78	0,86	0,57
	6PGD-B2	0,15	0,18	0,10	0,28	0,16	0,02	0,09	0,20	0,05	0,11	0,26	0,22	0,14	0,33
	6PGD-B3	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,02	0,00	0,00	0,00

Table 2b. Allele frequencies of 14 gene loci in the 13 studied populations of Black pine.
Tabel 2b. Frecvența alelelor locilor celor 14 gene, în cele 13 populații studiate de pin negru.

Provenances / Allele frequencies		FRA	SPA	CAL	COR	AUS	BUL	YUG	ROD	THAS	MET	SAM	MIT	KAL
PGI-B	PGI-B1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00
	PGI-B2	0,15	0,20	0,02	0,04	0,02	0,00	0,04	0,05	0,04	0,00	0,00	0,00	0,09
	PGI-B3	0,00	0,03	0,01	0,04	0,02	0,00	0,00	0,01	0,04	0,00	0,00	0,04	0,02
	PGI-B4	0,31	0,15	0,24	0,06	0,33	0,33	0,24	0,25	0,37	0,37	0,20	0,20	0,18
	PGI-B5	0,54	0,17	0,54	0,58	0,50	0,36	0,39	0,41	0,43	0,38	0,47	0,51	0,28
	PGI-B6	0,00	0,01	0,00	0,02	0,00	0,05	0,00	0,00	0,06	0,04	0,02	0,00	0,02
	PGI-B7	0,00	0,44	0,19	0,25	0,13	0,26	0,33	0,28	0,03	0,21	0,31	0,25	0,41
	PGI-B8	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
IDH-A	IDH-A1	1,00	1,00	1,00	0,92	1,00	1,00	1,00	0,94	1,00	1,00	1,00	1,00	1,00
	IDH-A2	0,00	0,00	0,00	0,08	0,00	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00
PGM-A	PGM-A1	0,00	0,00	0,00	0,00	0,00	0,08	0,04	0,00	0,08	0,00	0,00	0,03	0,00
	PGM-A2	1,00	1,00	1,00	1,00	1,00	0,92	0,96	0,97	0,92	1,00	1,00	0,97	1,00
	PGM-A3	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00	0,00
MNR-A	MNR-A1	0,00	0,00	0,16	0,00	0,55	0,42	0,47	0,53	0,36	0,66	0,45	0,23	0,34
	MNR-A2	0,95	1,00	0,84	1,00	0,41	0,53	0,48	0,47	0,54	0,30	0,49	0,77	0,36
	MNR-An	0,05	0,00	0,00	0,00	0,04	0,05	0,05	0,00	0,10	0,04	0,06	0,00	0,30
ACP-A	ACP-A1	0,02	0,00	0,17	0,10	0,02	0,04	0,00	0,00	0,00	0,04	0,09	0,00	0,00
	ACP-A2	0,94	1,00	0,69	0,72	0,94	0,92	0,94	0,98	1,00	0,90	0,90	0,99	1,00
	ACP-A3	0,02	0,00	0,08	0,14	0,04	0,00	0,04	0,00	0,00	0,01	0,00	0,00	0,00
	ACP-An	0,02	0,00	0,06	0,04	0,00	0,04	0,02	0,02	0,00	0,05	0,01	0,01	0,00
LAP-A	LAP-A1	0,94	0,90	1,00	0,99	0,96	0,99	0,99	0,98	1,00	0,97	1,00	0,93	0,98
	LAP-An	0,06	0,10	0,00	0,01	0,04	0,01	0,01	0,02	0,00	0,03	0,00	0,07	0,02
LAP-B	LAP-B1	0,00	0,04	0,00	0,00	0,01	0,00	0,01	0,00	0,02	0,03	0,00	0,03	0,00
	LAP-B2	0,89	0,90	0,98	1,00	0,99	0,97	0,97	0,95	0,97	0,89	1,00	0,92	1,00
	LAP-B3	0,11	0,06	0,02	0,00	0,00	0,03	0,02	0,05	0,01	0,08	0,00	0,05	0,00
GDH-A	GDH-A1	0,00	0,00	0,02	0,03	0,00	0,00	0,03	0,00	0,04	0,00	0,00	0,00	0,00
	GDH-A2	1,00	1,00	0,98	0,97	1,00	0,99	0,96	1,00	0,96	0,91	1,00	0,99	1,00
	GDH-A3	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,00	0,00	0,09	0,00	0,01	0,00
MDH-A	MDH-A1	0,00	0,00	0,00	0,01	0,01	0,01	0,07	0,00	0,00	0,16	0,00	0,00	0,00
	MDH-A2	1,00	1,00	1,00	0,99	0,99	0,97	0,93	1,00	1,00	0,84	1,00	1,00	1,00
	MDH-A3	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00
MDH-B	MDH-B1	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,00
