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INHERITANCE OF ISOZYME VARIATION
AND HETEROZYGOSITY IN PINUS PONDEROSA

By

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B.S., University of Massachusetts, 1974

Presented in partial fulfillment of the requirements for the degree of

Master of Arts

UNIVERSITY OF MONTANA

1980

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Botany

Inheritance of Isozyme Variation and Heterozygosity in
Pinus ponderosa (58 pp.)

Directors: Fred W. Allendorf and Sherman J. Preece

Techniques are presented to detect 23 isozyme loci in ponderosa pine. The segregation of allelic variants is examined directly in the haploid, meiotically derived megagametophyte tissue from seeds. Approximately 7 seeds per tree from 47 trees located in 10 stands in the northern Rocky Mountains were screened for variation in 12 enzymes. Additional seeds were examined from selected trees to confirm the inheritance of observed electrophoretic variants at 13 polymorphic loci. Linkage relationships could be tested for 56 pairs of loci. Significant nonrandom segregation was consistently detected for three pairs of loci: ADH-1:AAT-2, ADH-1:PGI-1, LAP-2:6PG-1. Preliminary estimates of population parameters reveal a relatively high average heterozygosity ($H = 0.123$). An estimated G_{ST} of 0.12 suggests a high level of genetic variation within stands.

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PREFACE

The research reported here is intended to provide methods to examine enzyme variation in Ponderosa pine and provides a preliminary assessment of its extent and genetic control. The literature was surveyed in 1977 and is current to that year. Seed material was provided by the Inland Empire Tree Improvement Cooperative. Funds for this study were made available under the McIntire-Stennis program of the Forest, Range and Conservation Experiment Station of the School of Forestry, University of Montana.

I gratefully acknowledge the generous support and encouragement of the members of my committee, Drs. Bilderback, Blake and Preece. I especially thank Dr. Allendorf for his patience and direction. I also wish to thank J. Madsen for his helpful suggestions.

Chapter I

INTRODUCTION

During the past ten years, the technique of gel electrophoresis has developed to the point where genetic variation in the molecular structure of specific enzymes and proteins can be routinely screened in natural populations. Multiple molecular forms of enzymes are called isozymes. Few studies of isozyme variation have dealt with forest trees (reviewed by Feret and Bergman 1976) although the information which could be obtained through such studies would be valuable to forest geneticists. Research on isozyme polymorphisms in forest trees is currently being pursued in several laboratories.

Variation in quantitative traits has been the only practical approach open to the study of tree genetics. The identification of isozyme loci in trees provides forest geneticists with an alternative method to assess genetic variation. Genetic differences between stands are now studied in provenance trials lasting many years. Isozyme studies may be able to provide similar information in considerably less time. There are few known, reliable, single locus marker genes in trees. Marker genes are useful tools not only in breeding studies, but also in the description of the mating system and local population structure which is an important consideration for selecting wild trees (Ledig 1974).

Variation in the amino acid composition of enzymes can be detected by electrophoresis if the amino acids which differ do not have the same electrical charge. Selander (1976) discussed electrophoretic

variation of enzymes in natural populations. He estimated that approximately 27% of point mutations can be detected by electrophoresis.

In natural populations of animals and plants, the proportion of heterozygous enzyme loci per individual averages from five to fifteen percent. Populations are generally polymorphic at ten to fifty percent of their enzyme loci. Unless trees are radically different from other plants and animals already studied, trees should have a considerable store of single locus enzyme variation.

No report to date has demonstrated isozyme variation in forest trees comparable to the variation observed in other plants and animals. With the exception of a study of Picea sitchensis (Simonsen and Wellendorf 1975) no attempt to survey genetic variation at a large number of isozyme loci in a tree species has appeared in the literature. The study reported here was undertaken to assess the extent, accessibility and genetic control of isozyme variation in ponderosa pine (Pinus ponderosa), an important western conifer.

The most practical tissue for conifer enzyme studies is the seed. Seeds have a low phenolic content which simplifies extraction procedures. Although seed crops are produced sporadically in trees, extensive collections of seed are made for research and reforestation projects. The most significant advantage to the use of seed material is a unique feature of gymnosperm biology, the presence of gametophytic tissue in the seed.

In conifer seeds, the megagametophyte serves as a food storage tissue. It is derived from a single haploid megaspore through a

complex developmental sequence (Foster and Gifford 1974). In comparison with pollen grains and with the female gametophyte of angiosperms, the conifer female gametophyte has a massive amount of haploid tissue. Analysis of isozyme loci in megagametophytes can provide a wealth of genetic information. An open-pollinated family (the seeds of a single tree) provides a situation similar to that exploited in fungal genetics, where haploid spores produced by meiosis in a single thallus are plated and grown until they can be scored for various traits.

The haploid genetics of conifer isozymes has been confirmed by studies relating isozyme segregation in megagametophytes with parental diploid genotypes and the inheritance of isozymes in offspring (Bartels 1971, Bergmann 1971, Lundkvist 1975, Rudin 1975 and 1977). The megagametophytes of a single tree can be expected to show a one-to-one ratio for the two alleles at an heterozygous locus. Attempts have been made to detect linkage with gametophytic tissue in conifers (Lundkvist 1974, Rudin 1975, Simonsen and Wellendorf 1975), however, few loci have been studied. Bergmann (1974) detected linkage between two esterase loci in the megagametophytes from a single tree. The statistical basis of linkage detection and gene mapping is the same for both haploids and diploids (Strickberger 1976). Meiotic products are scored for parental and nonparental genotypes, and recombinant frequencies calculated. The haploid tissue of conifer megagametophytes has not been utilized to full advantage in previous studies. Analysis of a large number of specific enzyme loci in this haploid tissue should provide valuable information concerning the organization of conifer genes and chromosomes.

The objectives of this investigation were (1) to develop techniques allowing the examination of genetic variation at many isozyme loci in ponderosa pine, (2) to describe the inheritance of isozyme variation found in ponderosa pine, and (3) to estimate the heterozygosity of ponderosa pine. The study was conducted in three phases. First, seed samples were run on starch gels with several different electrophoresis buffer systems and stained for a large number of enzymes. After the methods were established, seeds from 47 trees were screened for isozyme variation. This phase was undertaken to establish the extent of isozyme variation in ponderosa pine and to identify trees possessing variants suitable for genetic analysis. The final phase involved analysis of the segregation of isozyme variants in individual trees to ascertain the genetic basis of the variation and search for linkage between loci.

