basis (under the environmental conditions studied) of 0.78, 0.74, 0.66 and 0.77 respectively.

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Determination of the Origin of an Isolated Group of Trees of Pinus nigra through Enzyme Gene Markers

By S. Fineschi

Institut für Forstgenetik und Forstpflanzenzüchtung,
Forstliche Biometrie und Informatik der Universität Göttingen

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Summary

Pinus nigra is a collective species which consists of four subspecies. Previous taxonomic studies on European black pine have used biochemical methods: monoterpene contents and isoenzyme systems. The problem of a differentiation between the subspecies *laricio* and *nigricans* was here examined using isoenzyme gene markers.

In order to characterize the allelic structure on the basis of shikimate dehydrogenase (SKDH), eleven populations of *Pinus nigra* Arn. were studied *(Table 1)*.

Zone starch gel electrophoresis of haploid endosperms of dormant seeds was used. Results were analyzed by genetic distance *(Table 3, 4)*: they show that Corsican provenances of laricio pine clearly differ from the other populations in the high frequency of the B_1 allele *(Table 2)*.

A differentiation was successful within laricio pine, between the Corsican and the Calabrian populations. Therefore, it was possible to analyze an unknown population of *Pinus laricio* (Monti Pisani area) to determine if this tree sample belongs to the Corsica or the Calabria group. Since the results obtained on the genetic structure of the trees from the Monti Pisani area were similar to the allelic frequency in the Corsica populations, it is probable that this group of laricio pines originates from Corsica.

Key words: Pinus nigra, taxonomy, isoenzymes, SKDH.

Zusammenfassung

Pinus nigra ist eine aus 4 Unterarten bestehende Kollektivart. Frühere taxonomische Untersuchungen bei der europäischen Schwarzkiefer bedienten sich biochemischer Methoden: Monoterpengehalte und Isoenzymsysteme. Das Problem einer Differenzierung zwischen den Unterarten *laricio* und *nigricans* wurde hier mit Hilfe von Enzymgenmarkern bearbeitet.

Zur Charakterisierung der allelischen Struktur an den Shikimat-Dehydrogenasen (SKDH) kodierenden Genloci wurden 11 Populationen von *Pinus nigra* Arn. untersucht *(Tab. 1)*.

Die Labormethodik verwendete Stärkegel-Zonenelektrophorese der haploiden Endosperme ruhender Samen. Die Ergebnisse wurden mit Hilfe genetischer Abstandsmaße analysiert *(Tab. 3, 4)*: Sie zeigen, daß die korsischen Herkünfte der Unterart *laricio* von den übrigen Populationen durch die hohe Frequenz des Allels B_1 unterschieden sind *(Tab. 2)*.

Innerhalb der Unterart *laricio* war eine Differenzierung zwischen den korsischen und den kalabrischen Populationen erfolgreich. Daher war es möglich, ein fragliches Vorkommen von *Pinus laricio* in den Pisaner Bergen auf seine Zugehörigkeit zu der korsischen bzw. der kalabrischen Gruppe zu bestimmen. Da die genetischen Merk-

male der Bäume aus den Pisaner Bergen den Allelhäufigkeiten in den korsischen Populationen besonders entsprachen, stammt diese Gruppe von Kiefern wahrscheinlich aus Korsika.

Introduction

In the literature, the genetic differentiation of species has always been discussed with the help of different methods and characteristics. The present paper deals with the problem of identifying the origin of some trees of European pine, *Pinus nigra* Arn., a species which is spread across Central and Southern Europe, using biochemical methods.

According to Fukarek (1958), *Pinus nigra* Arn. is a collective species consisting of four "small species": *P. clusiana* Clem. ex Arias.; *P. laricio* Poir. in Lamk.; *P. nigricans* Host.; *P. pallasiana* Lamb.

In Italy, two subspecies are present: *P. nigricans* var. *austriaca* (Eastern Alps) and var. *italica* (Appennines); *P. laricio* var. *calabrica* (Calabria and Sicily). A detailed account of the distribution in Italy was presented by Morandini (1966).

The various subspecies differ in certain morphological characteristics, such as the form of the trunk, crown, needles and cones, as well as in edaphic characteristics: *P. laricio* grows on siliceous soils while *P. nigricans* is found on calcareous soils.