	MDH-B2	0,00	0,00	0,02	0,00	0,02	0,02	0,00	0,00	0,01	0,00	0,00	0,00	0,00
	MDH-B3	0,15	0,08	0,31	0,13	0,48	0,49	0,35	0,45	0,34	0,43	0,29	0,36	0,06
	MDH-B4	0,00	0,01	0,00	0,01	0,02	0,01	0,00	0,01	0,00	0,02	0,00	0,01	0,19
	MDH-B5	0,85	0,91	0,67	0,81	0,48	0,46	0,65	0,45	0,65	0,54	0,59	0,51	0,75
	MDH-B6	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,04	0,00	0,01	0,12	0,08	0,00
	MDH-B7	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00
MDH-C	MDH-C1	0,04	0,01	0,00	0,01	0,02	0,01	0,06	0,00	0,00	0,00	0,00	0,00	0,00
	MDH-C2	0,01	0,03	0,05	0,00	0,01	0,00	0,00	0,01	0,00	0,00	0,00	0,01	0,60
	MDH-C3	0,64	0,56	0,68	0,83	0,46	0,38	0,35	0,49	0,26	0,31	0,29	0,34	0,44
	MDH-C4	0,00	0,00	0,04	0,00	0,27	0,01	0,00	0,00	0,00	0,00	0,05	0,04	0,00
	MDH-C5	0,00	0,23	0,02	0,00	0,00	0,02	0,00	0,01	0,01	0,02	0,00	0,01	0,19
	MDH-C6	0,31	0,17	0,20	0,16	0,23	0,54	0,59	0,49	0,73	0,67	0,66	0,60	0,31
	MDH-C7	0,00	0,00	0,01	0,00	0,01	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00
MDH-D	MDH-D1	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	MDH-D2	1,00	1,00	1,00	0,99	0,94	1,00	1,00	1,00	1,00	0,83	0,84	0,93	0,92
	MDH-D3	0,00	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,17	0,16	0,07	0,08
	MDH-Dn	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
PGD-A	6PGD-A1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,15	0,03	0,20	0,05	0,07	0,04
	6PGD-A2	0,00	0,00	0,00	0,00	0,01	0,13	0,05	0,08	0,27	0,08	0,01	0,01	0,05
	6PGD-A3	0,60	0,74	0,60	0,48	0,81	0,69	0,74	0,69	0,65	0,57	0,60	0,81	0,72
	6PGD-A4	0,06	0,18	0,18	0,09	0,11	0,02	0,06	0,02	0,03	0,09	0,02	0,04	0,04
	6PGD-A5	0,34	0,08	0,22	0,43	0,06	0,16	0,15	0,06	0,02	0,06	0,32	0,07	0,15
PGD-B	6PGD-B1	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00
	6PGD-B2	0,66	0,73	0,64	0,58	0,78	0,52	0,61	0,65	0,42	0,54	0,28	0,46	0,79
	6PGD-B3	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	6PGD-B4	0,00	0,00	0,03	0,00	0,00	0,05	0,01	0,00	0,03	0,00	0,06	0,03	0,21
	6PGD-B5	0,19	0,27	0,27	0,42	0,16	0,41	0,35	0,34	0,55	0,46	0,55	0,43	0,00
	6PGD-B6	0,09	0,00	0,06	0,00	0,01	0,02	0,02	0,01	0,00	0,00	0,11	0,08	0,00
	6PGD-B7	0,00	0,00	0,00	0,00	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

All the loci tested were polymorphic for both species, except of the IDH enzyme system, which was monomorphic for Scots pine. The increased allele number, found in the present work, is attributed to the presence of rare alleles (alleles with frequencies less than 0.05). Concerning the unique alleles (appearing in only one population and in frequency greater than 0.1), they were detected mainly in Black pine and particularly in the Balkan populations (MNR-An in the KAL population with a frequency of 30%, MDH-B4 in the same population with 19% frequency, MDH-A1 in the population MET with 16% frequency).