Chapter II

MATERIALS AND METHODS

In this chapter, seed source, sample preparation and statistical considerations are discussed under the heading 'Samples', electrophoresis methods and enzyme stains under 'Electrophoresis', and identification of isozyme variants under 'Nomenclature'.

SAMPLES

Ponderosa pine seeds analyzed in this study were collected in western Montana, northern Idaho, and eastern Washington between 1968 and 1971 for the Inland Empire Tree Improvement Committee. Seed trees were selected for good growth characteristics, bole form, and freedom from disease and insect attack. In the ninety-three stands sampled, collections were usually made from four or five trees. The seeds from each tree were kept separate so each seed lot can be considered an open-pollinated family. Madsen (1975) analyzed the two year performance of each family in a provenance growth trial and helped to choose ten stands representative of the genetic variation in height growth and other traits in ponderosa pine in the northern Rocky Mountains (Figure 1). An effort was made to include stands from a wide geographic range, high and low elevations and most habitat types, as well as stands containing families showing fast and slow growth.

Seeds from each family were hydrated and stratified prior to preparation for electrophoresis. This process consisted of soaking

Figure 1. Locations of Selected Ponderosa Pine Stands in the Northern Rocky Mountains.

the seeds in cold running water for about four hours and refrigeration for two to four weeks in plastic bags filled with moist vermiculite. Simonsen and Wellendorf (1975) used dry Picea sitchensis megagametophytes and found weak or no activity for most enzymes tested. Stratified seeds have a higher rate of respiration than dry seeds, evidence of increased metabolic activity and perhaps larger amounts of enzyme. The megagametophytes dissected from individual seeds were crushed in four to six drops (about one-half milliliter) of distilled water. Sufficient homogenate was obtained from each seed to moisten three to six sample wicks for electrophoresis. Preliminary work with embryos showed that more than four drops of water diluted some of the samples too much. Freezing and storage (-40 C) did not appear to affect enzyme activity.

Megagametophytes from six to eight seeds were analyzed for each enzyme in each of the 47 families studied. Assuming one-to-one segregation of allozyme alleles in heterozygous trees, the probability of misclassifying a heterozygote at a particular locus as a homozygote is $1 - 0.5^{n-1}$ where n equals the number of megagametophytes analyzed per family. Tigerstedt (1973) discussed the effects of this method of classification on population parameters. Where M is the proportion of heterozygotes misclassified, the expected Hardy-Weinberg proportions are affected as follows:

	<u>Genotypes</u>			
	<u>A₁A₁</u>	<u>A₁A₂</u>	<u>A₂A₂</u>	<u>Total</u>
Observed	A	2B	C	N
Expected (proportion)	$p^2 + pqM$	$2pq(1-M)$	$q^2 + pqM$	

Allele frequency estimates via the gene counting method are not affected by misclassification so $p = (A+B)/N$. In this study, M is less than 0.032.

Thirteen families with high heterozygosities or rare alleles were selected for segregation and linkage analysis. A sample size of forty megagametophytes per family is sufficient to detect linkage between two loci with 34% recombination or less ($p = 0.05$) using a chi-square test (Appendix C). This seemed an appropriate level for surveying linkage because forty samples could be loaded on one gel and at least ninety-six samples would be required to detect linkage with 40% recombination.

ELECTROPHORESIS

Twelve and one-half percent starch gels one-fourth to one-half inch thick were poured for electrophoresis. Two buffer systems, both discontinuous, were employed:

I. described by Ridgway et al. (1970)

Gel Buffer: 99% 0.03M tris, 0.005M citrate pH 8.5

1% tray buffer

Tray Buffer: 0.06M lithium hydroxide, 0.30M boric acid,
pH 8.1

II. described by Fildes and Harris (1966)

Gel Buffer: 5.0mM histidine, pH 7.0

Tray Buffer: 0.41M sodium citrate, pH 7.0

System I was run at 250-300 volts for two to three hours until the borate front had migrated three to four centimeters. System II was run at 60 ma. per gel (about 100 volts) for three to four hours. Bags of "Blue Ice" on plexiglass plates were set on the gels to keep them cool while running.

Enzyme stains and methods used are described by Allendorf et al. (1977). Eighteen enzymes were tested for activity on several buffer systems. Twelve enzymes showed resolution adequate for scoring purposes on buffer system I or II (Table I). The staining buffer solution used for eleven of the enzymes was 0.2M tris-HCl, pH 8. Another buffer, 0.2M tris-citrate, pH 7, was used in the staining solution for leucine aminopeptidase. The stain for 6-phosphogluconate dehydrogenase was made as a concentrate (about 20ml) and applied drop-wise to the gel slices to conserve reagents. The volume of the other staining solutions was about 50 ml. Dehydrogenase stains all contained 2.5 mg NAD or NADP, 2.5 mg NBT or MTT and 5 mg PMS. MgCl_2 (2 ml of a 2% solution) was added to stains for NADP-dependent enzymes. The gels were developed at 35°C in darkness.

NOMENCLATURE

The nomenclature of isozymes follows Allendorf and Utter (1978). An abbreviation is chosen to designate each enzyme; when underlined, these same abbreviations represent the loci coding for

these proteins. In the case of multiple forms of the same enzyme, a hyphenated numeral is included; the form with the least anodal migration is designated one, the next two and so on. Allelic variants are designated according to the relative electrophoretic mobility. One allele (generally the most common one) is arbitrarily designated 100. This unit distance represents the migration distance of the isozyme coded for by this allele. Other alleles are then assigned a numerical value representing their mobility relative to this unit distance. Thus, an allele of the least anodal malic dehydrogenase (MDH) locus coding for an enzyme migrating 12% further than the common allele product would be designated MDH-1(112). When no enzyme activity can be observed, the allele is designated 100n. If the activity of the allelic product, as determined by staining intensity, differs significantly from an allele with identical mobility, it is designated by an r following the mobility designation. Multiple comigrating allelic products which are identifiable by activity differences are designated as r1, r2, etc.

Chapter III

RESULTS

The open-pollinated families from the 47 trees studied were surveyed for electrophoretic variation in twelve enzymes (Table I). Variation was detected in 60% of the isozymes and most families possessed variants for at least one isozyme. The isozyme variation found is presented diagrammatically in Figure 2 and photographs of gels stained for most of the polymorphic enzymes are included in Appendix D.

