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VARIATION IN ALLOZYMES AND STOMATAL SIZE IN PINYON (*PINUS EDULIS*, PINACEAE), ASSOCIATED WITH SOIL MOISTURE[1]

JEFFRY B. MITTON, MICHAEL C. GRANT, AND AYAKO MURAYAMA YOSHINO

Department of Environmental, Population, and Organismic Biology, Campus Box 334, University of Colorado, Boulder, Colorado 80309

Microgeographic allozyme variation was examined in pinyon pine, *Pinus edulis*, among five collection sites in Owl Canyon, Colorado. Relatively dry and moist sites were identified by associated plant communities and the sizes and densities of trees. Three moist sites and two dry sites were compared, and because all sites were within 600 m of one another, isolation by distance was not considered as a viable explanation of genetic differentiation between sites. Allelic frequencies at glycerate dehydrogenase (*Gly*) differed by 14% between moist and dry areas, and the pattern of microgeographic variation found here—allele 3 higher in frequency on dry sites—was consistent with previous studies of microgeographic variation in contrasting moist and dry sites. Trees within one of the dry sites were examined to test the hypothesis that stomata sizes and densities are heterogeneous among *Gly* genotypes. Heterozygotes had the longest and widest stomata; the stomatal area of heterozygotes was 28% greater than the stomatal area of homozygotes. Whereas the stomatal areas of the two homozygotes were similar, their shapes did not overlap when projected on a bivariate plot of length and width. These results suggest that stomatal shape may play a role in adapting pinyon to heterogeneity in soil moisture.

Key words: allozymes; microgeographic variation; pinyon; Pinaceae; *Pinus edulis*; soil moisture; stomata.

Natural selection can adapt populations to specific environmental conditions, and can establish and maintain genetic differentiation among populations (Antonovics and Bradshaw, 1970; Antonovics, 1971) even when the differentiation is opposed by gene flow (reviewed in Linhart and Grant, 1996). Microgeographic variation is most clearly attributed to natural selection when it occurs repeatedly within the distances of typical gene flow. Adaptive microgeographic variation for dozens of characters has been documented in plant species growing in many different types of heterogeneous habitats (e.g., McNeilly, 1968; Antonovics and Bradshaw, 1970). Additional examples of microgeographic variation are presented in Linhart and Grant (1996).

Although the extensive gene flow mediated by windborne pollen minimizes the likelihood of detecting microgeographic variation in conifers, examples of microgeographic variation in response to environmental heterogeneity have been reported. For example, the peroxidases of both Engelmann spruce (*Picea engelmannii*) and subalpine fir (*Abies lasiocarpa*) exhibit strong clinal variation across tree line in the Colorado Front Range (Grant and Mitton, 1977). Kinetic analyses revealed that the thermal optima differ among the enzymes of the peroxidase polymorphism in ponderosa pine, *Pinus ponderosa*, and these differences may explain changes in genotypic frequencies with elevation, with slope aspect, and with success at invading grasslands (Mitton et al., 1977; Beckman and Mitton, 1984). The *Pgm* locus in Engelmann spruce is consistently differentiated in adjacent wet and dry sites (Stutz and Mitton, 1988; Mitton et al., 1989).

Pinyon pine or pinyon (*Pinus edulis* Engelm) exhibits microgeographic variation between starkly contrasting soil types (Cobb, Mitton, and Whitham, 1994). The contrasting soils are normal sandy-loam and a deep bed of cinders produced by the eruption of Sunset Crater, near Flagstaff, Arizona. The cinder soil imposes both water and nutrient stresses on plants (Mopper et al., 1991). In replicated comparisons, the frequency of the slow allele (*S*) at the glycerate dehydrogenase locus (*Gly*) was higher on cinder soils than on adjacent sandy-loam soils (Cobb, Mitton, and Whitham, 1994; Mopper et al., 1991). In addition, Cobb, Mitton, and Whitham (1994) found significant differences in the growth rate and fitness of *Gly* genotypes on cinder soils. They reported that the growth rates were highest in *SS* homozygotes, intermediate in heterozygotes, and lowest in *FF* homozygotes. Probabilities of survival on the cinder soils followed the same pattern as the growth rates. These data suggest microgeographic variation in pinyon might be detectable among environmental patches differing in the availability of water. Given these results, we predicted that (1) allelic frequencies at *Gly* vary among environments differing in water availability and (2) the slow allele would reach its highest frequencies in dry environments.

