The Journal of Heredity 70:86–89. 1979.

Allozyme polymorphisms detected in mature needle tissue of ponderosa pine

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TYPICALLY, plant population geneticists sample populations by collecting seeds from natural populations, germinating the seeds in the laboratory, and electrophoretically determining the genotypes from these open-pollinated families. There are advantages and disadvantages to this technique. One advantage is that a few seedling genotypes from each family in a sample are generally sufficient to estimate the maternal genotype that bore the family, the frequency of alleles in the pollen pool that was effective in pollination, and the effective rate of outcrossing[3]. In addition, pollen, seedling and maternal frequencies may be used to estimate genotypic components of viability and fertility differences[4]. Finally, the mature tissues of many plant species contain chemical compounds that were evolved as defenses against herbivory and that also frustrate the biologist trying to extract enzymes for genetic analyses. These compounds are often absent or have low concentrations in juvenile tissues[11]; hence population biologists work primarily with seedlings grown in greenhouses.

On the other hand, this method is inconvenient and laborious and there are biases associated with the collection of seed from natural populations. For example, for a number of species of conifers, populations are characterized by years of heavy cone production interspersed with years of light cone production. Cone production may vary from 0 percent to 100 percent in a population over just a few years. In addition, not all of the mature individuals set seed in many populations. Thus, the trees producing cones one year may not be representative of the whole population[7,8]. Furthermore, seeds may only be available for sampling for brief periods of time during the year, which may present logistic or scheduling problems.

Presented here is a method to extract enzymes from adult leaf tissue of a diversity of trees. The advantages of this method are that samples may be collected at any time during the year regardless of the reproductive status of the tree or the maturity of the leaf tissue. This method has been used to detect protein polymorphisms in ponderosa pine, *Pinus ponderosa* (see cover plate), lodgepole pine, *P. contorta*, Engelmann spruce, *Picea engelmannii*, subalpine fir, *Abies lasiocarpa*, and quaking aspen, *Populus tremuloides*. In addition, evidence for the Mendelian nature of six protein polymorphisms in ponderosa pine will be presented.

Materials and Methods

The method for preparing adult somatic plant tissue is modified from the method of Kelly and Adams[6]. Mature or juvenile needle tissue may be prepared for electrophoresis in the following way. Tissue is ground to a fine, dry powder in a mortar and pestle under liquid nitrogen. We generally pour the liquid nitrogen into a mortar, and cut needles from a sprig so that they fall into the liquid nitrogen, and then grind until the liquid nitrogen evaporates. If the material is not finely ground more nitrogen is added, and the material is ground again. As the liquid nitrogen evaporates from the finely ground tissue, grinding solution (below) is added to the powder, and this material is worked with a spatula to make a thick slurry. Care should be taken not to allow the powder to come to room temperature; best results are obtained when the grinding solution partially freezes when it contacts ground tissue. The slurry is transferred to a test tube which is stored in an ice bath until centrifugation. The material is centrifuged at low speed in a refrigerated centrifuge for about 5 minutes. The supernatant is applied to wicks and subjected to horizontal starch gel electrophoresis.

To prepare one liter of grinding solution, place 0.93 gm of germanium dioxide in 650 ml of distilled water, and boil the solution until the germanium dioxide is completely dissolved. Allow this solution to cool to room temperature, and add 3.0 gm of diethyldithiocarbamic acid, 44.0 gm of polyvinylpyrrolidone, 44.0 gm of sodium ascorbate, 3.3 gm of sodium metabisulfite, 12.1 gm of sodium borate, and 88 ml of 0.16 m pH 7.0 phosphate buffer. Just prior to use, 88.0 ml of dimethyl sulfoxide, 8.0 ml of 1-phenoxyethanol and 1.8 ml of β-mercaptoethanol are added to the solution, and it is brought to a