In taxonomic studies on black pine (Arbez and Millier 1971; Arbez *et al.* 1974), the subspecies *laricio* was divided into two varieties, var. *corsicana* and var. *calabrica,* as a result of anatomical and biochemical research. The latter research was based on the identification of monoterpenes and was therefore partly affected by environmental factors.

On the contrary, the method used in the present paper to differentiate the various subspecies and the populations within one subspecies possesses the advantage of not being limited in its results by any environmental influence.

In addition to the differentiation of the provenances, a population of unknown origin must be studied in order to determine whether it can be assigned to one of the provenances, i.e. originates from it. The unknown population dealt with in the present paper is a small group of trees (about ten individuals) of the subspecies *laricio* which, according to old bibliographical data (Savi 1798; Longo 1904, 1920), were discovered in a wood of *P. pinaster* of the Monti Pisani area, about 100 km west of Florence. Only three trees of the group have reached adulthood. This probably results in a high rate of self-pollination, and the percentage of empty seeds is very high.

It is of interest to learn the variety of *P. laricio* to which this group of trees belongs, since it is situated well outside the typical geographical range of *P. laricio*. A previous attempt to determine its origin by means of tests on the physiological characteristics (germination tests) and on the monoterpene contents was unsuccessful (Fineschi 1980).

The assignment of this tree sample to one population or a group of populations is attempted here with the help of biochemical characters. These biochemical characters are so-called isoenzymes, which are largely independent of environmental conditions, and which are controlled by individual gene loci.

The aim of this investigation is to identify one or several isozyme systems useful for the differentiation of populations which enable a qualitative or quantitative comparison of the samples examined. Once such a system has been described, the identification or, better, the differentiation of the populations through the frequencies of its genes is possible. Following successful differentiation of the populations, the determination of the genetic structure must be used to assign the Monti Pisani sample to one of them.

Materials and Methods

Materials

Eleven populations of the subspecies *laricio* and *nigricans,* present in Italy, were examined *(Tab. 1)*[1]).

Applying the technique developed by Bartels (1971 a, b) and Bergmann (1971), endosperms of dormant seeds were tested for several isozyme systems. The seed samples of the eleven populations originated from many trees and have been mixed, while the seeds of the unknown tree sample were collected from two individual trees. However, the direct comparison of the Monti Pisani sample on one hand and the eleven populations on the other is only partly possible, since in the first case only the genotypes of two individuals can be detected, while in the other case only the allelic structure of the female contribution to seed production is detectable.

Biochemical methods

For each seed, endosperm tissue was separated from the embryo and from the seed coat. Then, each endosperm was homogenized in Tris-HCl buffer pH 7.5.

Starch gel electrophoresis was performed in 12.5% gel with a continuous Tris citrate buffer pH 7.5.

[1]) The seeds from Corsica have been kindly supplied by Dr. Bonnet-Masimbert, the seeds from Yugoslavia by Dr. Vidaković. The remaining material comes from the nursery of the Azienda di Stato Foreste Demaniali (Pieve S. Stefano). The seeds of the Monti Pisani area were personally collected by the author in the winter of 1981—1982. Unfortunately, one of the three adult trees did not bear any cones that year, and the study is therefore limited to two trees.

Tab. 1. — Geographical location of the provenances.

species	provenance	abbr.	Latitude	Longitude	Altitude above sea level in m
1 P.laricio corsicana	Valdoniello	Co1	42°19'N	8°54'E	1015-1325
2 P.laricio corsicana	Marmano	Co2	42°01'N	9°10'E	1000
3 P.laricio corsicana	Vizzavona	Co4	42°06'N	9°07'E	940-1160
4 P.laricio corsicana	Noceta	Co5	42°09'N	9°09'E	820-895
5 P.laricio calabrica	Sila Cosenza (Tasso)	Ca1	39°15'N	16°20'E	1000
6 P.laricio calabrica	Sila Catanzaro	Ca2	38°58'N	16°35'E	1000
7 P.laricio calabrica	Sila Cosenza (Monaco)	Ca3	39°15'N	16°20'E	1000
8 P.nigricans italica	Villetta Barrea	VB14	42°47'N	13°46'E	1000-1200
9 P.nigricans italica	Villetta Barrea	VB83	42°47'N	13°46'E	1000-1200
10 P.nigricans austriaca	Monte Terlago	MT	46°05'N	11°05'E	697
11 P.nigricans austriaca	Slovenia	YU			
13 P.laricio	Monti Pisani	P1	43°05'N	10°15'E	125-195
14 P.laricio	Monti Pisani	P2	43°05'N	10°15'E	125-195

The separation took place after four hours with a voltage of 160 V.