Additionally, previous work with ponderosa pine on a broad geographic scale demonstrated that there were heritable differences in stomatal sizes between geographic races (Grant, Linhart, and Monson, 1989). Differences between races of ponderosa pine appear to be associated with differences in moisture, for pines derived from comparatively drier habitats in the Rocky Mountains exhibited significantly lower stomatal densities than those derived from parents found in more mesic environments in

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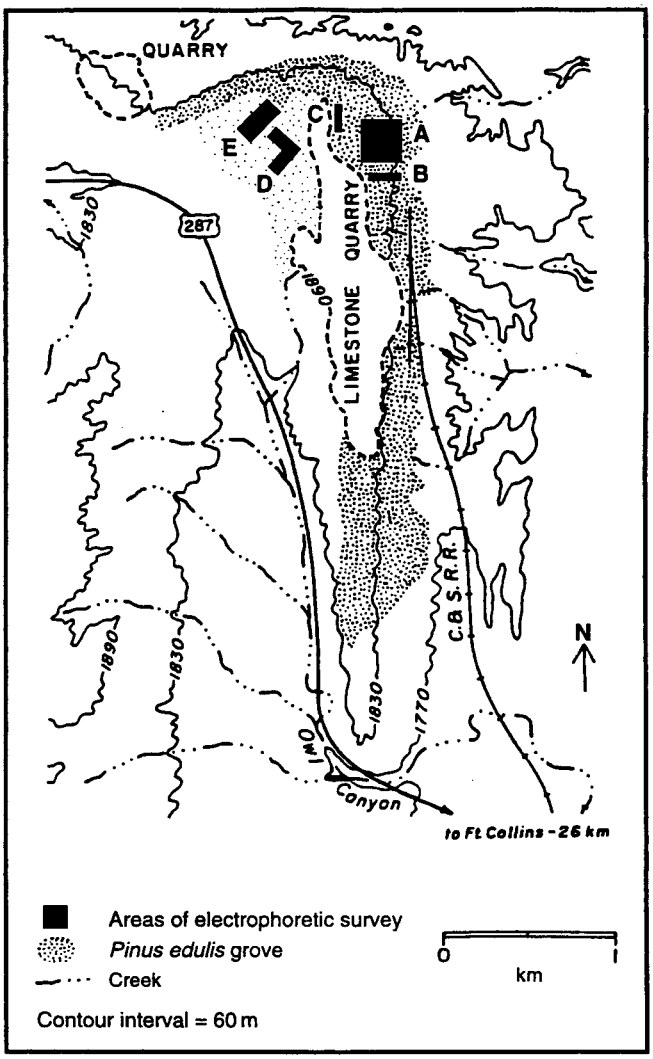

Fig. 1. Pinyon pine sample sites at Owl Canyon, 26 km north and west of Fort Collins, Colorado.

the Sierra Nevada. We set out to determine whether similar patterns could be detected on a microgeographic scale.

We tested these predictions in an isolated population of pinyon at Owl Canyon, Colorado (Betancourt et al., 1991; Premoli, Chischilly, and Mitton, 1994), near its northern limit of distribution along the Front Range. Judging from the density, the sizes of trees, and associated vegetation, local sites within the population vary in water availability. We tested for heterogeneity of allelic and genotypic frequencies among relatively moist and dry sites. We also compared the size, shape, and density of stomata among the *Gly* genotypes.

