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Genetic Differences among three Small Stands of *Pinus pungens*

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Summary. Analysis was made of isoenzyme and morphological differences among three small adjacent stands of *Pinus pungens* Lamb. and progeny derived from the stands. Results indicated that although significant differences were observed in measured characteristics among stands, progeny derived from the stands did not differ significantly. Analysis of gene frequencies for two esterase phenotypes indicated all three stands arose from a single panmictic population. The results of the analysis might be explained by environmental effects on the characteristics measured *in situ*, by genetic drift or by natural selection.

Introduction

It has been well documented that variation in enzyme mobility rates on an electrophoretic medium is correlated with the allelic constitution of a particular group of plants (Scandalios, 1969). Specifically in trees this has been demonstrated by Conkle (1971, 1972), Feret and Stairs (1971), Feret (1972) and Miyazaki and Sakai (1969). Consequently, there is no reason why variation in isoenzyme frequency among populations of trees (grown in a common environment) can not be interpreted in a genetic sense. *In situ* measurements of isoenzyme variation in natural stands of trees may also be equally valid if environmental gradients are not large, or if it can be assumed that the isoenzymes analyzed are environmentally stable under a given set of experimental conditions. Despite the possible problems of environmental stability, *in situ* isoenzyme analyses of natural forest-tree stands contributes significantly to knowledge of the population structure in natural tree populations (Sakai and Park, 1971; Sakai, Miyazaki and Matsuura, 1971).

In an attempt to elucidate some aspects of the population structure of *Pinus pungens* Lamb., three stands and progeny from the stands were studied using electrophoretic analysis and a variety of quantitative morphological characteristics.

Materials and Methods

Fifteen to seventeen trees from each of three small (15–20 trees) stands of *P. pungens* were used for the experiment. Stands were from 18 to 42 km apart in the Applachian Mountains of southwest Virginia. Vegetation surrounding the area sampled was composed primarily of oak-hickory forests with only a very few isolated and small stands of *P. pungens* intermixed. Needle tissue was sampled and seed collected from each tree during the winter of 1971.

Methods of needle peroxidase and esterase analysis have been previously described (Feret, 1971). Macrogametophyte esterase analysis was performed using seed collected from individual trees within each of the three stands. The gametophyte tissue was excised from the embryo and seed coat when the radicle had emerged 5–10 mm from a germinating seed. The macrogametophyte tissue was then homogenized using a small glass mortar and pestle and the entire homogenate electrophoresed.

Morphological variation was investigated both among stands and among progeny derived from stands. Needle characteristics (length, number of stomate rows and serrulations) were measured on 20 needles from each tree within each stand and measurements of seedling morphological variation were made using 4–6 seedlings/tree/stand. Characteristics measured in the seedling populations included number of cotyledons, cotyledon length, seedling height, and primary needle length.

Seedlings were grown in the greenhouse on a 15 hour daylength in 3″ peat pots. Temperatures ranged from 75–85 °F during the day and 72–75° at night. Seedling needle tissue was sampled for electrophoresis 10 weeks from germination.

Results

Needle Analysis. Nine needle peroxidase isoenzymes were detected and designated as PN1 to PN9 (Table 1). All trees sampled possessed PN2, PN4, PN5, PN8 and PN9 while the remaining needle peroxidases were present in varying frequencies in the three stands (Table 1). Needle esterases numbered five and were designated as EN1 to EN5. Esterases EN1, EN4 and EN5 were present in needle tissue of all trees sampled. EN2 and EN3 were present in low frequencies in each stand with the exception of EN2 in stand B which occurred in all 17 trees sampled (Table 1).

Comparison of isoenzyme frequency differences among the three stands was made using the simultaneous test tables for chi square developed by Jensen, Beus and Storm (1968) (Table 2). The results of the chi square analysis indicated that stands A and B were significantly different in frequency of occurrence for PN7 and EN2; B and C for EN2, while no frequency differences were observed between stands A and C.

