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Observations on the Genetic Structure and Mating System of Ponderosa Pine in the Colorado Front Range

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Summary. Variation of peroxidase enzymes is analyzed both in mature needle tissue and in open-pollinated seedling families of ponderosa pine, *Pinus ponderosa*, and is identified as being controlled by a single Mendelian locus. Variation at this locus, analyzed in 1,386 individuals, is used in the analysis of population differentiation and the mating system. Significant variation of gene frequencies is detected over distances of several hundred meters, and is found to be associated with slopes of different aspects. Ponderosa pine is wind-pollinated, and an analysis of the mating system indicates that the level of outcrossing is greater than 90 %. Selection specific for different environments is evidently strong enough to overcome the homogenizing force of migration and produce population fissuring in ponderosa pine.

Key Words: Selection - Migration - Peroxidase - Ponderosa Pine

Introduction

Differentiation of natural populations is viewed as a dynamic process involving the opposing forces of migration and selection. Traditionally, migration has been viewed as an overpowering force, so that differentiation was not expected unless barriers to migration arose (Mayr 1963; Kimura and Ohta 1971). More recently, observations have accrued that indicate that selection in natural populations is of a magnitude that can override the homogenizing forces of migration, even when migration is high (Williams et al. 1973; Jain and Bradshaw 1966; Antonovics et al. 1971; Snaydon and Davies 1972; Schaal 1975; Koehn and Mitton 1972; Linhart 1974). Nevertheless, enhanced differentiation is expected when migration is impeded either by the mating system (Allard 1975; Solbrig 1972) or by low mobility (Berger 1973; Selander and Kaufman 1975). Thus, selection and migration may have impacts of similar magnitudes upon population structure, and differentiation in one species may not be predictive for the population structure of a sympatric species with a slightly different mating system or dispersal rate.

To shed further light on the process of population differentiation, we are conducting studies on a long-lived perennial, *Pinus ponderosa* Laws., the ponderosa pine of western North America. This is a wind-pollinated gymnosperm found in a diversity of natural environments. This first report deals with Mendelian variation of peroxidase enzymes, and the use of this variation to analyze micro-geographic variation and to estimate parameters of the mating system. Many enzymes can be detected in tissues such as endosperm, pollen, and seedlings, but reliance upon flowering may bias sampling. Therefore, this analysis utilized only the fastest anodal peroxidase, which can be detected reliably in adult or seedling needle tissues. At this time, this system is the only one for which we have extensive data. To study micro-geographic variation, gene frequencies and genotypic proportions of trees from adjacent, contrasting environments have been estimated.

The wind-borne pollen of ponderosa pine is expected to provide effective migration over vastly greater distances than the 50-200 m separating the N- and S-slope populations sampled at each location. But the migration (pollen clouds can be seen in the air during anthesis) may not be effective if the trees are substantially self-pollinating, or if there are differences in phenology (eg. pollen anthesis and female receptivity) between sub-populations. Forest trees are known to be predominantly outbred (Stern and Roche 1974), but it is also known that this parame-

Table 1. Comparison of physical and biotic conditions on N- and S-facing slopes and ridge tops of Lower Sugarloaf Mountain (elev. 2130 m) and ridge top at Glacier Lake (elev. 2590 m) (From Marr 1961 and personal observation)