MONOMORPHIC ISOZYMES

Several isozymes showed no variation in the families surveyed. Mannose-6-phosphate isomerase (MPI), phosphoglucomutase (PGM), and sorbitol dehydrogenase (SDH) each produced a single monomorphic band of enzyme activity. Peptidase (PEP) gels had two monomorphic bands. Glucose-6-phosphate dehydrogenase (G6P), leucine aminopeptidase (LAP), and phosphoglucose isomerase (PGI) gels each had two zones of enzyme activity, one polymorphic and one monomorphic.

The SDH enzyme presented a potentially confusing situation in that sorbitol appears to serve as substrate for ADH, replacing ethanol. Using sorbitol as a substrate with buffer I, gels resulted in a single monomorphic band of activity. Ethanol produced no staining when used as a substrate on buffer I gels. SDH (on buffer I) is distinct from ADH (on buffer II) since ADH is polymorphic and no

Table I. Buffer Systems Providing Best Resolution, Locus Designations, and Alleles Detected for the Twelve Enzymes Used in this Study

Enzyme	Buffer System	Locus	Alleles		
			A ₁	A ₂	A ₃
Alcohol dehydrogenase	II	ADH-1 ADH-2	100 (100)*	120	100r
Aspartate aminotransferase	I	AAT-1 AAT-2 AAT-3	100 100 100	175 120 84	76
Glucose-6-phosphate dehydrogenase	I	G6P-1 G6P-2	(100 100	120)*	
Isocitric dehydrogenase	II	IDH	100	87	
Leucine aminopeptidase	I	LAP-1 LAP-2	100 100	100n	
Malic dehydrogenase	II	MDH-1 MDH-2 MDH-3 MDH-4	(100)* 100 100 100	110 62 90	100n
Peptidase	I	PEP-1 PEP-2	100 100		
6-Phosphogluconic dehydrogenase	II	6PG-1 6PG-2	100 100	112	
Phosphoglucose isomerase	I	PGI-1 PGI-2	100 100	120	
Phosphoglucomutase	I	PGM	100		
Phosomannose isomerase	I	PMI	100		
Sorbitol dehydrogenase	I	SDH	100		

*not used in population analysis because of insufficient activity for reliable scoring

Figure 2. Diagrammatic Representation of Allelic Variation Found for Isozyme Loci in Ponderosa Pine (anodal end is topmost in all cases, numbers below refer to allelic designations in Table I, all bands are shown for each enzyme but nonspecified loci are represented by their common allele).

polymorphism was detected in SDH, and because of the substrate specificity of SDH on buffer I.

POLYMORPHIC ISOZYMES

Isocitric dehydrogenase (IDH) gels had a single polymorphic zone of enzymatic activity. Embryos displayed three-banded patterns in heterozygotes, which is in agreement with the dimeric structure of IDH in other organisms. Faint IDH patterns could be detected on many gels stained for NADP-dependent enzymes. Citric acid was probably the substrate utilized.

G6P, LAP, and PGI each had one polymorphic zone of activity, besides a monomorphic one. Electrophoretic variants in one zone did not affect the other zone. This independence of expression is evidence that the isozymes in the two zones are encoded by two separate loci (e.g., LAP-1 and LAP-2). G6P-1 did not stain consistently so could not be analyzed in all families. It was found that adding NADP to the gel before degassing encouraged more intense staining (Brewer 1970). The PGI-1 isozyme zone was wide and stained faintly.

The variation in each of the two polymorphic isozyme bands on 6-phosphogluconate dehydrogenase (6PG) gels was controlled by single loci. Two families were polymorphic for both loci. The 112 variant in 6PG-1 overlapped the 94 variant in 6PG-2. The difference in migration between the two variants in 6PG-2 was slight and distinguishing genotypes in diploid tissue would be difficult.

Aspartate aminotransferase (AAT) isozyme patterns consisted of four bands, one of which was cathodal (AAT-C). AAT isozymes in

spinach leaves have been shown to be organelle specific (Huang et al. 1976) with one isozyme band being associated with either mitochondria, chloroplasts, peroxisomes, or cytoplasm. In ponderosa pine, the three anodal isozymes were controlled by three distinct loci, AAT-1, AAT-2, and AAT-3. Variation in AAT-C matched that in AAT-1 almost perfectly (cathodal AAT migrates unevenly), but the two were polymorphic only in family 113. AAT-1 and AAT-C might be two closely linked loci, but it is more likely that AAT-C is an artifact or post-translational modification of AAT-1. The same situation was found in Pinus rigida (Guries 1978a) and it is improbable that such tight linkage be found in separate species. AAT-C was not counted as an individual locus. In embryos, AAT isozymes sometimes showed three-banded patterns suggesting that the AAT molecule is a dimer.

Alcohol dehydrogenase (ADH) isozyme patterns have been found to be controlled by two loci in Lupinus, Helianthus, and other plant species (Marshall et al. 1974 and Torres 1976). The enzyme molecules are dimers and three zones of enzyme activity appear on ADH-stained gels. The intermediate zone is produced by a heterodimer consisting of one subunit from each of the two loci. The other two zones are produced by a homodimer for the two loci. ADH in Pinus attenuata embryos (Conkle 1971) and Pinus ponderosa megagametophytes has an isozyme pattern which appears consistent with this interpretation.

ADH isozyme patterns from ponderosa pine megagametophytes consisted of three zones of activity, but two of the zones were faint or absent on most gels. Only ADH-1 stained consistently and darkly.

In many plants, the expression of ADH varies in different tissues, often with only one locus transcribed (Torres 1976). It may be that only one ADH locus is expressed in megagametophytes. Two of the ADH-1 alleles detected coded for multiple banded ADH-1. The cause of this phenomenon was not investigated but might be due to the enzyme molecules binding different amounts of some substance (Johnson 1978). The third allele coded for an enzyme molecule with reduced activity (under the assay condition).

MDH isozyme patterns were complex, but interpretation was greatly facilitated by the segregation of genetic variants in individual families. MDH isozymes in other plants have been found to be organelle specific, and this is probably the situation in ponderosa pine as well. Ponderosa pine MDH patterns were similar to those reported for Sitka spruce (Simonsen and Wellendorf 1975). MDH-1 did not stain consistently and was faint or absent on part or all of most gels. MDH-1 is not included in the analysis.