After the separation, the gel slices were stained in a staining solution (60 ml Tris HCl pH 8.5; 70 mg Shikimic acid; 15 mg MTT; 8 ml NADP solution; 2 ml PMS solution.

— Determination of the genetic distance

The genetic differentiation of the populations is indicated by the presence of differing allelic frequencies in the various populations. To be able to compare the various populations with reference to a single gene locus using a single parameter, the genetic distance between each pair of populations can be calculated as a function of the allelic frequencies.

The genetic distances at each of the investigated loci were calculated according to GREGORIUS, formula (1974):

$$d_o = \frac{1}{2} \sum_{i=1}^{n} |X_i - Y_i|$$

(X_i and Y_i indicate the frequency of the allele i in populations X and Y, respectively; n ist the number of alleles of that gene locus).

The method for the evaluation of data consists of the quantitative differentiation of the populations based on the genetic distance. It must be noted that a qualitative differentiation is impossible in this case, because the seeds examined are only a random sample of every population and do not necessarily contain all of the existing alleles.

The limited number of individuals in the unknown tree sample and the fact that the genotypes of the two individuals must be compared with the allelic structure of the female contribution to seed production of the populations make a particular procedure necessary. In this case, an assignment is possible only if the allelic frequencies of the populations allow one to draw a clear conclusion about the expected frequencies of genotypes. If, for instance, a certain allele appears very frequently in a population, most individuals can be expected to be homozygous for this allele.

Results

Several isozyme systems have been investigated in seeds of *P. nigra*, however, it turned out that the shikimate dehydrogenase (SKDH) system displays the best differentiation of the provenances here studied. Therefore we focused primarily on this system which is controlled by two gene loci (BERGMANN *et al.* in prep.). *Tab. 2* shows the allelic frequencies at the two isozyme loci observed in the eleven populations. *Tab. 3* and *4* show the values of the genetic distances of the populations at the locus SKDH-A and locus SKDH-B. For locus A, the populations are all relatively homogeneous, and a differentiation is hardly detectable. On the contrary, for locus B there are large distances between the Corsica populations and all other populations. In particular, the Corsica populations show a higher frequency of the allele B_1, not only as compared to the subspecies *nigricans* (populations 8 to 11), but also as compared to the Calabria group and therefore to its subspecies *laricio*. Comparison of the Corsica populations with the subspecies *nigricans* at locus B shows that the Corsica group more closely resmbles the variety *italica* than the variety *austriaca*.

Homogeneity at both gene loci as seen in the Corsica populations can also be observed within each of the varieties *austriaca* and *italica*. These two varieties differ from each other, however, at locus B, so that within their common subspecies, *nigricans*, diversities are present.

The Calabria group (populations 5, 6, 7) is relatively heterogeneous, the population Ca2 being markedly different from the other two. At the locus B, Ca2 is closer to the populations of the subspecies *nigricans* than are Ca1 and Ca3. The allele B_3 is present only in the varieties *calabrica* of *P. laricio* and *italica* of *P. nigricans* and is the most frequent allele in the populations Ca1 and Ca3.

It must be noted that the results for the two gene loci do not contradict each other.

Tab. 2. — Relative frequency of alleles at the SKDH loci.

allelic frequency / population	A_1	A_2	A_3	sample size A	B_1	B_2	B_3	B_0	sample size B
Co1	.067	.933	0	134	.948	.051	0	0	136
Co2	.062	.937	0	48	.875	.104	0	.020	48
Co4	.149	.851	0	47	.958	.041	0	0	48
Co5	.266	.733	0	45	.755	.244	0	0	45
Ca1	.147	.803	.049	61	.036	.290	.672	0	55
Ca2	.193	.722	.035	57	.098	.804	.098	0	51
Ca3	.107	.875	.018	52	.114	.386	.500	0	44
VB14	.096	.887	.016	62	.258	.645	.097	0	62
VB83	.241	.724	.034	57	.210	.737	.053	0	57
MT	.015	.984	0	64	0	1	0	0	67
YU	.072	.891	.036	83	.023	.977	0	0	87

