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Allozyme Differentiation in the Genus Pinus

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Abstract

Needle samples from 17 species of pine taken from the collection of botanical gardens were analyzed at 11 enzyme loci. In all 57 alleles were found among 156 trees of the analyzed species, and only 7 of them were common to the two subgenera, *Pinus* and *Strobus*. The values of NEI's distance between species belonging to these subgenera vary from 1.42 to 2.66 (with average value 2.17) and are very great for intra-genus gene differentiation (maximal estimates of the distance in plants do not exceed 1.5). Large samples taken from natural populations of *Pinus sibirica* and *P. sylvestris* were analyzed at 22 loci to estimate bias in genetic distances influenced small samples from the botanical gardens.

Key words: subgenus *Pinus*, subgenus *Strobus*, alloyzme polymorphism, genetic divergence, genetic distance, phylogeny.

Introduction

The genus *Pinus* includes more than 100 species, more than any other gymnosperm. Pines are one of the main forest tree species and account for as much as 70% of all woodland areas. Rational management and usage of forests should be combined with measures aimed to preserve the biological diversity (NAMKOONG, 1983). One of these measures is the study of taxonomy and phylogenetic relationships of species. The systematics of pines has been extensively studied in this century and has been repeatedly reconstructed (MIROV, 1967). The classification of LITTLE and CRITCHFIELD (1969) is now widely recognized.

At the same time there are some problems in the taxonomy of pines. One needs to estimate genetic differentiation among pines, especially between two main subgenera, *Strobus* (white pines) and *Pinus* (hard pines). In addition, the taxonomic status of some species in the subsection *Sylvestris* needs to be verified (KORZUBOV and MURATOVA, 1986). Similar problems occur for the subsection *Contortae* (WHEELER et al., 1983; CRITCHFIELD, 1985).

The analysis of allozyme variability can allow an estimation of genetic differentiation among pine species, since the genetic variability of pine is high (MITTON, 1983). However, studies of allozyme variation in pine have dealt mainly with species of the subgenus *Pinus* (WHEELER et al., 1983; CONKLE et al., 1988; MILLAR et al., 1988; KARAMANGALA and NICKRENT, 1989). The other big subgenus, *Strobus*, is little studied (KRUTOVSKII et al., 1990). Therefore the aim of this study was to estimate genetic variability within the genus *Pinus*, especially to emphasize the genetic difference between these two subgenera.

Table 1. — Taxonomical positions, areals and sourses of samples. MGB, Main botanical garden of Russian Academy of Sciences; NBG, Nikitsky botanical garden.

Sign	Species	Number of trees	Areals	Collection
	Subgenus Strobus Sectio Strobus Subsectio Cembrae			
ko	P. koraiensis	10	South-East Asia	MBG
ce	P. cembra	10	Balkans	MBG
si	P. sibirica	10	Siberia	MBG
pu	P. pumila	10	South-East Asia	MBG
	Subsectio Strobi			
pe	P. peuce	10	Balkans	MBG
gr	P. griffithii	6	South-East Asia	NBG
st	P. strobus	10	North-East America	MBG
	Subgenus Pinus Sectio Pinus Subsectio Sylvestres			
re	P. resinosa	10	North America	MBG
de	P. densiflora	4	South-East Asia	NBG
sy	P. sylvestris	10	Eurasia	MBG
mu	P. mugo	10	South, Middle Europe	MBG
th	P. thunbergii	10	South-East Asia	NBG
ni	P. nigra	6	South Europe	NBG
pa	P. pallasiana	10	Crimea	NBG
ha	P. halepensis	10	South Europe	NBG
	Subsectio Ponderosae			
po	P. ponderosa	10	North-West America	NBG
	Subsectio Contortae			
ba	P. banksiana	10	North America	MBG

Materials and Methods

Needle samples from 17 species of pine were obtained from the collection of the Central Botanical Garden of the Russian Academy of Sciences (Moscow) and from the collection of the Nikitsky Botanial Garden (Yalta, Crimia). The species and their taxonomic positions (following LITTLE and CRITCHFIELD (1969)) are listed in *table 1*. Sample sizes were 10 trees for each species, with the exception of *Pinus densiflora, P. griffithi, and P. nigra,* which were represented in these collections by 4, 6 and 6 trees, respectively.

In addition, samples from natural populations of *P. sylvestris* and *P. sibirica* were collected in Western Siberia northward from 60^0 latitude. Altogether, 100 trees of Scots pine and more than 1500 trees of Sibirian cedar pine were taken for investigation.