Variation at MDH-4 did not affect the other MDH isozymes. The pattern of the three most cathodal (excluding MDH-1) isozymes resembled the three-banded pattern of heterozygotes for a dimeric enzyme. Since the tissue is haploid, the variation appears to be controlled by two loci which form an heterodimer. The MDH-3 (100) isozyme had a mobility similar to the MDH-4 isozyme and was obscured by it. The MDH-3 isozyme was consistently visible only in those families possessing a slow variant. Two variants were found for the MDH-2 isozyme, MDH-2 (110) and MDH-2 (110n). The reduced activity

variant lacked activity as an homodimer but the heterodimer present stained lightly. The MDH-2 (110) variant had only a very small mobility difference most easily detected in the heterodimer band. This posed problems in interpretation. Some of the variants assigned to MDH-2 may have actually been MDH-3 variants. However, one tree was found segregating for both MDH-3 and MDH-2, although it was difficult to distinguish some gamete types.

INHERITANCE OF VARIATION

Fourteen families were chosen for segregation analysis and to test the linkage relationships between loci. A tree heterozygous at a particular locus can be expected to produce gametes (megagametophytes) carrying the alternative alleles in a one-to-one ratio. Of the 53 segregation ratios studied (Table II), only three were significantly different from the expected one-to-one. These results confirm the genetic basis of all the electrophoretic variation reported here and is evidence that the isozyme variants are inherited as single gene traits.

Of the 78 possible two-locus combinations of the thirteen polymorphic loci, families were available to test over half of them for nonrandom joint segregation, i.e., linkage. These results are presented in Appendix B and summarized in Table III. The linkage survey detected one linkage group consisting of three loci (ADH-1, AAT-2, PGI-1) (Table IV). The combinations ADH-1:AAT-2 and ADH-1:PGI-1 were confirmed in three separate families each. Unfortunately, there were

Table II. Segregation of Isozyme Alleles

Locus	Family	Alleles	Segregation	χ^2_1 d.f. ^a
ADH-1	8	100/120	23:28	0.49
	9	"	23:27	0.32
	10	"	21:19	0.10
	22	"	24:22	0.09
	113	"	15:25	2.50
	351	"	20:15	0.71
	390	"	26:18	1.45
	111	100/100n	19:21	0.10
	388	"	22:25	0.19
AAT-1	113	100/175	24:15	2.08
AAT-2	8	100/120	17:34	5.67*
	9	"	30:20	2.00
	113	"	15:24	2.08
	502	"	17:23	0.90
AAT-3	111	100/84	16:24	1.60
	429	"	19:21	0.10
	502	100/76	19:21	0.10
G6P-1	505	100/120	20:20	0.00
IDH	8	100/87	13:27	4.90*
	10	"	20:20	0.00
	111	"	22:18	0.40
	113	"	21:19	0.10
	351	"	13:22	2.31
	388	"	20:20	0.00
	429	"	19:21	0.10
	430	"	25:15	2.50
	505	"	21:19	0.10
LAP-2	10	100/100n	19:21	0.10
	114	"	30:30	0.00
	388	"	20:20	0.00
MDH-2	10	100/110	19:20	0.03
	351	"	16:19	0.26
	390	"	20:18	0.11
	430	"	18:22	0.40
	489	"	21:19	0.10
	111	100/100n	20:20	0.00
MDH-3	8	100/62	15/16	0.90
	489	"	21:19	0.10

Table II. (continued)

Locus	Family	Alleles	Segregation	$\chi^2_{1 \text{ d.f.}}$ ^a
MDH-4	505	100/90	20:19	0.03
6PG-1	114	100/112	24:36	2.40
	351	"	17:18	0.03
	429	"	22:18	0.40
6PG-2	8	100/94	23:17	0.90
	113	"	20:20	0.00
	351	"	16:19	0.26
	388	"	29:11	8.10**
	429	"	23:17	0.90
	430	"	22:18	0.04
	502	"	17:23	0.90
	505	"	18:20	0.10
PGI-1	22	100/120	21:25	0.35
	388	"	20:17	1.04
	390	"	20:24	0.36

^acritical values for chi-square, 1 d.f.: 0.050 level = 3.84*
0.010 level = 6.64**
0.001 level = 10.83***

Table III. Summary of Joint Segregation Analysis for 13 Polymorphic Loci (numbers above the diagonal represent the number of gametes / number of trees examined; below the diagonal, R indicates no evidence of nonrandom assortment, I designates inconsistent evidence, and a numerical value signifies consistent evidence for nonrandom assortment with the value specifying the average portion of recombinant gametes for that pair of loci (see Table IV); a dot indicates no data for that pair of loci).

	AAT-1	AAT-2	AAT-3	ADH-1	G6P-1	IDH	LAP-2	MDH-2	MDH-3	MDH-4	6PG-1	6PG-2	PGI-1
AAT-1	*	39/1	.	39/1	.	39/1	.	37/1	.	.	.	37/1	.
AAT-2	R	*	40/1	140/3	.	37/1	.	37/1	31/1	.	.	119/3	.
AAT-3	.	R	*	40/1	.	80/2	.	40/1	.	.	40/1	80/2	.
ADH-1	R	.27	R	*	.	235/6	80/2	190/5	31/1	.	35/1	155/4	137/3
G6P-1	.	.	.	.	*	40/1	.	.	.	39/1	.	38/1	.
IDH	R	R	R	R	R	*	79/2	191/5	31/1	39/1	75/2	283/7	40/1
LAP-2	.	.	.	R	.	R	*	39/1	40/1	.	60/1	40/1	40/1
MDH-2	R	R	R	I	R	R	R	*	40/1	.	36/1	114/3	38/1
MDH-3	.	R	.	R	.	R	R	R	*	.	40/1	31/1	.
MDH-4	.	.	.	.	R	R	.	.	.	*	.	37/1	.
6PG-1	.	.	R	R	.	R	.33	R	R	.	*	75/2	.
6PG-2	R	R	R	R	R	R	R	I	R	R	R	*	40/1
PGI-1	.	.	.	.26	.	R	R	R	.	.	.	R	*

Table IV. Joint Segregation Results Which Suggest Linkage (for a pair of loci, A;B, R denotes the fraction of recombinants assuming the largest linkage class ($A_1B_1+A_2B_2$ or $A_1B_2+A_2B_1$) is the parental type; the standard error of R is S_R ; the X^2 for linkage has 1 d.f.)