Conditions	Sugarloaf			Glacier Lake
	N	Ridge	S	Ridge
Physical				
Air Temperatures Dec.-Feb. Ranges (°C)				
Maxima	12 to 14	14 to 16	17 to 21	12 to 17
Mean Daily Max	3 to 6	4 to 7	6 to 10	2 to 5
Minima	-18 to -17	-19 to -17	-18 to -17	-20 to -21
Mean Daily Min.	- 8 to - 3	- 8 to - 1	- 7 to 2	-11 to - 7
Soil Temp. at 15 cm Dec.-Feb. Ranges (°C)				
Maxima	- 1 to 1	0 to 1	8 to 10	- 1 to 2
Mean Weekly Max.	- 2 to - 1	0 to 0	3 to 5	- 2 to - 1
Minima	- 5 to - 3	- 4 to - 2	- 2 to - 1	- 6 to - 3
Mean Weekly Min.	- 3 to - 2	- 2 to - 1	- 1 to 1	- 4 to - 3
Total Months when Minimal Soil Temperatures < 0°C				
at 15 cm depth	4	3	0	4
at 33 cm depth	4	3	0	4
Months of Snow Depth of 15 cm or more	6	2	3	6
Total Yearly Precipitation (mm of rainfall)	513	513	498	538
Total winds Dec.-Feb. (km)	21,381	30,244	19,130	37,576
Biotic				
Total Trees per 20 × 20 m quadrat	75	49	42	variable
Shrubs: % area covered	7.1	1.3	0.8	9.4
Herbs: % area covered	6.0	12.2	18.0	9.4
Species distribution				
Juniperus virginiana		X	X	X
Yucca glauca			X	
Opuntia rafinesquei			X	
Pseudotsuga menziesii	X	X		X
Amelanchier alnifolia	X			
Populus tremuloides	X	X		X
Pinus ponderosa	X	X	X	X
Pinus flexilis				X

ter of the mating system is subject to natural selection in plants (Kahler et al. 1976).

Diversifying selection in the adjacent environments was expected on the basis of manifest differences in biotic and abiotic components of the environment (Table 1). There are clear differences in air and soil temperatures, snow accumulation, and sunlight intensity with aspect. The distribution of some plant species also changes dramatically with aspect. Marr (1961) has shown that species characteristic of mesic habitats (eg. various mosses, *Populus tremuloides*, *Amelanchier alnifolia*) are often restricted to N-facing slopes, while species of more xeric habitats (eg. *Yucca glauca*, *Opuntia rafinesquei*) are restricted to S-facing slopes. We expected that factors influencing the distribution and abundance of species would also influence the genetic constitution of populations. We also expected that genetic differences between populations on N- and S-facing slopes would be consistent with, but smaller than differences between populations in more xeric and mesic sites separated by greater distances.

Materials and Methods

A. Field Collections

Trees were selected at random and sampled for needles in four localities for comparison of allele frequencies in subpopulations of N- and S-facing slopes. Seventy to ninety-six trees per slope were collected at each locality. The localities (all in Boulder County) included Left Hand Canyon, Coal Creek Canyon, Boulder Canyon and a ridge on the lower portion of Sugarloaf Mountain called Site A by Marr (1961). The comparison between N and S slopes is a comparison between relatively mesic versus xeric habitats. To extend these observations to greater geographic and environmental contrasts, three additional collections were made. One hundred and ninety trees were collected at an elevation of 2590 m, at a cool mesic site near Glacier Lake, called Site B by Marr (1961). This collection was made to compare the results to populations at similar altitudes at Left Hand Canyon and Coal Creek Canyon; furthermore, the trees sampled were shedding pollen and genotypes were derived from pollen samples. Ninety-six trees were collected at a relatively xeric habitat at a grassland-forest ecotone near Eldorado Springs, Boulder, Colorado, altitude 1760 m. Finally, 41 trees were collected in the easternmost outlier of ponderosa pine in Colorado, a very xeric site at Cheyenne Lookout, near Limon, 170 km south-east of Boulder. Environmental data collected at A and B by Marr (1961) illustrate the differences in climatic and biotic variables which can be expected between N- and S-facing slopes and between elevations (Table 1). The variables cited in the table exemplify the differences in physical conditions between the various sites. Only December to February data are shown because during these months the sun is lower on the horizon and north-south differences are maximal and produce relatively mesic conditions on N-facing slopes (Marr 1961).

Maternal trees used to analyze the mating system were taken from the ridge top of lower Sugarloaf Mountain between northern and southern exposures.