Proteins were extracted from needle tissues in the presence of polyphenol inhibitor. Following the method of LOOMIS and BATTAILE (1966), we used insoluble polyvinylpyrrolidone (PVP). We ground 100 mg of mature needles in a mortar with liquid nitrogen mixed with PVP. Needle/PVP ratios have been defined for each species individually. A portion of extraction buffer was then added to make a "slurry". The extraction buffer was composed of 1 M sucrose, 5.7 mM L-ascorbic acid, 8.3 mM DL-cisteine, 0.02 M dithiotreitol, and 1.5 mM-aminocaproic acid dissolved in electrode buffer diluted 1:1.7. To 100 ml of this solution we added 1 ml of Tween-80. Extraction time was 14 to 18 hours at 4^0C. After extraction, the slurry was filtered through the nylon in the centrifuge at 6000 rpm.

Vertical-slab polyacrylamide gel electrophoresis was conducted in Tris-EDTA-Boric system (PEACKOK et al., 1965) with slight modifications. The composition of the buffers are the following: electrode buffer (pH 8.0) 0.115 M Tris, 0.005 M EDTA, 0.16 M boric acid; gel buffer (pH 8.6) 0.115 M Tris, 0.005 M EDTA, 0.12 M boric acid. Gel concentrations were 7%. Usual recipies have been taken for histochemical staining (HARRIS and HOPKINSON, 1976).

For different species, electrophoretic mobilities of protein variants were compared with each other in adjacent tracks of gel slabs. Cluster analysis was performed by the UPGMA method using NEI's genetic distance measure (SNEATH and SOKAL, 1973).

Results

Table 2 presents the genetic data for 17 pine species that could be obtained by the conditions of protein extraction and electrophoretic resolution. *Pinus pinea, P. sabiniana,* and *P. attenuata* were also studied, but their electrophoretic patterns were not sufficiently clear for genetic interpretation. Of 12 tested enzymes, 6 were excluded because of unsatisfactory resolution. Each of the 17 species was analyzed for 6 enzymes, apparently coded by 11 loci. The following variability was observed among the species under consideration.

Enzyme SKDH (E. C. 1.1.1.25): two loci were scored; 12 alleles were found for *Skdh-1* and 7 for *Skdh-2.*

Enzyme PGM (E. C. 2.7.5.1) has two loci; 8 alleles were found at *Pgm*-1 and 8 at *Pgm*-2.

Enzyme SOD (E. C. 1.15.1.1): four loci were found; Sod-1 has two alleles, and the loci *Sod-2* — 2, *Sod-3* — 3 and *Sod-4* each have 4 alleles.

Enzyme IDH (E.C. 1.1.1.42): only one locus (*Jdh-1*) was analyzed: 3 alleles were found.

Enzyme DIA (E.C. 1.6.4.3): one locus (*Dia-2*), with 3 alleles, was analyzed.

Enzyme GOT (E.C. 2.6.1.1): one locus (*Got-2*) was analyzed; 5 alleles were found.

Thus, we could analyze genetic variability at 11 loci. The distribution of allele band mobility and allele frequencies are given in *figure 1* and *table 2.*

The locus *Skdh-1* was the most variable (12 allozyme variants among studied samples). Minimal variation was

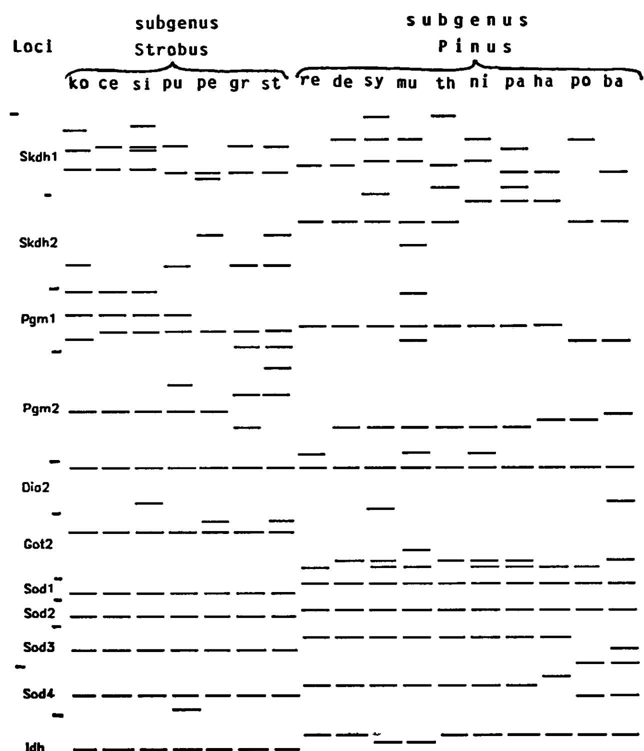

Figure 1. — The distribution of allele band mobility among species analyzed.