Loci	Family	A_1B_1	:	A_1B_2	:	A_2B_1	:	A_2B_2	X^2_{link}	$R \pm S_R$
ADH-1:AAT-2	8	12	:	11	:	5	:	23	7.08*	.29 $\pm$.06
	9	19	:	4	:	11	:	16	8.00**	.30 $\pm$.06
	113	11	:	4	:	4	:	20	13.56***	.21 $\pm$.07
ADH-1:PGI-1	22	7	:	17	:	14	:	8	5.57*	.33 $\pm$.07
	388	18	:	4	:	2	:	23	26.06***	.13 $\pm$.07
	390	16	:	10	:	4	:	14	5.82*	.32 $\pm$.07
LAP-2:6PG-1	114	7	:	17	:	23	:	13	6.67**	.33 $\pm$.06
MDH-2:6PG-2	113	8	:	6	:	10	:	14	0.95	.42 $\pm$.08
	351	8	:	8	:	8	:	11	0.26	.46 $\pm$.08
	430	4	:	14	:	18	:	4	14.40***	.20 $\pm$.06
MDH-2:ADH-1	10	10	:	10	:	9	:	10	0.03	.49 $\pm$.08
	111	6	:	13	:	14	:	7	4.90*	.33 $\pm$.07
	113	11	:	6	:	3	:	18	10.53**	.24 $\pm$.07
	351	8	:	12	:	8	:	7	0.71	.43 $\pm$.08
	390	12	:	9	:	8	:	9	0.42	.45 $\pm$.08

no families found possessing suitable genotypes for three-point mapping. A second linkage group (LAP-2:6PG-2) could be tested in only one family so could not be confirmed. Suggestive results were found with two pairs of loci. Two of the five ratios tested for ADH-1:MDH-2 were significantly nonrandom and for MDH-2:6PG-2, one of the three ratios was highly nonrandom (probability of random segregation less than 0.001). In both cases the other ratios gave no indications of linkage. The sum of the chi-squares with the appropriate degrees of freedom was significant for both pairs of loci, but the significance was due almost entirely to one ratio in each pair so could not be considered conclusive evidence for linkage.

POPULATION ANALYSIS

The genotypic data from the ten stands was pooled for population analysis. The observed genotypic numbers were not significantly different from Hardy-Weinberg expectations (Table V). A chi-square value for testing departure from H-W proportions can be expressed as a function of the generalized inbreeding coefficient F (Wright 1965) as follows: $\chi^2 = F^2 N$ (Li and Horvitz 1953). There was a small proportional excess of heterozygotes at nine of the twelve polymorphic loci surveyed.

No obvious patterns of variation were observed in the geographic distribution of alleles at the polymorphic loci. The small sample size prohibits accurate comparisons. Genetic divergence among stands was analyzed using a series of 2×2 contingency chi-squares on the

observed allele frequencies at the three most polymorphic loci (ADH-1, IDH, 6PG-2). Only four of the 135 pairs showed any significant differences ($P = 0.05$). Average heterozygosity was estimated using pooled data from 20 loci, twelve identified by segregation analysis and eight presumed monomorphic loci. The expected heterozygosity (H_e) is defined by Nei and Roychoudhury (1974) as the average heterozygosity per loci (h), where $h = 1 - \sum p_i^2$, and p_i is the frequency of the i th allele at a particular locus. The expected heterozygosity of Pinus ponderosa in the northern Rocky Mountains is estimated to be 0.127. The observed average heterozygosity (H_o) is slightly larger, 0.138. The distribution of heterozygosity (h) among loci (Figure 3) shows that most loci have a low heterozygosity as is typical of many organisms.

Table V. Pooled Population Data for 12 Polymorphic Loci (allele designations refer to Table I; F is the generalized inbreeding coefficient for departure from Hardy-Weinberg proportions)

Locus	Genotypes: observed (expected)				F	Frequency of Alleles		
	A ₁ A ₁	A ₁ A ₂	A ₂ A ₂	A ₁ A ₃		A ₁	A ₂	A ₃
ADH-1	16 (17.1)	21 (20.2)	7 (6.9)	3 (2.9)	-.049	.596	.372	.032
AAT-1	46 (46.0)	1 (1.0)	0 (0.0)	---	0.000	.989	.011	---
AAT-2	42 (42.2)	5 (4.6)	0 (0.2)	---	-.045	.947	.053	---
AAT-3	35 (35.9)	4 (3.4)	0 (0.9)	8 (6.7)	-.134	.872	.043	.085
IDH	15 (14.2)	21 (22.6)	11 (10.2)	---	+.109	.543	.457	---
LAP-2	44 (44.1)	3 (2.8)	0 (0.1)	---	-.022	.968	.032	---
MDH-2	27 (29.4)	19 (14.5)	0 (2.2)	1 (1.0)	-.257	.787	.202	.011
MDH-3	40 (40.4)	7 (6.2)	0 (0.4)	---	-.069	.926	.074	---
MDH-4	46 (46.0)	1 (1.0)	0 (0.0)	---	0.000	.989	.011	---
6PG-1	44 (44.1)	3 (2.8)	0 (0.1)	---	-.022	.968	.032	---
6PG-2	17 (18.2)	24 (21.5)	6 (7.2)	---	-.069	.617	.383	---
PGI-7	42 (42.2)	5 (4.6)	0 (0.2)	---	-.045	.947	.053	---

Figure 3. Distribution of Heterozygosity for Ponderosa Pine Isozyme Loci.

Chapter 4

DISCUSSION

The isozyme polymorphisms found in this study indicate a considerable store of single-locus genetic variation in ponderosa pine enzymes which can be detected using standard electrophoretic techniques. Although the seed collections analyzed were part of a diverse population sample, the small number of trees representing each stand made precise population comparisons inappropriate. The focus of the study was on individual variation and isozyme variability at the species level.

THE HAPLOID GENETICS OF PONDEROSA PINE ISOZYMES

Of the 53 allozyme (allelic isozymes) segregation ratios observed in the megagametophytes of 14 families, only three ratios were significantly different from the expected one-to-one. Two of the altered ratios were in one family (8). Morphological markers in conifers (usually deviant seedling types) often show disturbed segregation ratios in controlled crosses (Sorensen 1966). Altered allozyme segregation ratios in megagametophytes do not appear common.