B. Electrophoresis

Peroxidase activity for several putative loci is evident in the preparations of adult needle tissue, seedling tissue, pollen, and gametophyte tissue. This report deals with the peroxidase activity that migrates fastest anodally, and is present in adult needles, seedling tissue, and pollen. The migration rates in these tissues are the same. The bands appearing in this area fit the expectation of variation due to a Mendelian locus (see results). These bands appear reliably on gels from extracts of fresh tissue, tissue frozen at -60°C for more than one year, and tissue collected at different times of the day or year. Samples were prepared for electrophoresis by cutting needle tissue in lengths of 3 to 5 mm, immersing it in an equal volume of distilled water, and disrupting cells with ultrasonic sound for approximatley 10 seconds. Preparation of tissue by grinding with mortar and pestle was avoided because this method produces additional bands that obscure the simple pattern of variation. Polyvinylpolypyrrolidone may be added to concentrate the sample, but does not seem to be necessary.

Samples were absorbed onto filter paper, inserted into horizontal starch gels, and subjected to electrophoresis in a discontinuous tris-citric acid buffer system (gel buffer pH 7.7, .15 M tris, .003 M monohydrate citric acid; electrode buffer pH 7.5, .031 M sodium hydroxide, .300 M anhydrous boric acid). After the borate front had moved 6 cm past the origin, gels were sliced and stained by the method of Shaw and Prasad (1970).

C. Genetic Analyses

Variation postulated to arise from the segregation of the three alleles of a locus was tested in open-pollinated families to determine if it was strictly Mendelian. Trees were identified as being a particular genotype, and seeds collected from that tree were stratified for 4 weeks, germinated, grown in pots in the greenhouse, and then identified for their peroxidase genotypes.

Homogeneity of gene frequencies was tested with the method of Workman and Niswander (1970), and gene frequencies in the pollen and the level of selfing were estimated from seedling progeny arrays by the method of Brown and Allard (1970). Alleles were designated Per-2^1, Per-2^2, and Per-2^3, with Per-2^1 being the fastest migrating of the three alleles. The migration rates of these alleles, relative to the borate line, are 0.77, 0.71 and 0.64 respectively. The Per-2^1 allele was absent in 5 of 11 collections, and never attained a frequency greater than 0.036. Because of its rarity and for easons discussed below, the rate Per-2^1 allele has been pooled with the allele Per-2^3.

Results

Table 1 shows that there are differences in physical conditions between north- and south-facing slopes which result in the former being much more mesic and supporting a different flora from the latter. On the ridge top, conditions tend to be intermediate between those of the two slopes with the exception of degree of exposure to winds; winds are more severe on ridge tops. At higher altitudes such as Glacier Lake, more mesic conditions and higher winds prevail.

In all progeny analyses, the peroxidase variation behaved as proper Mendelian units; for example, trees of genotypes Per-2^{22} produced Per-2^{12}, Per-2^{22} and Per-2^{23}, but never Per-2^{33} progeny. Trees of genotype Per-2^{23} could produce progeny of genotypes Per-2^{12}, Per-2^{22}, Per-2^{23}, or Per-2^{13}. All but Per-2^{13} were observed among the progeny of such trees.

The four comparisons of adjacent north- and south-facing slopes disclosed significantly different gene frequencies between slopes in Left Hand Canyon and

Table 2. Allele and genotypic frequencies of a peroxidase locus at several localities sampled

Sample locality	Aspect	Elevation (m)	Genotypes		
			22	23	33
Glacier Lake		2590	135 (129.7)	44 (54.5)	11 (5.7)
Left Hand Canyon	North Facing	≃2440	60 (60.4)	11 (10.1)	0 (0.4)
	South Facing	≃2440	44 (45.1)	26 (23.8)	2 (3.1)
Coal Creek Canyon	North Facing	≃2440	62 (61.8)	26 (26.4)	3 (2.8)
	South Facing	≃2440	55 (56.1)	24 (21.8)	1 (2.1)
Lower Sugarloaf Mountain	North Facing	2130	41 (42.2)	29 (26.6)	3 (4.2)
	South Facing	2130	23 (31.6)	52 (34.8)	1 (9.6)
Boulder Canyon	North Facing	1738	54 (56.5)	37 (32.0)	2 (4.5)
	South Facing	1738	40 (45.6)	51 (39.7)	3 (8.6)
Eldorado Springs		1760	26 (35.0)	64 (45.9)	6 (15.1)
Cheyenne Lookout		1770	2 (7.5)	31 (20.1)	8 (13.4)