Table 1. *Frequency of peroxidase (PN) and esterase (EN) isoenzymes in trees of stands A, B and C and peroxidases in seedlings (SPN) derived from A, B and C*

Stand	Trees Analyzed	Number of trees possessing: PN1	PN2	PN3	PN4	PN5	PN6	PN7	PN8	PN9
A	17	6	17	10	17	17	17	15	17	17
B	17	12	17	8	17	17	14	6	17	17
C	15	10	15	6	15	15	15	9	15	15

Stand	Trees Analyzed	Number of trees possessing: EN1	EN2	EN3	EN4	EN5
A	17	17	4	2	17	17
B	17	17	17	1	17	17
C	15	15	2	2	15	15

Seedling Group	Seedlings Analyzed	Number of seedlings possessing: SPN1	SPN2	SPN3	SPN4	SPN5	SPN6
A	32	32	32	32	10	16	32
B	32	32	32	32	9	13	32
C	32	32	32	32	14	13	32

Analysis of isoenzyme frequency differences among the three stands was also made using Berry's D^2 measure of divergence (Berry, 1961; Sakai and Park, 1971). D^2 analysis was not as critical as the chi square analysis. Stand A and B differed significantly in frequency of PN1, PN6, PN7 and EN2, while stands B and C differed for both PN6 and EN2. As with chi square analysis, no differences in isoenzyme frequency were found between stands A and C.

Six peroxidase isoenzymes were found in the needle tissue of seedlings derived from the three stands (designated as SPN1 to SPN6). Only two peroxidases varied (SPN4 and SPN5) in the seedling populations (Table 1). Chi square and D^2-analysis indicated no significant among stand differences in SPN4 or SPN5 frequencies (Table 2).

Macrogametophyte Analysis. Of nine esterases isolated from macrogametophyte tissue, only one varied and it was designated as FGE6. The variation observed for FGE6 indicated that its presence or absence was controlled by a single locus. Since female gametophyte tissue is haploid, FGE6 variation could be interpreted in a mendelian sense. Some parent trees in each population produced macrogametophyte populations with complete absence of FGE6 while other parent tree gametophyte populations all possessed FGE6. Still other parent trees produced macrogamete populations which segregated in a 1:1 ratio for the presence or absence of FGE6 (Table 3).

Where segregation occurred within a parent-tree population of macrogametophytes chi square analysis was performed (Table 3). There was no reason to reject the hypothesis that FGE6 is controlled by a single locus with two alleles. In Table 4 the two alleles were designated as E6A for the "active" allele and E6I for the "inactive" allele. Stand A contained 13 homozygous E6A/E6A individuals, 3 heterozygous E6A/E6I individuals and 1 tree homozygous for E6I. Similar patterns of variation were found in stands B and C.

From the segregration patterns observed for the presence and absence of FGE6, gene frequencies were calculated and used to test the hypothesis that E6A and E6I were in Hardy-Weinberg equilibrium both within each of the three stands and among all three stands (Table 5). All chi square tests indicated that there was no significant deviation from Hardy-Weinberg equilibrium in any stand and pooled data indicated that the three stands taken together were also in Hardy-Weinberg equilibrium.

Morphological Variation. All morphological characteristics measured varied significantly among trees and half-sib progeny within each stand. However, significant among stand differences were not observed for all characters (Table 6). Needle lengths in stand B were significantly shorter than needles

Table 2. *Results of chi square and D^2 analysis of needle peroxidase (PN), esterase (EN) and seedling needle peroxidase (SPN) frequencies*