Table 3a. Polymorphism parameters estimated for the 14 studied populations of *Pinus sylvestris*.
Tabel 3a. Parametrii estimați ai polimorfismului pentru cele 14 populații studiate de *Pinus sylvestris*.

Populations	A/L	Ae*	P%	He
XAN	2,64	1,35	85,71	0,263
TRA	2,50	1,33	85,71	0,247
LEY	2,29	1,29	71,43	0,228
FLO	2,29	1,31	71,43	0,235
ALM	2,29	1,34	85,71	0,256
ELA	2,50	1,29	85,71	0,226
LAI	2,64	1,31	85,71	0,237
PER	2,21	1,3	85,71	0,235
JUN	2,64	1,31	92,86	0,239
MAC	2,14	1,25	71,43	0,206
BER	2,79	1,41	92,86	0,294
DEV	2,93	1,38	92,86	0,275
SWE	2,79	1,33	92,86	0,260
SPA	2,14	1,27	71,43	0,213
Mean	**2,48**	**1,32**	**83,67**	**0,244**

* The monomorphic locus IDH included

Table 3b. Polymorphism parameters estimated for the 13 studied populations of *Pinus nigra*.
Tabel 3b. Parametrii estimați ai polimorfismului pentru cele 13 populații studiate de *Pinus nigra*.

Populations	A/L	Ae	P%	He
FRA	2,21	1,26	64,29	0,208
SPA	2,21	1,23	50,00	0,191
CAL	2,57	1,33	64,29	0,249
COR	2,57	1,27	78,57	0,215
AUS	2,93	1,32	78,57	0,244
BUL	3,00	1,38	85,71	0,276
YUG	2,64	1,35	78,57	0,260
ROD	2,57	1,35	64,29	0,258
THAS	2,71	1,32	85,71	0,245
MET	2,71	1,46	85,71	0,316
SAM	2,86	1,37	85,71	0,274
MIT	2,36	1,34	57,14	0,255
KAL	2,36	1,28	57,14	0,246
Mean number	**2,59**	**1,33**	**71,98**	**0,249**

The four parameters of population genetic diversity (A/L, Ae, P, He) for each species are presented in tables 3a, 3b. Black and Scots pine showed on average, a high diversity rate, which, compared to that of other conifers, justifies well why the two species belong to the most polymorphic conifers. Mean heterozygosity was 0,244 and 0,249 for Scots and Black pine, respectivelty. For Scots pine, the Berovo population had the highest value (0,294), while that of Mala Krusa, the lowest (0,206). As for Black pine, the highest heterozygosity value (0,316) was observed at the Greek population of Kalambaka (Central Greece) and the lowest one (0,191) at the population of Spain. The other three parameters (A/L, Ae, P) followed similar trends for both species, reaching their highest and lowest values at the same populations. It is worth mentioning the fact that the populations of Spain showed relatively low diversity rates (He: 0,213 for Scots pine and 0,191 for Black pine), probably due to their isolation and consequently to the low gene flow while, on the contrary, the populations of the Balkan Peninsula had higher values.

The differentiation coefficients Gst for each species are presented in table 4. Between the two, it was found that Black pine had at least twice higher Gst than Scots pine (8,8% and 3,8%, respectively). The above is considered an unexpected result, firstly, because Scots pine has a multiple geographic expansion compared to that of Black pine and thus, a greater differentiation among its populations would be more plausible.

The genetic distances shown in Tables 5a and 5b for both species, also, confirm the larger genetic differentiation among the populations of Black pine. Concerning Scots pine populations, table 5a shows that the largest genetic distances were observed among the Iberian and the other Scots pine populations, fact which indicates that the particular peninsula represents original ancient Tertiary gene pools. The above is well explained by the discontinuity and existing high altitude of the Pyrenees contributing to this isolation. Similar observations were recorded for the Black pine populations. In particular, the Spanish and Corsican populations exhibited the largest genetic distances from the majority of the other studied populations (table 5b).