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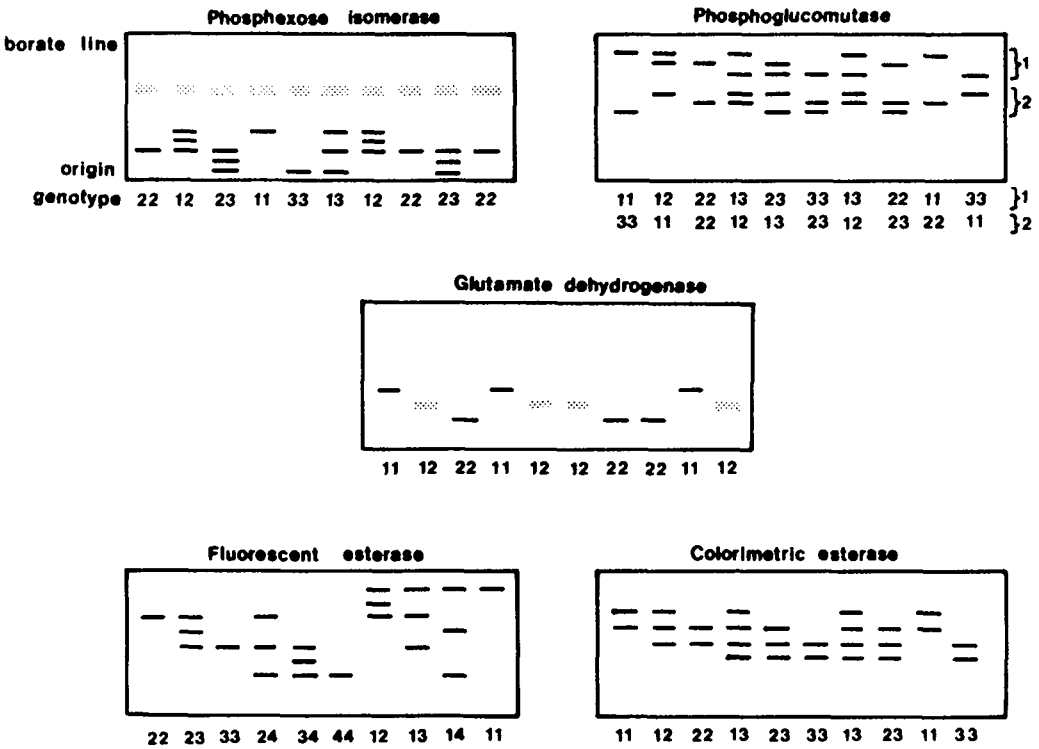

FIGURE 1—Patterns of variation in ponderosa pine in five enzyme systems.

final volume of 1 liter. The solution is placed in an ehrlenmeyer flask, which is closed with a rubber stopper, and then placed on ice. The grinding solution seems to deteriorate with time, so we prepare it the day it is used.

Satisfactory resolution for all of the enzymes presented here is achieved with the discontinuous buffer of Mitton *et al.*[9]. When gels are cooled in a refrigerator the borate line of this buffer system does not break through the origin smoothly and the subsequent zymograms are difficult or impossible to interpret. We find that this annoyance may be avoided by allowing the gels to cool to room temperature before loading.

Staining methods for phosphohexose isomerase, phosphoglucomutase, and glutamate dehydrogenase are modified slightly from those of Shaw and Prasad[12] by the addition of an equal volume of a 1 percent agar solution (Bactoagar). This solution is then poured over sliced gels. In addition, we stain for two esterase proteins, one detected colorimetrically, and one detected with fluorescence under ultraviolet light. The staining solution for the colorimetric esterase consists of 2 ml of 1 percent α-napthyl acetate in acetone and 20 mg of fast red TR salt in 90 ml of distilled water, buffered with 10 ml of 0.2 M sodium acetate pH 6.0. A stain solution for the fluorescent esterase is made by dissolving 5 mg of 4-methylumbelliferyl acetate in 3 ml of acetone, and then adding 12 ml of 0.18 M pH 5.0 sodium acetate buffer. This solution is then painted over a gel with either a paint brush or tissue; bands appear in the dark under fluorescent light within 10 minutes.

Results and Discussion

Extraction methods, banding patterns, and evidence for the Mendelian nature of the peroxidase variation are presented elsewhere[9]. Banding patterns for five other polymorphic enzyme systems are presented in Figure 1.

For each of the enzyme systems below, 12 open-pollinated families, consisting of the maternal tree and 16–45 seedlings, have been scored. Genetic interpretations are taken from the observed pattern of segregation of phenotypes.

Phosphexose isomerase

Three alleles of this dimeric protein appear to be segregating in populations near Boulder, Colorado. Heterozygotes have three bands, with the center band staining darkest. Genotypes may be difficult to assign, for shadow bands follow each of the bands shown in Figure 1. The slowest and fastest alleles are rare, and have never been seen in homozygous condition. Maternal trees homozygous for the allele with intermediate migration rate have identically homozygous offspring, bearing the same allele, and heterozygous offspring.

Phosphoglucomutase

Two variable areas of activity are designated PGM-1 and PGM-2. Patterns of variation in each appear consistent with a monomer segregating two or more alleles; an individual may have one or two bands at either position, but never three. Patterns of segregation among the 12 open-pollinated families scored for this locus are consistent with expected Mendelian patterns. A homozygous female may bear heterozygous and homozygous offspring, but the homozygous offspring are always carring the same alleles as the mother. Heterozygous mothers bear an assortment of heterozygous and homozygous offspring, at approximately the expected frequencies.

Glutamate dehydrogenase

One area of activity is evident for this protein, and either a single, well-resolved band of activity or a broad, diffuse band is exhibited by each individual. Progeny tests indicate that the single bands represent homozygotes, and diffuse stains are produced by heterozygotes. Thus, both homozygous genotypes have one well resolved area of activity, and the heterozygote has a diffuse band intermediate between the homozygotes. This unusual pattern has also been reported for glutamate dehydrogenase in maize[10] and in *Pinus rigida* and *Pinus taeda*[1].

Fluorescent esterase

The most conspicuous area of fluorescence with this stain exhibits considerable variation in populations near Boulder. Patterns of variation and patterns of segregation are consistent with a dimeric locus segregating four alleles. Heterozygotes have three bands, with the intermediate band having the greatest activity.