The biological basis for segregation of isozymes in megagametophytes is found in megasporogenesis. In pines, the megaspore mother cell divides during meiosis to form a linear tetrad of megaspores, only one of which (the basal megaspore) will develop into the megagametophyte (Foster and Gifford 1974). Normal segregation ratios will be obtained in each of the homologous chromosomes carrying the

two alleles has an equal chance of reaching the basal megaspore. This may not always be the case. Preferential segregation can occur when the functional megaspore is determined by its position in the meiotic tetrad (Grant 1975). A case of preferential segregation has been described in Zea mays where a linear tetrad also occurs (Rhodes 1952). Approximately 70% of the maize ovules produced by heterozygotes for knobbed chromosomes 10 contained knobbed chromosome 10. A locus on this chromosome showed a similar ratio. Another possible cause of disturbed segregation might be linkage to embryonic or gametophytic lethals.

The precision of gene maps produced by gametophytic analysis will probably be limited. The order of genes in individual trees can probably be reliably determined but map distances will lack accuracy. Recombination rates are affected by a variety of environmental, chemical, and genetic factors (Sera 1965). These variables are difficult to control and manipulate in trees. In conifers, mapping depends upon fortuitous combinations of heterozygous isozyme loci in individual trees.

Conifer chromosomes are very large, the chromosome number is small ($1N = 12$ in nearly all species) and conifers have high chiasmata frequencies (2.3 - 2.5) (Sax 1933 and Tigerstedt 1973). In ponderosa pine, two linkage groups were detected by analysis of joint segregation of isozyme loci in individual families. ADH-1 was linked to AAT-2 in three families and to PGI-1 in another three families. No family was heterozygous for all three loci so the order of the genes

could not be determined. Linkage between an AAT and a PGI locus has also been found in Pinus rigida (Guries 1978b). LAP-2 was linked to 6PG-1 in one family, but no families were available to confirm this linkage group. Success in detecting linkage can be attributed to the large number of two locus combinations tested. This study demonstrates the potential of using gametophytic analysis of isozymes for gene mapping.

GENETIC DIVERSITY OF PONDEROSA PINE ISOZYME LOCI

The genetic variation of isozymes and proteins in populations and species has commanded considerable attention and controversy recently (Selander 1976). Genetic variation (or diversity) can be measured in terms of heterozygosity, H , defined as the mean of h over all loci where $h = 1 - \sum x_i^2$ and x_i is the frequency of the i th allele at a particular locus (Nei and Roychoudhury 1974). Heterozygosity has been determined for only a few plant species. The heterozygosity of Pinus ponderosa is of interest for two reasons: (1) most of the plant species studied so far have been annuals or herbaceous perennials and the contrast in life histories might be reflected in the genetic diversity of the two groups, and (2) the assessment of genetic variation in forest trees can be valuable in the management and manipulation of this resource.

The ponderosa pine data should be adequate for an estimate of species heterozygosity. Lewontin (1974) listed requirements for reasonably reliable estimates of heterozygosity. In ponderosa pine,

a relatively large (20), unbiased and diverse (12 enzymes) sample of loci were examined in 47 individuals representative of a large geographic area. For heterozygosity estimates, the number of individuals sampled is not as crucial as the number of loci screened. The variance of heterozygosity within loci in most studies is much smaller than the variance between loci (Nei and Roychoudhury 1974). The heterozygosity of ponderosa pine in the northern Rockies is estimated to be .127 with a standard error of .040, based upon 20 loci.

Comparisons of heterozygosity estimates in different species cannot be very precise. Different studies survey different enzymes and not all enzymes are equally likely to be polymorphic. Levin (1975a) reviewed the degree of polymorphism for various plant enzymes. Only 15% of the species surveyed were polymorphic for glucose-6-phosphate dehydrogenase while 73% were polymorphic for esterase-1. Another problem is the large standard errors associated with most heterozygosity estimates, often 20-40% as large as the estimate itself. For comparative evolutionary studies, data from about 100 loci are needed (Lewontin 1974). The estimates available for plant species where more than ten loci have been examined (Table VI) should be taken only as first approximations.

Ponderosa pine has a relatively high heterozygosity, but it is not exceptionally different from other species. Mejnartowicz and Bergmann (1977) commented on the great variability of tree isozymes and the apparent "inability" to detect monomorphic isozymes in forest trees. However, most tree studies reported thus far have dealt with

nonspecific, highly polymorphic isozymes (e.g., esterase). Fowler and Morris (1977) found no isozyme variation in Pinus resinosa, a species which shows little variation in most other traits. In ponderosa pine, the "reverse J" distribution of heterozygosity (h) among loci (Figure III) is typical of that found in other organisms. Levin (1975b) concluded that while there may be correlations found between life-history or breeding system and heterozygosity within plant genera, no general relationship can be readily discerned.

The literature attempting to explain observed levels of heterozygosity is extensive and complex. Rather than reviewing the various hypotheses, the ponderosa pine data will be interpreted in terms of Soulé's (1976) paper. There are several features which favor ponderosa pine having a high level of heterozygosity. Ponderosa pine has an extensive range, occurring in 15 western states and British Columbia (Mirov 1967). The species has a large population size. Variation is generated by mutation but mutation rates are low, generally 10^{-4} - 10^{-6} . Large populations are needed to maintain variability generated by mutation. Mutant alleles have to be available before they can be acted upon by evolutionary processes. Polymorphisms are usually acquired at a very slow rate, so old lineages should have more polymorphisms than younger ones. Conifers are a very old lineage. The genera of the Pinaceae probably diverged about 135 MYA (million years ago) (Prager et al. 1976) but the age of species is more important as a determinant of heterozygosity and in this case, is unknown.