Tab. 3. — Genetic distances at the locus SKDH-A according to GREGORIUS (1974).

population	1 Co1	2 Co2	3 Co4	4 Co5	5 Ca1	6 Ca2	7 Ca3	8 VB14	9 VB83	10 MT	11 YU
1 Co1	----										
2 Co2	.005	----									
3 Co4	.082	.086	----								
4 Co5	.200	.204	.118	----							
5 Ca1	.130	.134	.049	.119	----						
6 Ca2	.161	.166	.079	.074	.045	----					
7 Ca3	.058	.062	.042	.160	.072	.103	----				
8 VB14	.046	.050	.052	.170	.084	.115	.012	----			
9 VB83	.209	.213	.127	.034	.094	.048	.151	.163	----		
10 MT	.052	.047	.133	.251	.181	.212	.109	.097	.260	----	
11 YU	.041	.046	.077	.194	.088	.121	.035	.024	.169	.093	----

Tab. 4. — Genetic distances at the locus SKDH-B according to Gregorius (1974).

population	1 Co1	2 Co2	3 Co4	4 Co5	5 Ca1	6 Ca2	7 Ca3	8 VB14	9 VB83	MT	11 YU
1 Co1	----										
2 Co2	.074	----									
3 Co4	.010	.083	----								
4 Co5	.193	.140	.203	----							
5 Ca1	.912	.859	.922	.719	----						
6 Ca2	.850	.798	.860	.658	.575	----					
7 Ca3	.835	.782	.845	.642	.173	.418	----				
8 VB14	.690	.638	.700	.497	.576	.160	.403	----			
9 VB83	.738	.685	.758	.545	.620	.112	.447	.092	----		
10 MT	.949	.896	.958	.756	.709	.196	.614	.355	.263	----	
11 YU	.926	.873	.935	.733	.686	.173	.591	.332	.240	.023	----

Both trees from the Monti Pisani region are homozygous for both loci. Their genotypes are A_2A_2/B_1B_1. Since the allele B_1 clearly prevails in the Corsica populations, it must be present mainly in homozygous form. The fact that the genotypes of the two trees are homozygous for the allele B_1 suggests that they be assigned to the Corsica group. Further assignment to a particular population of the Corsica group is difficult; however, they very probably originate from the population Co1 and/or Co4, since these two groups show the highest frequency of the allele B_1.

Discussion

In a study on four other gene loci (EST-B, ACP-A, LAP-A, LAP-B), Nikolić (1982) compared 28 populations of black pine. Using the same parameter, i.e., the genetic distance according to Gregorius, the single Corsica population examined was distinguished from the one Calabria population at the loci ACP-A and EST-B. These results concerned only the comparison of two populations; however at the locus SKDH-B studied here the four Corsica populations, though very similar to one another, must be clearly distinguished from the three Calabria populations, despite the heterogeneity shown by the Calabria group.

In a further study by Bonnet-Masimbert and Bikay-Bikay (1978) on 12 *laricio* populations (6 from Corsica and 6 from Calabria), a distinction between the two groups at the GOT loci was impossible.

In the present paper, the study was limited to two subspecies of black pine, *nigricans* and *laricio*. The first goal was the genetic differentiation of the various subspecies, above all within the subspecies *laricio*. The distinction observed confirms the results of Arbez and Millier (1971) about the anatomical characteristics of the needles and of Arbez *et al.* (1974) about the monoterpene contents. Further information was gained about the distinction within the subspecies *nigricans*, although the differentiation is not as clear as in the subspecies *laricio*.

These results contribute to the understanding of the systematics of black pine as a whole, which has already been the main object of the works by Vidaković (1955, 1957) and Gellini (1968).

The second goal, namely the classification of the seeds collected from two individual trees, could be reached due to the particular distinctions of the populations examined. Because of the remarkably high percentage of the allele B_1 in the Corsica group and of the homozygosity of the allele B_1 in the two trees examined in the Monti Pisani area, this isolated group of trees can probably be assigned to the provenance Corsica. However, it is hard to say in which one of the four Corsica populations it must be included, since the allelic frequency resulted from random samples. On the basis of the observed allelic frequencies, inclusion into the populations with highest frequency of the allele B_1 (Co1 and Co4) can be considered very probable.

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