Table 4. Comparative table of the genetic differentiation coefficient Gst per gene locus on the total of *Pinus sylvestris* and *Pinus nigra* populations.
Tabel 4. Tabel comparativ al coeficientului de diferențiere genetică Gst per locus gena, pe totalul populațiilor de *Pinus sylvestris* și *Pinus nigra.*

	Pinus sylvestris	***Pinus nigra***
Gene loci	**Gst**	**Gst**
PGI-B	0,043	0,057
IDH-A	0,000	0,048
PGM-A	0,029	0,050
MNR-A	0,044	0,220
ACP-A	0,050	0,089
LAP-A	0,038	0,036
LAP-B	0,027	0,035
GDH-A	0,043	0,054
MDH-A	0,036	0,095
MDH-B	0,033	0,094
MDH-C	0,037	0,134
MDH-D	0,034	0,098
PGD-A	0,049	0,069
PGD-B	0,062	0,148
Mean	**0,038**	**0,088**

Table 5a. Genetic distances (NEI, 1978) among the 14 studied Scots pine populations.
Tabel 5a. Distanțele genetice (NEI, 1978) între cele 14 populații studiate de pin scotian.

Population	**1. XAN**	**2. TRA**	**3. LEY**	**4. FLO**	**5. ALM**	**6. ELA**	**7. LAI**	**8. PER**	**9. JUN**	**10. MAC**	**11. BER**	**12. DEV**	**13. SWE**	**14. SPA**
1. XAN	*	.013	.008	.012	.006	.008	.007	.006	.006	.011	.007	.010	.013	.020
2. TRA		*	.016	.010	.013	.018	.016	.009	.012	.010	.007	.018	.011	.014
3. LEY			*	.009	.011	.009	.017	.010	.007	.021	.010	.013	.020	.028
4. FLO				*	.009	.016	.017	.009	.011	.019	.003	.021	.008	.016
5. ALM					*	.018	.016	.012	.011	.019	.009	.022	.013	.013
6. ELA						*	.006	.013	.003	.014	.014	.014	.015	.039
7. LAI							*	.012	.005	.007	.013	.011	.012	.030
8. PER								*	.008	.007	.007	.007	.012	.018
9. JUN									*	.012	.008	.012	.008	.028
10. MAC										*	.014	.010	.016	.024
11. BER											*	.013	.005	.012
12. DEV												*	.022	.033
13. SWE													*	.012
14. SPA														*

Table 5b. Genetic distances (NEI, 1978) among the 13 studied populations of Black pine.
Table 5b. Distanțele genetice (NEI, 1978) între cele 13 populații studiate de pin negru.

Population	**1. FRA**	**2. SPA**	**3. CAL**	**4. COR**	**5. AUS**	**6. BUL**	**7. YUG**	**8. ROD**	**9. THAS**	**10. MET**	**11. SAM**	**12. MIT**	**13. KAL**
1. FRA	*	.025	.013	.017	.053	.054	.050	.057	.063	.091	.068	.041	.051
2. SPA		*	.029	.034	.067	.069	.057	.067	.087	.110	.088	.052	.041
3. CAL			*	.012	.033	.041	.040	.041	.064	.076	.057	.037	.047
4. COR				*	.077	.080	.075	.080	.103	.127	.085	.066	.072
5. AUS					*	.026	.024	.018	.051	.040	.053	.037	.035
6. BUL						*	.004	.004	.010	.015	.014	.010	.033
7. YUG							*	.006	.014	.014	.014	.012	.021
8. ROD								*	.020	.014	.023	.015	.028
9. THAS									*	.020	.019	.016	.047
10. MET										*	.020	.031	.045
11. SAM											*	.016	.043
12. MIT												*	.040
13. KAL													*

On the other hand, the Scots pine populations with the highest affinity (D=0.003) were those of JUN (BUL) and ELA (Northern Greece), and BER (FYROM) and FLO (Northern Greece). Another remarkable observation is the relatively low genetic distances among populations of distant geographical regions such as Sweden and Balkan countries. As for Black pine populations, the lowest genetic distances (D=0.004) were observed between the Bulgarian (BUL) and the Jugoslavian (JUG) populations, as well as, between the ROD (Northern Greece) and the Bulgarian (BUL) ones.