Note: Numbers in parentheses are those expected under the assumptions of the Hardy-Weinberg model. X_A^2 tests the fit to Hardy-Weinberg expectations, and X_B^2 tests the homogeneity of gene frequencies between slopes of different aspect. X_C^2 tests the homogeneity of mean gene frequencies for all localities. *, **, *** indicate significance at the .05, .01, and .001 levels. x indicates a probability $> .05$ and $< .10$. Per 2^2 is the mean frequency of Per-2^2 at that locality.

on lower Sugarloaf. In Boulder Canyon the difference approaches significance ($.05 < P < .1$), but no difference is observed in Coal Creek Canyon (Table 2). In all cases where differentiation is evident, the disparity between slopes is similar. South-facing slopes have a higher frequency of the slowest migrating allele (Per-2^3).

An analysis of these repeated tests for differences of gene frequencies between slopes was performed following Sokal and Rohlf (1969 p. 581). This analysis indicates that there is probably heterogeneity for these differences between sampling localities (see Table 2) but that the overall effect of slope on gene frequencies at these four localities is significant ($P < .001$).

The Glacier Lake population, assayed on the basis of pollen samples, shows allelic and genotypic frequencies comparable to those in Left Hand Canyon and Coal Creek Canyon, both of which are at similar altitudes and are similarly mesic (Table 1). The Eldorado Springs population, at the grassland-forest ecotone, is the most xeric of the mountain sites, and shows a significant increase in Per-2^3 compared to the populations at higher altitudes. The Cheyenne Lookout population represents the most xeric of the sites sampled and is the easternmost ponderosa pine population in Colorado. Per-2^3 has by far the highest frequency in that population.

The fit of the observed genotypic distributions at each sampling locality to Hardy-Weinberg expectations is measured by X_A^2 in Table 2. Xeric, low elevation, or south-facing slopes show a significant excess of heterozygotes from expectations. These sampling localities include Cheyenne Lookout, Eldorado Springs, and the S-facing slopes of Boulder Canyon and lower Sugarloaf Mountain. At the higher elevation, more mesic sites, the S-facing slopes show slight but non significant excesses of heterozygotes;

for ponderosa pine, *Pinus ponderosa*

N	Per 2^2 freq. ± S.E.	X^2_A	X^2_B	Per 2^2 freq. ± S.E.	X^2_C
190	.826 ± .019	7.1**		.826 ± .019	
71	.923 ± .022	0.5	10.0***	.857 ± .021	
72	.792 ± .034	0.6			
91	.824 ± .028	0.1	0.1	.830 ± .020	
80	.837 ± .029	0.8			
73	.760 ± .035	0.6	4.7*	.701 ± .027	114.1***
76	.645 ± .039	18.5***			
93	.780 ± .030	2.3	3.3x	.738 ± .023	
94	.697 ± .034	7.6**			
96	.604 ± .035	14.9***		.604 ± .035	
41	.427 ± .055	12.2***		.427 ± .055	

Table 3. Results of analysis of progeny arrays of ponderosa pine from the ridge top of Lower Sugarloaf Mountain, Colorado f(2) is the frequency of allele Per-2^2, and t is the rate of outcrossing

	Genotypes			f(2)	t
	Per-2	Per-23	Per-3		
Maternal Trees	25	8	0	Maternal 0.879 ± 0.04	0.958 ± 0.51
Seedlings	298	72	6	Pollen Pool 0.820 ± 0.28	

Note: The standard errors for the allele frequencies are calculated for observed frequencies of maternal genotypes, and taken from the joint estimation procedure for the pollen allele frequencies and rate of selfing.