Table 2. — Allele frequencies at 11 loci for 17 species of genus Pinus.

Locus	Allele	Allele frequency																
		Subgenus Strobus							Subgenus Pinus									
		ko	ce	si	pu	pe	gr	st	re	de	sy	mu	th	ni	pa	ha	po	ba
Skdh-1	110	-	-	-	-	-	-	-	-	-	0.05	-	0.35	-	-	-	-	-
	107	-	-	0.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	106	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	103	-	-	-	-	-	-	-	-	0.17	0.85	0.56	-	0.92	-	-	-	-
	100	-	0.87	0.10	0.15	-	0.50	0.95	-	-	-	-	-	-	-	-	-	-
	99	0.20	-	0.50	-	-	-	-	-	-	-	-	-	-	0.50	-	-	-
	95	-	-	-	-	-	-	-	-	-	0.10	0.44	-	0.08	-	-	1.00	-
	94	-	-	-	-	-	-	-	1.00	0.83	-	-	0.55	-	-	-	-	-
	93	0.70	0.12	0.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	92	-	-	-	0.85	0.80	0.50	0.05	-	-	-	-	-	-	0.35	1.00	-	1.00
	87	-	-	-	-	0.20	-	-	-	-	-	-	-	-	-	-	-	-
	84	-	-	-	-	-	-	-	-	-	-	-	0.10	-	0.15	-	-	-
Skdh-2	108	-	-	-	-	-	-	-	-	-	0.15	-	-	-	-	-	-	-
	106	-	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00	1.00	-	-
	100	-	-	-	-	-	-	-	1.00	1.00	0.85	0.95	1.00	-	-	-	1.00	1.00
	96	-	-	-	-	1.00	-	0.05	-	-	-	-	-	-	-	-	-	-
	93	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-
	87	1.00	-	-	1.00	-	1.00	0.95	-	-	-	-	-	-	-	-	-	-
	79	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgm-1	111	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-
	105	0.75	0.94	0.95	0.50	-	-	-	-	-	-	-	-	-	-	-	-	-
	101	-	-	-	-	-	-	-	1.00	1.00	1.00	0.75	1.00	1.00	1.00	1.00	-	-
	100	-	0.06	0.05	0.33	1.00	0.50	0.10	-	-	-	-	-	-	-	-	-	-
	97	-	-	-	-	-	-	-	-	-	-	0.20	-	-	-	-	1.00	1.00
	96	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	94	-	-	-	-	-	0.50	0.90	-	-	-	-	-	-	-	-	-	-
	93	-	-	-	0.17	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgm-2	132	-	-	-	-	-	-	0.20	-	-	-	-	-	-	-	-	-	-
	119	-	-	-	0.35	-	-	-	-	-	-	-	-	-	-	-	-	-
	115	-	-	-	-	-	0.50	0.80	-	-	-	-	-	-	-	-	-	-
	110	1.00	1.00	1.00	0.65	1.00	-	-	-	-	-	-	-	-	-	-	-	-
	109	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00	-
	100	-	-	-	-	-	0.50	-	-	1.00	1.00	0.95	1.00	0.25	1.00	-	-	-
	92	-	-	-	-	-	-	-	1.00	-	-	0.05	-	0.75	-	-	-	-
Dia-2	100	1.00	1.00	0.85	1.00	1.00	1.00	1.00	1.00	1.00	0.80	1.00	1.00	1.00	1.00	1.00	1.00	0.35
	88	-	-	0.15	-	-	-	-	-	-	-	-	-	-	-	-	-	0.65
	85	-	-	-	-	-	-	-	-	-	0.20	-	-	-	-	-	-	-
Got-2	119	-	-	-	-	0.80	-	0.05	-	-	-	-	-	-	-	-	-	-
	100	1.00	1.00	1.00	1.00	0.20	1.00	0.95	-	-	-	-	-	-	-	-	-	-
	95	-	-	-	-	-	-	-	-	-	-	0.30	-	-	-	-	-	-
	88	-	-	-	-	-	-	-	-	1.00	0.45	-	1.00	0.75	0.40	-	-	1.00
	72	-	-	-	-	-	-	-	1.00	-	0.55	0.70	-	0.25	0.60	1.00	1.00	-
Sod-1	100	-	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1-00	1.00	1.00	1.00	1.00
	97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-
Sod-2	100	-	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-
Sod-3	100	-	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	0.20
	89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	0.80
Sod-4	104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-
	100	-	-	-	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-
	91	1.00	1.00	0.95	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	1.00	1.00
	76	-	-	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Idh	103	-	-	-	-	-	-	-	1.00	1.00	-	-	1.00	1.00	1.00	1.00	1.00	1.00
	101	-	-	-	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-

found for loci *Sod-1* and *Sod-2*, and they were monomorphic across all species, within each subgenus.