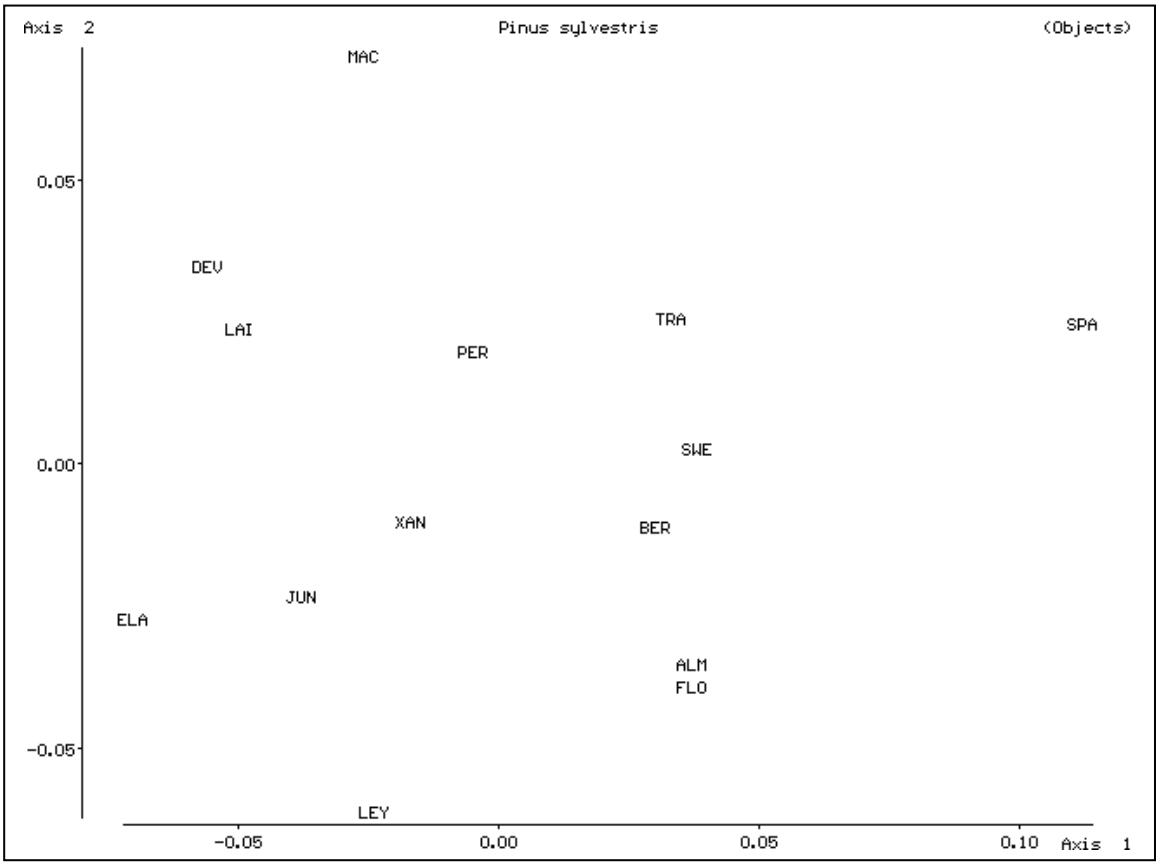


Fig. 1a. Principal coordinate analysis (PcoA) of Scots pine populations based on the genetic distances.
Fig. 1a. Principal coordinate analysis (PcoA) of Scots pine populations based on the genetic distances.