MATERIALS AND METHODS

The sites sampled at Owl Canyon are illustrated in Fig. 1. The data for site A are from Betancourt et al. (1991), and they contain a sample of the largest and oldest individuals. Site B has a set of large trees growing along an intermittent stream that drains to the east. Site C contains stunted, wind-twisted trees growing along a ridge immediately west of site A. Sites D and E are on the rolling fields below the escarpment, to the west of sites A, B, and C. Site D contains 50 trees permanently numbered for this study. Sites E (100 m northwest of D) and D are ecologically similar.

Table 1. Allelic and genotypic frequencies at the *Gly* locus in pinyon pine, *Pinus edulis*, at five sites at Owl Canyon, Colorado.

Site	Gly genotypes 22	23	33	$p \pm 1$ SE	χ^{2a}	F
A	26	21	4	0.72 ± 0.045	0.00	−0.012
B	16	14	0	0.77 ± 0.055	1.15	−0.304
C	22	19	3	0.72 ± 0.048	0.08	−0.062
D	14	26	10	0.54 ± 0.050	0.11	−0.047
E	18	27	5	0.63 ± 0.048	1.25	−0.158

Note: F is the inbreeding coefficient, and p is the frequency of the *Gly* 3 allele. Allelic frequencies are heterogeneous among the sites ($\chi^2 = 16.61$, $P < 0.05$); χ^{2a} tests the fit of genotypic frequencies to Hardy-Weinberg equilibrium expectations.

Sites D and E are characterized as dry due to their low density of small trees, the predominance of mountain mahogany, *Cercocarpus montanus*, and the presence of both yucca, *Yucca glauca*, and prickly pear cactus, *Opuntia compressa*. The presence of these plants suggests relatively low availability of water. Sites A, B, and C are within 300 m of one another and are characterized as relatively moist, because they have high densities of trees, the trees at sites A and B are large for pinyon pines, and these sites lack the three species of plants characteristic of relatively dry sites.

Needle samples from sites B, C, D, E were collected in 1995. Needles were ground with a mortar and pestle under liquid nitrogen, then worked to a slurry with the extraction buffer of Mitton et al. (1979). Homogenates were kept frozen in an ultra-cold freezer until thawed for electrophoresis. Starch gel electrophoresis was carried out with a discontinuous buffer system (Poulik, 1957) for ~2.5 h at 50 milliamps. *Gly* is a dimer, so heterozygotes are triple banded and homozygotes single banded. Three alleles segregate at this locus, and they are numbered 1, 2, and 3, with 1 having the fastest migration rate, and 3 the slowest. Allele 2 here corresponds to allele F, and allele 3 to allele S in Cobb, Mitton, and Whitham (1994).

Homogeneity of allelic and genotypic frequencies among sites were tested with row-by-column tests of independence (Spiess, 1977).

Twenty-nine of the marked trees from site D were resampled in 1996 for analyses of stomata size and density. Fourteen of these were heterozygotes, and 15 were homozygotes. Needle tissue was prepared for the scanning electron microscopy by extracting wax with heptane overnight. Six needles were randomly chosen from each tree, and from the central portion of each needle, five randomly chosen stomata were measured to the nearest 10^{-5} m along the longest axis (length) and then perpendicular to that axis at the widest point (width). The density of stomata was measured on each needle for three fields, 7.5×10^{-8} m^2 in area. Values for the length, width, and density of stomata were the average of 30 measurements, five measurements per needle for six needles for each individual.

RESULTS

The *Gly* locus segregates three alleles at Owl Canyon; the average frequencies of alleles 1, 2, and 3 are 0.02, 0.65, and 0.33, respectively. Because allele 1 is not sufficiently frequent for statistical tests of genotypic frequencies, alleles 1 and 2 were pooled (Table 1). None of these population samples have genotypic frequencies that depart from Hardy-Weinberg equilibrium expectations. Allelic frequencies are heterogeneous among the population samples ($\chi^2 = 12.54$, $P < 0.02$).