Table VI. Estimates of Genic Heterozygosity in Plant Species (modified from Levin 1975a)

Species*	Number of Loci	Heterozygosity(H)
<u>Avena barbata</u>	16	.03
<u>Gaura brachycarpa</u>	12	.06
<u>G. demareei</u>	18	.05
<u>G. longifolia</u>	18	.07
<u>G. suffulta</u>	12	.03
<u>G. triangulata</u>	12	.08
<u>Liatris cylindracea</u>	27	.06
<u>Lycopodium lucidulum</u>	18	.06
<u>Denothera argillicola</u>	20	.08
<u>De. biennis</u>	20	.10
<u>De. hookeri</u>	20	.00
<u>De. parviflora</u>	20	.16
<u>De. strigosa</u>	20	.03
<u>Phlox cuspidata</u>	16	.01
<u>P. drummondii</u>	16	.04
<u>Pinus ponderosa</u>	20	.13
<u>Silene maritima</u>	21	.15
<u>Stephanomeria exigua</u>	11	.12
	—	—
Mean	18	.07

*References for estimates: Baker et al 1975, Gottlieb 1973, Gottlieb and Pilz 1976, Levin 1975, Levin and Crepet 1973, Levin and Levy (cited in Levin 1975b in prep.), Levy and Levin 1975, Marshall and Allard 1970, Schaal 1974.

Slowly evolving lineages should also have higher levels of heterozygosity than rapidly evolving ones. Directional selection tends to reduce variability. Prager et al. (1976) investigated rates of evolution in conifers and concluded that rates of conifer protein evolution determined from antigenic distances are comparable to rates determined for other groups of organisms. Approximately a 1% change in amino acid sequence occurs per 9 MY, but rates of anatomical and chromosomal evolution are very slow. Rate of change in chromosome number in conifers is 0.04 changes per lineage per 100 MY, about 100 times slower than in mammals. Karyotype is fairly constant and chromosomes in interspecific hybrids often pair well at meiosis. Chromosomal evolution is thought to involve changes in the patterns of gene regulation, and gene regulation is probably more important in evolution than changes in structural genes (Wilson 1976).

A particularly important factor in the determination of levels of heterozygosity is the length of time since the species or lineage last passed through a "bottleneck", where substantially reduced population size can cause a loss of variability. Unfortunately, "bottlenecks" can be invoked to explain away most of the variation in species heterozygosities. Three variables, mutation rate, population size, and time, probably determine levels of heterozygosity but population size is difficult to quantify and time is nearly impossible. These problems also confound the theories relating heterozygosity to ecological niche.

The adaptive significance of isozyme variation is unclear. The allelic forms of some isozymes have been shown to differ in their

Km's and temperature and pH optima (Johnson 1976). Isozyme alleles sometimes have a distribution related to environmental parameters. Lewontin (1974) discussed patterns of geographical variation in Drosophila pseudoobscura, an extensively surveyed species. There was little geographic differentiation in most polymorphic isozyme loci, and this seems typical of many species. Most isozyme variation occurs within populations. Some workers do not believe selection plays a major role in the maintenance of isozyme polymorphisms. If selection coefficients are on the order of mutation rates, the frequencies of various alleles could be maintained by an equilibrium between mutation and selection (reviewed by Selander 1976). Another point of view holds that selection rarely acts upon single loci. Rather, the units of adaptation are balanced combinations of loci (Carson 1976).

The isozyme data collected in this study provide some information about the genetic structure of the ponderosa pine population in the northern Rocky Mountains. Gene frequency differences between populations cause genotypic data pooled from several populations to show a deficit of heterozygotes (Wahlund effect). The population data in Table V actually reveal a slight excess of heterozygotes, suggesting little population differentiation has occurred among the ponderosa pine stands.

Nei (1973) describes a method of analyzing genetic diversity. It is similar to Wright's (1965) F-statistics, but is designed for inference about the whole genome, not individual loci. Considering

the small sample size, Nei's method is the most appropriate to apply to the data. Genetic diversity can be partitioned into within and between population components to provide an assessment of the extent of genetic differentiation among the populations examined. For ponderosa pine in the northern Rocky Mountains:

$$G_{ST} = D_{ST}/H_T = .015/.123 = .122$$

This G_{ST} estimate may be interpreted as meaning that approximately 12% of the genetic variation detected by electrophoresis is associated with genetic differences between stands. However, the standard error of G_{ST} (.19), calculated following Chakraborty (1974), suggests that the statistical significance of the estimate may be questionable.

The population structure revealed by isozyme methods is in basic agreement with the quantitative genetic results of Madsen and Blake's (1977) study of two-year height growth of ponderosa pine seedlings. They attributed 71.5% of the variability in height growth to within and among family variation and the remaining 28.5% to among stand variation. The stands chosen for isozyme analysis were representative of the range of quantitative variation found by Madsen and Blake. Both studies show that ponderosa pine in the northern Rocky Mountains is not strongly differentiated geographically.

IMPLICATIONS FOR FOREST TREE IMPROVEMENT

Isozyme studies may have practical implications for tree improvement programs. If the genetic variation revealed by electrophoretic techniques is representative of variation throughout the whole genome,

isozyme analysis could complement quantitative genetics in assessing the genetic variability of species of interest (Allendorf and Utter 1978). For ponderosa pine, estimates of the distribution of genetic variation within and between stands provided by quantitative (Madsen and Blake 1977) and by electrophoretic studies were in general agreement. Further, both studies detected relatively large amounts of genetic variation. An isozyme study of red pine (Fowler and Morris 1977) detected no variation at an estimated 9 loci (50-100 independently sampled genes per locus). Correspondingly, red pine is considered to be remarkably uniform with respect to several quantitative traits (Fowler and Lester 1970). The data from conifers supports the idea that isozyme variation might serve as an indicator of variability throughout the whole genome.

Conventional methods to estimate heritability and the distribution of additive genetic variation are expensive and require many years to obtain results. Forest geneticists may be able to identify species and populations with large amounts of additive genetic variation through isozyme studies at considerable savings of time and expense. However, the relationship between additive genetic and isozyme variability must be established empirically. Isozyme studies on material already described quantitatively will be valuable. Brown et al. (1978) studied barley (Hordeum) in Israel by both isozyme and morphometric methods. The distribution of genetic variation in isozymes was found to be the reverse of that for morphometric spike characters. They proposed several hypotheses which could explain

the results but which is correct is a matter of speculation. Clearly, more joint studies are needed before a relationship between additive genetic and isozyme variation can be established.