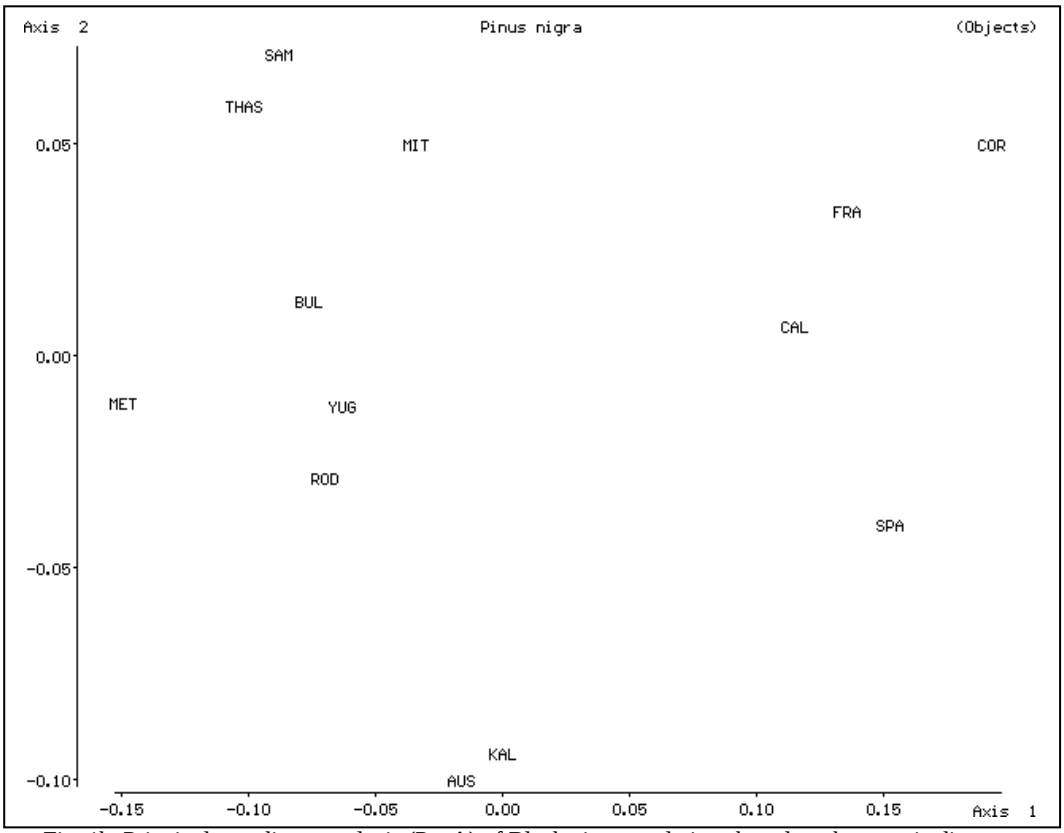
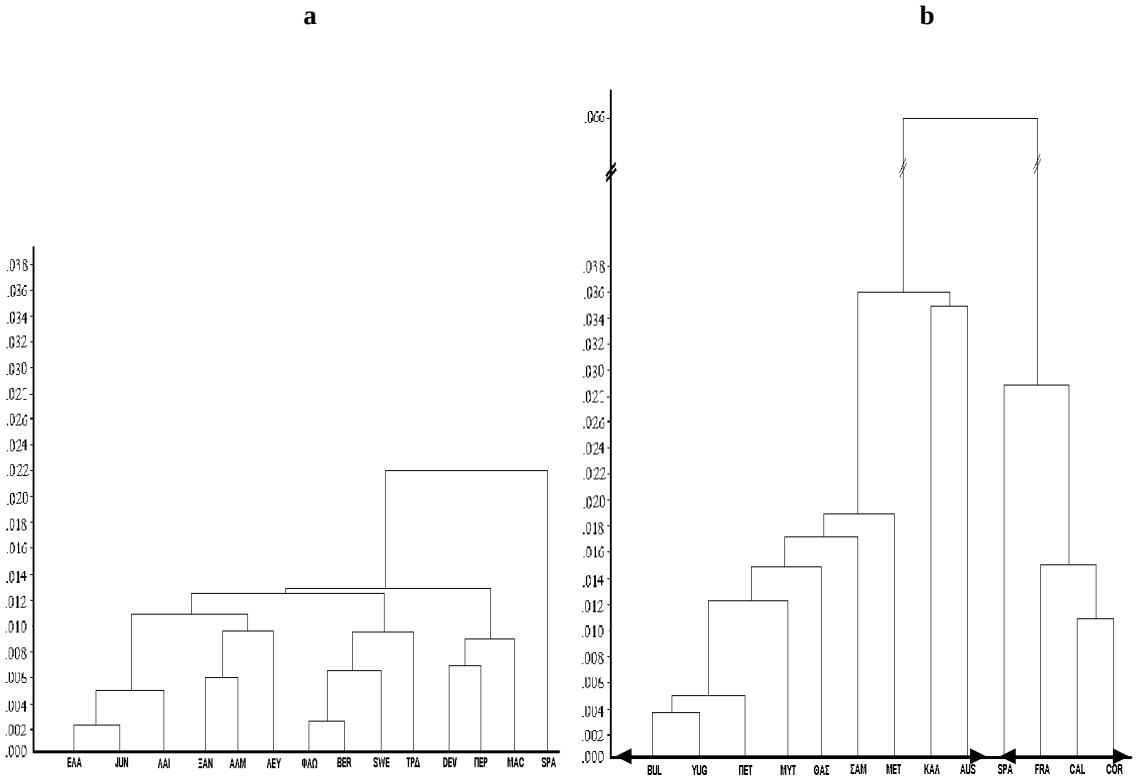