Isoenzyme		Population Comparison A–B Chi. Sq.	A–B D^2	B–C Chi. Sq.	B–C D^2	A–C Chi. Sq.	A–C D^2
PN	1	4.250 NS	0.427*	0.057 NS	−0.118 NS	3.137 NS	0.299 NS
	3	0.014 NS	0.002 NS	0.161 NS	−0.106 NS	1.129 NS	0.621 NS
	6	3.290 NS	0.650*	2.921 NS	0.642*	0.000 NS	0.000 NS
	7	10.008*	1.247*	1.953 NS	0.131 NS	3.388 NS	0.313 NS
EN	2	17.934*	4.367*	21.351*	5.653*	0.080 NS	−0.044 NS
	3	0.000 NS	−0.072 NS	0.001 NS	−0.067 NS	0.161 NS	−0.125 NS
SPN	4	0.075 NS	−0.058 NS	1.697 NS	0.050 NS	1.067 NS	0.010 NS
	5	0.568 NS	−0.300 NS	0.000 NS	0.000 NS	0.568 NS	−0.030 NS

* indicates means differ at 95% probability level.

Table 3. *Results of chi square analysis of presence and absence of female gametophyte esterase 6 (FGE6)*

Stand	Tree No.	Macro-gametes Analyzed	# Complete*	# Incomplete**	Chi. Sq.+	P
A	1–8, 10–13, 15	20	20	0	—	—
	9	20	0	20	—	—
	14	40	22	18	0.40	.5–.7
	16	40	17	23	0.90	.3–.5
	17	40	18	22	0.40	.5–.7
B	1, 3–7, 9, 13–17	20	20	0	—	—
	2	20	0	20	—	—
	8	40	20	20	0.00	>.99
	10	40	18	22	0.40	.5–.7
	11	40	22	18	0.40	.5–.7
	12	40	20	20	0.00	>.99
C	1, 2, 5–7, 14, 15	20	20	0	—	—
	3, 9	20	0	20	—	—
	4	40	22	18	0.40	.5–.7
	8	40	18	22	0.40	.5–.7
	10	40	20	20	0.00	>.99
	11	40	21	19	0.10	.7–.8
	12	40	16	24	1.60	.2–.3
	13	40	20	20	0.00	>.99

+ Assuming 1:1 segregation.
* Complete = FGE1–FGE9
** Incomplete = FGE1–FGE5, FGE7–FGE9

Table 4. *Frequency of homozygous E6A/E6A, heterozygous E6A/E6I and homozygous E6I/E6I individuals within each of the three populations A, B and C*

Stand	# Trees Analyzed	Number of trees possessing genotype:		
		E6A/E6A	E6A/E6I	E6I/E6I
A	17	13	3	1
B	17	12	4	1
C	15	7	6	2
$\Sigma = 49$		32	13	4

in stands A and C. Number of stomatal rows were significantly greater in trees of stand A than B or C while number of needle margin serrulations were not different among stands. In the seedlings derived from the trees in the three stands no significant among stand differences were observed for any of the characteristics measured (Table 6).

Discussion

The discovery of an esterase gene marker in the gametophyte tissue with gene frequencies conforming to Hardy-Weinberg equilibrium provides evidence to suggest that all three stands were formed from a single panmictic population. In spite of this, it appears that the three stands are significantly different in both isoenzyme frequencies and morphological characteristics. Similar among stand differences have been shown by other researchers (Sakai and Park, 1971; Sakai, Miyazaki and Matsuura, 1971). The causal mechanisms responsible for the apparent genetic differentiation among stands may be threefold:

1. Differences observed may be due to environmental modification of isoenzyme and morphological characteristics.
2. Random drift may account for the differences.
3. Microsite characteristics may have been sufficiently different within each stand to cause differential selection pressures on genetically similar seedling populations resulting in genetic differentiation among stands.