Altogether, 57 alleles were found among 156 trees of the analyzed species. Only one allele, Dia-2-100, was present in all species of both subgenera, *Pinus* and *Strobus*. Six other common alleles were shared by some species of both subgenera (*Table 3*). Hence, only 7 alleles of the 57 observed were common to the two subgenera under comparison.

Table 3. — Common alleles for subgenera *Pinus* and *Strobus*.

Common alleles	Species	
	Strobus	*Pinus*
Dia-2[100]	All	All
Dia-2[88]	si	ba
Skdh-1[99]	ko, si	pa
Skdh-1[92]	pu, pe, gr, st	pa, ha, ba
Pgm-2[100]	pe	de, sy, mu, th, ni, pa
Sod-3[95]	All	ba
Sod-4[91]	All	ba, po

Five allozymes (*Got*-2-100, *Sod*-1-97, *Sod*-2-98, *Sod*-4-91, and *Idh*-100) were present in all species of white pine (*Strobus*), but were not found in the subgenus *Pinus*. In contrast, two allozymes (*Sod*-1-100 and *Sod*-2-100) were common in each species of hard pine, (subgenus *Pinus*), but were not present in *Strobus*.

The quantitative differences among the species were expressed by a matrix of NEI's genetic distances, which were then transformed into a dendrogram by the UPGMA method (*Fig.* 2). The dendrogram is in agreement with the known taxonomy of the genus *Pinus*.

In *figure* 2, two large isolated clusters correspond to the subgenus *Pinus* and the subgenus *Strobus*. In *Strobus*, one can see a cluster corresponding to subsection *Cembrae* and a more differentiated group of species belonging to the subsection *Strobi*. For the subgenus *Pinus*, one cluster corresponds to the subsection *Sylvestres* and the other unites the two subsections *Ponderosae* and *Contortae*.

Discussion

Analyzing protein variability allows phylogenetic relationships among taxa to be successfully investigated (GOTTLIEB, 1977; CRAWFORD, 1983,1985). However, this method inay give rise to two potential statistical problems. The first arises from low sample size. NEI and ROYCHOUDHURY (1974) showed that the strength of interspecific comparisons depends on the number of loci examined. They estimated the minimal number of loci to be a few dozen. Some authors have suggested even lower values, for example, 10 (FERGUSON, 1980) or 11 (GOTTLIEB, 1981). All of these authors concluded that the number of individuals does not significantly affect the estimates of genetic identity of the species compared, and only a few individuals need to be sampled from different populations (NEI, 1978; GORMAN and RENZI, 1979). Our sample sizes satisfy these requirements.

Another statistical problem is connected with a possible bias attributable to the choice of the populations sampled. For instance, spatial differentiation of the species under comparison could make a sample taken from one natural population unrepresentative. Fortunately, this problem is not too important for pines, since the intraspecies gene differentiation is not large. Even for widely distributed pine species, the spatial genetic subdivision is low (GULLBERG et all., 1985; GURIES and LEDIG, 1982), although some important statistically significant differences can be found ROBERDS et al., 1987). However it is important for estimating the difference between the two subgenera, *Pinus* and *Strobus*, since the genetic distance between them is