Fig. 1b. Principal coordinate analysis (PcoA) of Black pine populations based on the genetic distances.
Fig. 1b. Analiza coordonată principală (PcoA) a populațiilor de pin negru, pe baza distanțelor genetice.



ssp. *nigra* ssp. *salzmannii*

Fig. 2a, 2b. Dendrograms of a) 14 Scots pine and b) 13 Black pine populations, based on the UPGMA method.
Fig. 2a, 2b. Dendrogramele pentru: a) 14 populații de pin scotian şi b) 13 populații de pin negru, întocmite prin utilizarea metodei UPGMA.

Principal Coordinate analysis (PcoA) and the dendrograms (based on the cluster analysis of genetic distances), of the two species are shown in figures 1a, 1b and 2a, 2b respectively. By comparing the respective figures, it can be seen that each species follows almost the same genetic variation pattern in both analyses (PcoA, dendrograms). As indicated, the populations of Scots pine possess a tendency to form smaller pairs and groups while the opposite occurs in the case of Black pine. This is attributed to the closer phylogenetic relation that exists among the Scots pine populations and to the larger differentiation observed among the populations of Black pine. The differentiation patterns of Black pine populations follow an East-West geographic distribution model (e.g. ssp. nigra ssp. salzmannii), while the differentiation model of the Scots pine populations does not express a clear trend.

DISCUSSION AND CONCLUSIONS

Biochemical markers and specifically, isozymes were used successfully in the last decades both in phylogenetic and evolutionary studies of conifers (HAMRICK et al., 1979, HAMRICK et al., 1981, MITTON 1983, GULLBERG et al. 1985, HAMRICK and GODT 1990, BERGMANN 1991, SCALTSOYIANNES et al., 1997, SCALTSOYIANNES, 1999). Pines are, on average, among the most genetically diverse plants, both among and within populations, as measured by quantitative traits (CORNELIUS 1994), and diversity at isozyme loci (HAMRICK et al., 1979, HAMRICK and GODT 1990, LEDIG, 1998)

In the present study 14 Scots and 13 Black pine populations, from the entire expansion area of the two species in Europe, were analyzed for allozyme variation in 14 loci. Our data revealed that, all the tested loci, were polymorphic for both species, except of the IDH enzyme system, which was monomorphic for Scots pine. A previous comparative study of the two species (Pasagiannis *et al*., 2000) on the Balkan peninsula populations, reported the same number of gene loci for the particular nine enzyme systems, coded by 49 and 56 alleles for Scots and Black pine, respectively. It appears that both species in the Balkan Peninsula accumulate a great load of their gene pool. The above is in accordance with previous studies conducted on the two species in question (SCALTSOYIANNES et al. 1994, MOULALIS et al. 1996).

Between the two species, unique alleles were detected mainly in the Balkan Black pine populations.

The fact that Black and Scots pine belong to the most polymorphic coniferous species is well explained by the high average diversity rates, found in the present work, compared to that of other conifers. Similar diversity rates were, also, found by other researchers (GONCHARENKO et al. 1994, SILIN and GONCHARENKO 1996, LEDIG, 1998).