Because sites A, B, and C are in proximity, and their

Table 2. *Gly* frequencies in pooled samples of pinyon pine, *Pinus edulis*, from moist and dry sites, Owl Canyon, Colorado.

Site	Gly genotypes 22	23	33	$p \pm 1$ SE	χ^2	F
(Moist) A + B + C	64	54	7	0.73 ± 0.028	1.03	−0.091
(Dry) D + E	32	53	15	0.59 ± 0.035	0.84	−0.092

Note: F is the inbreeding coefficient, and p is the frequency of the *Gly* 3 allele. Allelic frequencies are heterogeneous between moist and dry sites ($\chi^2 = 10.19$, $P < 0.001$), and genotypic frequencies are also heterogeneous between moist and dry sites ($\chi^2 = 10.94$, $P < 0.01$).

plant communities are similar, and allelic frequencies do not differ between them ($\chi^2 = 0.60$, $P > 0.50$), sites A, B, and C were pooled to represent the moist area. Similarly, because the allelic frequencies at sites D and E are not significantly different ($\chi^2 = 1.67$, $P > 0.50$), and these sites are similar ecologically, sites D and E were pooled to represent the dry area (Table 2). Both the allelic frequencies ($\chi^2 = 10.2$, $P < 0.001$) and the genotypic frequencies ($\chi^2 = 10.9$, $P < 0.01$) are significantly different between the moist and dry areas. The difference in allelic frequencies is ~14%. The difference in the frequency of allele 3 between the two areas matches the pattern of variation seen by Cobb, Mitton, and Whitham (1994) and by Mopper et al. (1991); allele 3 is relatively more common in dry areas.

Kruskal-Wallis tests of stomatal lengths, widths, and densities revealed differences among the genotypes (Table 3). Lengths are significantly heterogeneous among the genotypes ($P < 0.001$). Simultaneous comparisons revealed stomatal length differed between genotypes 23 and 22 ($P < 0.01$) and the difference betwee the 22 and 33 homozygotes approached significance ($P < 0.07$). Similarly, the widths were significantly heterogeneous among the genotypes ($P < 0.01$). Simultaneous comparisons revealed that only the difference between the 23 and 33 genotypes reached statistical significance ($P < 0.01$). The densities of stomata did not differ among the three genotypes.

Stomatal areas were calculated from the mean values for the genotypes, using the formula area = πbh, where π is 2.13, and b and h are half the lengths of the major and minor axes of an ellipse. The stomatal areas of the 22 and 33 homozygotes were very similar, but the stomatal areas of the 23 heterozygotes were 28% greater than the stomatal areas of the homozygotes.

Ratios of the mean lengths to the mean widths revealed heterogeneity in the shape of the stomata among the genotypes; the ratios are 1.28, 1.45, and 1.63 for the 22 homozygotes, 23 heterozygotes, and 33 homozygotes, respectively (Table 3). A bivariate plot of stomatal lengths and widths (Fig. 2) revealed that the shapes of the 22 and 33 homozygotes do not overlap. Thus, stomata of the three common genotypes are distinctly different. The stomata of the heterozygotes are intermediate in shape, but largest in area. The stomata of the homozygotes are similar in area, but the stomata of 22 homozygotes are nearly circular, whereas the stomata of 33 homozygotes are long and narrow.