The current study does not permit definition of those factors responsible for the observed stand differences. Environmental factors may of course

Table 5. *Results of chi square analysis for Hardy-Weinberg equilibrium of gene frequencies E6A (p) and E6I (q)*

Stand	No. of Individuals (N)	Frequency E6A (p)	Frequency E6I (p)	Obs. p^2N	Exp. p^2N	Obs. $2pqN$	Exp. $2pqN$	Obs. q^2N	Exp. q^2N	X^2	df	P
A	17	0.853	0.147	13	12.38	3	4.26	1	0.37	1.48	1	.3–.5
B	17	0.823	0.177	12	11.51	4	4.95	1	0.53	0.62	1	.7–.8
C	15	0.667	0.333	7	6.66	6	6.66	2	1.66	1.04	1	.5–.7
A + B + C										3.14	5	.5–.7

Table 6. *Results of analysis of morphological characteristics of P. pungens parent trees and their progeny*

Group	Character	Stand mean value, stand identification, and results of range test*		
Parent Trees	Needle length (mm)	70.9 (C)	69.9 (A)	65.0 (B)
	# Rows of Stomates on Needle:			
	Upper Surface	11.6 (A)	10.9 (B)	10.6 (C)
	Lower Surface	17.6 (A)	16.4 (B)	16.4 (C)
	Serrulations on Needle Edge (#/cm)	25.4 (A)	25.0 (B)	26.7 (C)
Seedlings	# Cotyledons	5.97 (A)	6.14 (B)	5.88 (C)
	Cotyledon Length (mm)	32.1 (A)	32.7 (B)	31.3 (C)
	Seedling Height (mm)	52.9 (A)	52.2 (B)	49.3 (C)
	Needle Length (mm)	16.4 (A)	15.8 (B)	17.4 (C)

* means with a common underline do not differ at a probability of 95% or more.

influence biochemical and morphological *in situ* measurements. However, the stands sampled were all on similar SW exposures and soil types. Altitudinal differences among stands varied only 10 to 60 m. Thus, it would seem likely that environmental differences among stands would not have a great effect on the measurements made.

Random drift may be a plausible mechanism to account for the observed differences. *P. pungens* is a pioneer species in the Applachians, usually becoming established on burned areas. It is conceivable that the stands studied were each derived from only a very few "seed" trees. Consequently, the presumed genetic differences among stands may simply be due to sampling error in a single generation. If indeed the stands were each derived from many original "seed" trees, microsite selection pressures at each stand location may have been sufficiently different to cause genetic differentiation. Alternatively, differences observed may be due to a combination of these factors.

The results of morphological and isoenzyme analysis of progeny from the three stands indicated no significant among stand differences, although significant half-sib family differences were evident for all characteristics measured. There are at least two plausible interpretations of this data. It is possible that the three mature stands studied were insufficiently isolated to prevent pollen exchange among stands. If indeed the three stands were panmictic, then seedlings grown under greenhouse conditions in the absence of natural selection pressure would not be genetically different. Conversely, if the stands are sufficiently isolated t prevent random pollen exchange, it is also likely that among stand progeny differences would not exist. This would be particularly true considering the evidence presented earlier indicating that the mature stands might have differentiated because of selection pressure exerted over a single sporophyte generation. Relaxation of selection pressure under greenhouse conditions would decrease or eliminate stand differences even if they did exist in nature. In addition, the juvenile characteristics measured in progeny may have little correlation with the mature characteristics measured in the parent trees.

The results of this study illustrate some of the problems associated with the use of isoenzyme frequency data for determination of neighborhood size in forest tree populations. The illucidation of frequency differences in needle isoenzymes provides a convenient basis for defining genetically differentiated subpopulations, but little is learned of the dynamic nature of such differentiation. In *P. pungens* it appears that genetic differentiation is only temporary. Although other authors have explained isoenzyme frequency differences by pollen migration distances, (Sakai and Park, 1971) such explanations assume *a priori* that the differences observed are stable from generation to generation. It is apparent from this study that such differences may not be stable or caused by simple isolation followed by selection. This may be particularly true for pioneer tree species such as *P. pungens*. In pioneer species stand location may vary from generation to generation. This might result in periodic genetic encounters among nearby stands resulting in reduced among stand genetic differences. Further study of isoenzyme frequencies and isolation of marker genes in both pioneer and climax forest trees is needed before reliable models of genetic differentiation among adjacent forest tree subpopulations can be developed.

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