The isolation and consequently, the low gene flow of Black and Scots pine Spanish populations are considered responsible for the relatively low diversity rates, found. To the contrary, the populations of the Balkan Peninsula had

higher values for both species. High rates in the diversity parameters for the Balkan populations of both species were, also, recorded in previous works by PASAGIANNIS et al. (2000) and TSAKTSIRA et al. (1997), while low rates for the populations of the Iberian peninsula were found by PRUS-GLOWACKI and STEPHAN (1994) and PRUS-GLOWACKI et al. (2003) for Scots pine and by AGUINAGALDE et al. (1997), TSAKTSIRA and SCALTSOYIANNES (1998) for Black pine. The above, support the hypothesis of many researchers that the populations of the Iberian Peninsula were geographically isolated for considerable periods of time and were not affected by the glaciers, drastically (Mirov 1967, Vidakovic 1991, PRUS-GLOWACKI and STEPHAN 1994, SALVADOR et al., 2000). According to the previous authors and others (HUNTLEY and BIRKS 1983, BENNETT et al. 1991), Tertiary gene pools have survived in Iberian peninsula more or less unchanged through the glaciation period.

Gst was at least twice higher for Black pine compared to Scots pine, a paradox result considering the fact that Scots pine is more expanded than Black pine and thus, a greater differentiation among its populations would be expected. This is well explained by the different evolutionary course of the two species, especially during the post-glacial period (MIROV 1967, GULLBERG et al. 1985, VIDAKOVIC 1991, PRUS-GLOWACKI and STEPHAN 1994, MOULALIS et al. 1996). Similar Gst values were also found by GONCHARENKO et al. (1994), PRUS-GLOWACKI and STEPHAN (1994) for Scots pine and by TSAKTSIRA (1992), SCALTSOYIANNES et al. (1994) and SILIN and GONCHARENKO (1996) for Black pine.

The genetic distances of both species, also, reveal the larger genetic differentiation among the populations of Black pine. In particular, the majority of the studied populations exhibited the largest genetic distances from the Spanish and Corsican populations. Various morphological and biochemical data confirmed the distinctive differences of the above mentioned populations from other European ones (BARBERO et al., 1998).

As, for Scots pine populations, the largest genetic distances were detected among the Iberian and the other Scots pine populations, fact which indicates that the particular peninsula represents original ancient Tertiary gene pools. The discontinuity and existing high altitude of the Pyrenees contributes to this isolation. SINCLAIR et al., (1999), based on mitochondrial DNA variation analyses, further support the above statements.

By comparing the respective data of PcoA and dendrogram analyses for Black and Scots pine, we observed that each species follows almost the same genetic variation pattern in both analyses. Scots pine populations tend to form smaller pairs and groups, due to the closer phylogenetic relation existing among these populations. The opposite occurs in the case of Black pine, where larger differentiation was observed among the populations. Black pine populations follow an East-West geographic distribution model corresponding to two subspecies (ssp. nigra, ssp. salzmannii), while the differentiation model of the Scots pine populations does not express a clear trend. PASAGIANNIS et al. (2000), working on Balkan populations of the two species resulted in the same conclusions. These findings confirm previous statements on the different post-glacial evolution of these particular species in Europe. According to geobotanical and other studies (MIROV 1967, GULLBERG et al. 1985, MOULALIS et al. 1996), European populations of Scots pine, apart from those of the Iberian peninsula, draw back southwards in refugia, as these became subject to great pressure by glaciations, which occupied a significant area in Northern and Central Europe during the Tertiary period. All the currently existing European populations of Scots pine except those of the Iberian Peninsula are considered an end-product of the post-glacial expansion of the species, and therefore, they are relatively uniform genetically. The above hypothesis seems to be in accordance with the theory formulated by GULLBERG et al. (1985), that natural species, as Scots pine, strongly affected by the glaciations, now present little differentiation among their populations. To the contrary, other plant species, such as Black pine, that were not affected significantly by the glaciations preserve, even today, a genetic diversity that already existed during the Tertiary period and therefore, are characterized by higher interpopulation differentiation and higher number of rare alleles.

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