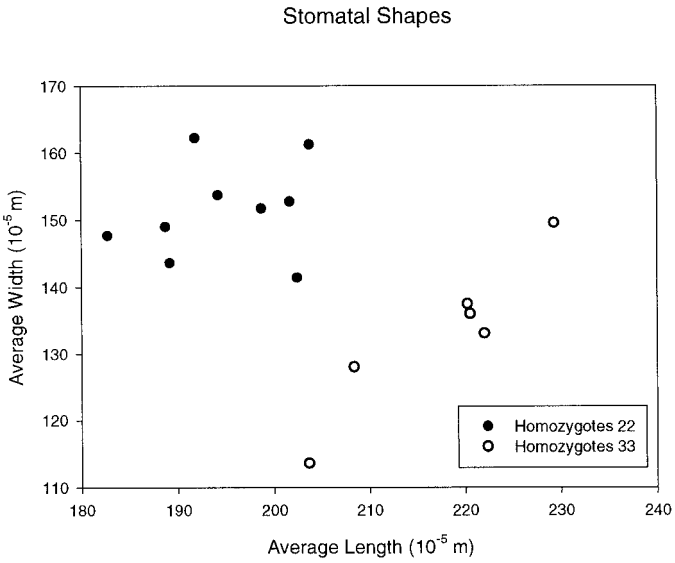

Fig. 2. Scattergram of the average lengths and widths of stomata of 22 and 33 homozygotes at the glycerate dehydrogenase locus in pinyon pine. Units are 10^{-5} m.

DISCUSSION

Since the distances among the collection sites (0.2–0.6 km, Fig. 1) are clearly smaller than (1) the distance of bird dispersal (12–22 km; Tomback and Linhart, 1990) and (2) the distance of pollen flow (Schuster, Alles, and Mitton, 1989; Hamrick, 1987), one would not expect to see any genetic differentiation caused by isolation. Yet consistent patterns of microgeographic variation have been reported in two studies at Sunset Crater, Arizona (Mopper et al., 1991; Cobb, Mitton, and Whitham, 1994)

Table 3. Stomatal lengths, widths, and densities for glycerate dehydrogenase genotypes at Owl Canyon, Colorado.

	Genotypes 22	23	33	χ^2	P
Length (10^{-5} m)	194.82a	232.27b	217.37a,b	19.61	***
SD	7.29	19.15	9.50		
Width (10^{-5} m)	151.45a,b	159.88b	132.91a	11.58	**
SD	7.06	23.78	11.85		
Density (no./7.5 × 10^{-8} m^2)	6.97a	6.68a	7.05a	2.16	NS
SD	0.36	0.56	1.00		
Length/width ratio	1.28	1.45	1.63		
Stomatal area (πbh)	23 161	29 151	22 678		

Note: SD is the standard deviation. χ^2 is the chi-square value from a Kruskal-Wallis nonparametric tests of homogeneity of means. *** indicates $P < 0.001$, ** indicates $P < 0.01$ and NS indicates "not significant." Mean values marked with different letters are significantly different.

and the present study. This repeated pattern of microgeographic variation between moist and dry sites is most parsimoniously attributed to natural selection favoring allele 3 in the relatively dry sites.

Whereas the microgeographic variation in Colorado pinyon pine at Sunset Crater (Mopper et al., 1991; Cobb, Mitton, and Whitham, 1994) was found over a striking difference in soil types, the environmental variation sampled here represents ecological heterogeneity typical in pinyon stands. Thus, this study extends the observations of Mopper et al. (1991) and Cobb, Mitton, and Whitham (1994) into the typical range of environmental variation experienced by pinyon pine. Furthermore, the soil contrasts in the studies at Sunset Crater confounded water and nutrient stresses. Results from the present study are consistent with the hypothesis that moisture is an important variable influencing the microgeographic differentiation of *Gly* frequencies.

The mechanism by which the microgeographic variation has been established is not known, but there are two clear possibilities, one focusing on metabolites, the other focusing on stomata. Cobb, Mitton, and Whitham (1994) pointed out that glycerate dehydrogenase produces serine, one of the precursors of glycinebetaine, which is accumulated in both plants and animals in response to drought stress. Differences among genotypes in pools of glycinebetaine may influence differences in cell turgor and the capability to sustain normal physiological processes during drought stress. The second possible cause for the microgeographic variation is the variation in stomatal size and shape among *Gly* genotypes. These morphological differences may influence needle water balance, adapting the genotypes to slightly different environments. It is also possible that both of these hypotheses are correct; the size and shape of stomata might reflect the turgor and metabolite concentrations in developing needles.

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