Colorimetric esterase

This esterase shows an unusual pattern in that homozygotes have two bands, and heterozygotes generally have four bands. This pattern is complicated when two alleles have similar migration, so that the slower band from a fast allele resolves on top of the faster band from the slower allele. These heterozygotes exhibit three rather than four bands. The Mendelian nature of this variation has not been confirmed in analyses of progeny, for this area of activity is not apparent in seedlings. Yet for other, more indirect evidence (appearance of all genotypes in expected frequencies), we propose that this pattern is generated by three alleles segregating at one locus.

Methods presented here allow an investigator to sample gene frequencies from natural populations during any season of the year and irrespective of whether that individual produced seed that year. An example is presented here from a site at Pawnee Buttes, an eastern extreme of the distribution of ponderosa pine in northeastern Colorado. The site, approximately 4 km NE of Boulder, is dry and exposed to strong winds. The year that we collected needle samples at this site, a small fraction of the individuals produced mature cones. If it had been necessary for us to rely on collection of viable seed, genotypic data for this site could not have been obtained. Genotypic frequencies, allelic frequencies, and chi-square tests of fit to Hardy-Weinberg expectations for the polymorphisms described here are presented in Table I. Excesses of heterozygotes occur at both the peroxidase locus and the fluorescent esterase locus, but the remaining loci approach Hardy-Weinberg expectations. An excess of heterozygotes has been noted previously[2,9] for peroxidase from ponderosa pine growing on south-facing slopes or on well drained soils in relatively warm environments.

Summary

A method for extraction of enzymes from mature leaves of a diversity of trees is described. The methods of tissue preparation allow samples to be collected at any time of year regardless of the reproductive state of the plant. We have used this method to detect six enzyme polymorphisms in ponderosa pine and we present evidence to show that the genes coding for these proteins are simple Mendelian loci.

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Table I. Genotypic and allelic frequencies for seven enzyme polymorphisms in ponderosa pine at Pawnee Buttes, Colorado

Enzyme	Genotypes						N	Allelic frequencies			χ^{2*}	*P*
	11	*12*	*22*	*13*	*23*	*33*		f(1) ± SE	f(2) ± SE	f(3) ± SE		
Peroxidase	0	0	11	0	36	4	51	0.000 ± 0.000	0.568 ± 0.049	0.432 ± 0.049	9.8	<0.01
Fluorescent esterase	1	13	13	4	17	3	51	0.186 ± 0.038	0.451 ± 0.049	0.363 ± 0.048	6.4	<0.05
Colorimetric esterase	24	7	0	9	0	0	40	0.801 ± 0.045	0.087 ± 0.031	0.112 ± 0.035	0.9	>0.50
Phosphoglucomutase-1	0	4	47	0	0	0	51	0.039 ± 0.019	0.961 ± 0.019	0.000 ± 0.000	0.0	>0.50
Phosphoglucomutase-2	2	6	38	0	5	0	51	0.098 ± 0.029	0.853 ± 0.035	0.049 ± 0.021	0.7	>0.50
Phosphohexose isomerase	0	1	30	0	1	0	32	0.016 ± 0.015	0.968 ± 0.022	0.016 ± 0.015	0.0	>0.50
Glutamate dehydrogenase	0	0	21	0	12	0	33	0.000 ± 0.000	0.819 ± 0.047	0.181 ± 0.047	0.5	>0.50

* Chi-square tests the fit of the observed genotypic distribution to that expected under Hardy-Weinberg expectations

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The AGA Has Moved

ON June 1, 1979 the offices of the American Genetic Association and the JOURNAL OF HEREDITY were moved from 1028 Connecticut Avenue, N.W., to 818 Eighteenth Street, N.W., Washington, D.C. 20006. Please address any correspondence relating to editorial or business matters to the new address. If you wish to telephone there is no change in the number ((202) 659-2096). We regret the delay in publication and related matters the relocation has caused, but it was not an action motivated by choice. The building that housed the association headquarters for 11 years was sold and soon thereafter destined for the wrecking crew. With 60 days notice to vacate, and affordable office space at a minimum in burgeoning and inflated D.C., finding new quarters was a bit of a problem that, though eventually solved, put some unavoidable obstacles in the way of progress. We regret any inconvenience or irritation the delays may have caused, especially to *Journal* contributors and readers, and offer our assurance that every effort is being made to regain lost ground as rapidly as possible.

—B.C. KUHN

1979 Key Lecture Announced

DR. VICTOR A. McKUSICK, director of the Moore Clinic, Department of Medicine, the Johns Hopkins Hospital University School of Medicine, Baltimore, Maryland, will deliver the 1979 Wilhelmine Key Lecture at the annual meeting of the American Society of Human Genetics to be held at the University of Minnesota October 10–13. Dr. McKusick's topic will be "Anatomy of the human genome." Persons who wish to hear the lecture should consult the program for date, location, and time.

The American Genetic Association sponsors the annual lecture in genetics from funds bequeathed to the association by Dr. Wilhelmine Key for that purpose. Dr. McKusick's address will be the sixteenth lecture in the series.