N-facing slopes do not. The population at Glacier Lake, based on pollen samples, has a deficiency of heterozygotes.

Table 3 shows genotypic and allelic frequencies of the maternal trees, genotypic frequencies of seedlings, allelic frequencies in the pollen pool, and the rate of outcrossing, estimated from progeny arrays by the method of Brown and Allard (1970). Within the sample of 33 families available for this analysis, 25 were homozygous Per-2^2, and 8 were heterozygous for alleles Per-2^2 and Per-2^3. No Per-2^3 homozygotes were observed and the estimated frequency of the common allele is .879 ± .04. The maximum-likelihood procedure of Brown and Allard (1970) estimates the frequency of the common allele in the effective pollen pool to be .820 ± .28, and the rate of outcrossing to be .958 ± .51.

Discussion

Theoretical discussions (eg. Mayr 1963; Kimura and Ohta 1971) present migration as a strong unifying factor, opposing diversifying selection. Empirical data suggest that gene flow in nature is often quite restricted, both in wind-pollinated (Colwell 1951; Wolfenbarger 1959) and animal-pollinated plants (Levin and Kerster 1968; Kerster and Levin 1969; Ehrlich and Raven 1969; Linhart 1973; Schaal 1975). These results have led Bradshaw (1972) to suggest that in plant populations demes are very small, often a few meters in diameter. The extent of gene flow does affect the magnitude of differentiation in *Drosophila* (Endler 1973), molluscs (Berger 1973) and plants (Jain and Bradshaw 1966; Barber 1975). Differentiation may be an ephemeral phenomenon, dependent upon the relative magnitudes of two variables, the rate of migration, and the intensity of selection.

There is no general agreement about the extent of potentially effective gene flow in conifers. Although the majority of pollen from a point source falls within a hundred meters (Libby et al. 1969; Levin and Kerster 1974), clouds of pollen have been reported to cover remarkable distances (Koski 1970; Tigerstedt 1973; Stern and Roche 1974; Hamrick 1976). The effectiveness of pollen flow is thought to be density-dependent even in wind-pollinated species (Gleaves 1973), with the effective distance of pollen flow decreasing with both the density of pollen and the number of pollen sources. Therefore, the clouds of pollen seen during anthesis in pine forests do not necessarily demonstrate high gene flow.

Pollen flow over great distances would not indicate high rates of gene flow in predominantly inbred species. In such species, gene flow is predominantly via seeds, not pollen. However, our estimate of the rate of outcrossing indicates that the trees on the ridge top of lower Sugarloaf Mountain are producing most of their progeny by outcrossing. Our observation is consistent with other observations on the level of outcrossing in forest trees (reviewed in Squillace 1974). The visible movement of pollen clouds, coupled with estimates of high rates of outcrossing, suggests the potential for high gene flow, at least between populations on adjoining slopes. In this context, the differentiation reported here over distances of approximately 100 m is striking. A reasonable conclusion extracted from these observations is that although pollen flow is substantial, it is overridden by diversifying selection. Additional evidence for this selection is found in the deviation from expected genotypic frequencies, with excesses of heterozygotes on S-facing slopes and in the most xeric environments.

The differentiation observed here may seem surprising in a population of mixed age classes of a perennial with a long generation time and high rates of outcrossing. A number of workers including Barber (1965), Callaham and Liddicoet (1961), Hamrick (1976) and Fryer and Ledig (1972) have found differentiation between tree populations which, by virtue of their proximity, could be interbreeding. However, we believe that our observation of genetic differentiation between adjacent microhabitats is somewhat novel, and indicates that this phenomenon, already reported in herbaceous plants, also occurs in trees. Similar differentiation over a few hundred meters has been found to be associated with growth forms of Engelmann spruce *(Picea engelmannii)* and subalpine fir *(Abies lasiocarpa)* near tree line in Colorado. Comparisons between krummholz, radially symmetrical spire trees and the intermediate flag trees show highly significant differences in allelic frequencies between these morphs (Grant and Mitton 1977).