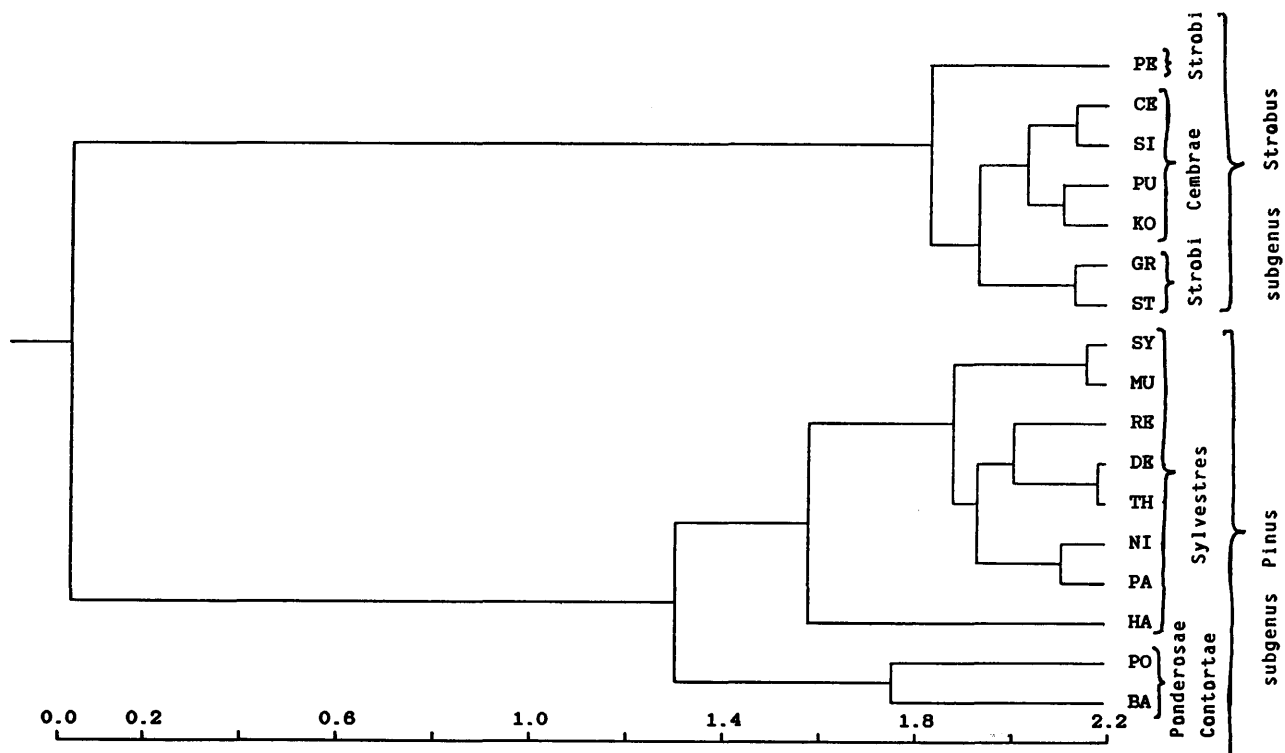

Figure 2. — UPGMA dendrogram using NEI's genetic distance matrix.

exclusively large (see below). However, another effect of influence of the choice of populations can lead to statistical bias in the estimates of genetic differences among species. Namely, in our case, with samples taken from botanical gardens, the species were represented by only a few individuals. These individuals probably have few alleles, probably, only those that are frequent in the species. Indeed, the average heterozygosity within investigated species was very low (H = 0.074), less than in natural populations. Hence, our samples could produce a bias in genetic variability, perhaps increasing the genetic distances between dissimilar species and decreasing the genetic distances between similar species with many alleles in common.

To check our estimates for two species, we analyzed new large samples taken from natural populations of *P. sibirica* and *P. sylvestris*, since the samples of these species taken from the botanical gardens showed maximal genetic distance (D = 2.66). In these more representative samples analyzed (N = 100 for *P. sylvestris* and N = 1500 for *P. sibirica*), the number of loci was 22. The value of NEI's genetic distance determined on the basis of these data is D = 2.39. Hence, there is some bias in the estimates of genetic distances, but it is not large for these two species. We found only 2 allozymes of 72 observed at 22 loci to be common for natural populations of *P. sylvestris* and *P. sibirica*. This level of genetic variability is in correspondence with the data collected in the botanical gardens: only one allozyme of the 32 observed was common for both species (*Table 2*).

Figure 2 shows that the species studied are genetically subdivided into two clusters corresponding to the subgenera *Pinus* and *Strobus*. The values of NEI's distance between species belonging to these subgenera vary from 1.42 (between *P. pumila* [subgenus *Strobus*] and *P. banksiana* [subgenus *Pinus*]) to 2.66 (between *P. sibirica* [*Strobus*] and *P. sylvestris* [*Pinus*]); their average value is 2.17. These values are large for intra-genus genetic differentiation. As a rule, genetic differences among congeneric species are smaller. CRAWFORD (1983), GOTTLIEB (1981), and ELISENS and CRAWFORD (1988) estimated that intra-genus genetic differentiation in plants does not exceed 1.5. For animal genera, maximal estimates of D are less than 2.0, with the exception of salamanders and Hawaiian Drosophila (NEI, 1987).

Thus, we can conclude that the genetic difference between the subgenus *Pinus* and the subgenus *Strobus* is great. This conclusion is in agreement with the results of immunological analysis that also indicates considerable difference between white and hard pines (PRAGER et al., 1976). It is also in accordance with paleontological investigations showing that when pines appeared in the Upper Triassic, they were already represented by the both forms, Haploxylon and Diploxylon.

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