Long-generation plants may not have the same response to environmental heterogeneity as short-generation plants, for the response to heterogeneity depends upon the length of cycles of environmental variation relative to generation time (Slobodkin 1964, 1968; Long 1970). Trees, however, generally have the vast majority of their mortality occurring in the seedling stage, and it is at just this stage of the life cycle that the environmental parameters that differ between N- and S-facing slopes (Table 1) would have the greatest effect on survival. Such selection can be intense in ponderosa pine; for example, in the southwestern USA, about 100 seeds are necessary to obtain one seedling under "moderately favorable field conditions" (Schubert 1974). Evidently the environmental heterogeneity sampled here is of sufficient magnitude and persistence to cause population differentiation in a life cycle and mating system least inclined to exhibit it.

There is a satisfying consistency to the patterns of allelic frequencies and genotypic distributions in our samples. The differences between our cool, moist (high elevation) and warm, dry (low elevation) sample localities extend our observations on the genetic differences between populations on slopes of different aspect. Specifically, allele Per-2^3 is in higher frequencies in warmer, more xeric environments, and as expected, differences between sample localities are greater than differences between slopes within a locality. The pattern of heterozygote excesses is also consistent with the patterns of geographic and microgeographic variation of allelic frequencies. The frequency of heterozygotes significantly exceeds equilibrium expectations in warm and dry localities and slopes, and this excess of heterozygotes generally contributes to the elevated frequency of Per-2^3 in xeric sites. Preliminary observations on the kinetics of the peroxidase genotypes provide a mechanistic basis for these patterns; genotypes differ in their activities at different temperatures, and these kinetic properties are consistent with the present observations. The kinetics data suggest similar performances for the enzymes coded by alleles Per-2^1 and Per-2^3. Per-2^1 is rare, and although present at Glacier Lake, Lower Sugarloaf Mountain, and Boulder Canyon, it never exceeds a frequency of .036. Consequently, we have pooled alleles Per-2^1 and Per-2^3 for statistical analyses.

There are several observations which do not neatly fit the general patterns of variation summarized above. Although differentiation of allelic frequencies between slopes of different aspect were noted at several localities, no difference was detected in Coal Creek Canyon. We have no ready explanation for the homogeneity of allelic frequencies at this site.

At lower Sugarloaf Mountain, the allelic frequencies of maternal trees and their progeny are not intermediate between the allelic frequencies of populations on either slope. This may be due to several reasons. One is that this sample may be biased, for it includes only cone-bearing trees. Another reason may be that the ridgetop may not be ecologically intermediate between the two slopes. As Table 1 shows, wind speeds are much higher on the ridgetop, and wind damage is much more extensive; perhaps due to this damage, there is an extensive infestation of dwarfmistletoe *(Arceuthobium vaginatum)* that does not extend onto either of the adjacent slopes or our other sites. In the southwest, such infestations are often heaviest on ridgetops (Hawksworth 1959). The Glacier Lake population stands out by having a significant deficiency of heterozygotes. Again, there is a possible bias in these data, for all individuals sampled bore male strobili. Similar deficiencies of heterozygotes have been noted in studies that relied on flowering individuals of *Pinus sylvestris* (Rudin et al. 1974) and *Eucalyptus obliqua* (Brown et al. 1975).

Finally, the standard errors on the allelic frequencies in the pollen pool and on the rate of outcrossing are surprisingly large. The magnitudes of these standard errors are not something inherent in the estimation procedure, but are a product of the data. In addition, the allelic frequency of the pollen does not match the frequency of the maternal genotypes or the resulting progeny. These problems may stem from various complexities of the population biology of ponderosa pine that do not precisely fit the assumptions of the model of Brown and Allard (1970). For example, the pollen reaching any individual tree may not be a random sample of the population, and seed development may not be independent of genotype (Sarvas 1962). In addition, the population may be composed of clusters of related individuals, rather than a random spacing of genotypes (Coles and Fowler 1976).

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