Chapter V

SUMMARY

The technique of gel electrophoresis has made possible the routine screening of single locus genetic variation in enzymes and proteins in natural populations. Few studies of isozyme variation have dealt with forest trees although alternate approaches to assess genetic variation would be of great value to forest geneticists. Conventional techniques to study tree genetics face the problems of long generations and the difficulties involved with the experiment breeding of trees. Most plant isozyme studies have examined only annuals or herbaceous perennials, despite the contrasting life histories of trees. The objectives of this study of Pinus ponderosa were 1) to develop electrophoretic methods suitable to resolve isozyme variation in megagametophyte tissue from seeds, 2) to establish the inheritance of any variation found, and 3) to estimate the heterozygosity of ponderosa pine.

Conifer megagametophytes tissue is derived from a single haploid megaspore, thus providing an opportunity to examine the segregation of isozyme variants without resort to breeding. A one-to-one ratio is expected from the two alleles at a heterozygous locus. Linkage can be established from the nonrandom segregation of pairs of loci. Megagametophyte tissue contains very low levels of the phenolic chemicals which make it difficult to extract active enzymes from other conifer tissues.

Twelve enzymes were found to resolve well on two electrophoresis buffer systems. One-to-one segregation of the variants in megagametophytes from individual trees established the single locus inheritance of thirteen distinct isozymes. No variants were found for eight isozymes; these were assumed to also be controlled by single loci. Nonrandom joint segregation was found for several pairs of loci. Consistent evidence for nonrandom segregation in several trees established a linkage group involving three loci; ADH-1, AAT-2, PGI-1.

Isozyme variation was surveyed in 47 families (seeds from individual trees) from ten stands of ponderosa pine in the northern Rocky Mountains. Genotyping six megagametophytes per family corresponds to a probability greater than 0.96 of correctly identifying heterozygous mother trees. The average expected heterozygosity for the 47 trees studied was 0.123, based on 20 isozyme loci. The variability detected was greater than that found for many plant species, but it was not exceptionally different from the other estimates.

The genotypic and gene frequency data give some indication of the geographical differentiation of the stands involved in this study. Pooling genotypic data from populations differing in gene frequency is expected to cause a deficit of heterozygous genotypes compared to Hardy-Weinberg proportions (Wahlund effect). The pooled genotypic data actually show a slight excess of heterozygotes. This evidence suggests little differentiation has occurred. The genic diversity of ponderosa pine isozyme loci was partitioned following the methods of Nei (1973). The G_{ST} estimate of 0.122 can be interpreted as

meaning that approximately 12% of the genetic variation detected by electrophoretic methods is associated with genetic differences between stands.

The ponderosa pine families analyzed were part of the collections studied by Madsen and Blake (1977). The families were chosen to be a geographically and ecologically diverse sample representative of the range of variation Madsen and Blake found in two-year height growth. The isozyme and quantitative results compare favorably. Both attributed most variability to within stand sources. Madsen and Blake estimated that 28.5% of the variation in height growth was associated with between stand differences. Both studies detected relatively large amounts of genetic variation. A contrast can be made with isozyme results reported for Pinus resinosa. No variation was found at an estimated nine isozyme loci and correspondingly red pine is considered to be remarkably uniform with respect to several quantitative traits (Fowler and Morris 1977 and Fowler and Lester 1970). The data from conifers thus lends support to the idea that isozyme variability might serve as an indicator of variability throughout the whole genome. Isozyme variability might serve as a valuable tool helping geneticists find species and populations which will demonstrate large amounts of additive genetic variation. The relationship between isozyme and additive genetic variation will have to be established empirically. Brown et al. (1978) reported that the distribution of isozyme and morphometric variation in barley (Hordeum) did not agree. More joint studies are needed to test the idea that isozyme variability can be used as an indicator of genome variability.

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APPENDIX

A. GENOTYPES^a OF PONDEROSA PINE TREES STUDIED

STAND AND FAMILY	LOCUS <u>ADH</u> 1	<u>AAT</u> 2	<u>AAT</u> 3	<u>IDH</u>	<u>LAP</u> 2	<u>MDH</u> 2	<u>MDH</u> 3	<u>6PG</u> 1	<u>6PG</u> 2	<u>PGI</u> 1
604										
6	<u>120</u>	.	76	90	.	110	.	.	94	.
7	<u>120</u>	.	.	.	.	.	75	.	94	.
8	120	120	.	90	.	.	75	.	94	.
9	120	120	.	90	.	.	.	.	94	.
10	120	.	.	90	100n	110	.	.	.	.
607										
21	<u>120</u>	.	.	<u>90</u>	.	.	.	.	94	.
22	<u>120</u>	.	.	<u>90</u>	.	.	.	.	.	120
23	120	.	.	.	.	110	.	.	94	.
24	.	.	.	<u>90</u>	.	.	.	.	.	.
25	.	.	.	<u>90</u>	.	.	.	.	94	.
620										
111	120	.	84	90	.	100n	.	.	<u>94</u>	.
112	120	.	.	90	.	110	.	.	.	.
113 ^b	120	120	.	90	.	110	.	.	94	.
114	.	.	.	<u>90</u>	100n	.	75	120	.	.
115	120	.	.	<u>90</u>	.	110	.	.	.	.
638										
242	<u>120</u>	.	76	.	.	.	.	.	.	.
243	<u>120</u>	.	.	.	.	.	.	.	94	.
244	<u>120</u>	.	.	90	.	.	.	.	<u>94</u>	.
245	<u>120</u>	.	.	<u>90</u>	.	.	.	.	<u>94</u>	.
658										
351	120	.	.	90	.	110	.	120	94	.
352	100n	.	.	.	.	.	75	.	.	.
353	.	.	.	<u>90</u>	.	.	.	.	.	120
354	.	.	.	<u>90</u>	.	.	.	.	.	.
355	120	.	84	90	.	.	.	.	.	.
664										
386	.	.	.	90	.	.	.	.	94	.
387	.	.	.	<u>90</u>	.	110	.	.	.	.
388	.	.	.	<u>90</u>	100n	.	.	.	94	120
389	.	.	76	<u>90</u>	.	110	.	.	94	.
390	120	.	.	.	.	110	.	.	<u>94</u>	120

APPENDIX A. (continued)

STAND AND FAMILY	LOCUS <u>ADH</u> 1	<u>AAT</u> 2	<u>AAT</u> 3	<u>IDH</u> .	<u>LAP</u> 2	<u>MDH</u> 2	<u>MDH</u> 3	<u>6PG</u> 1	<u>6PG</u> 2	<u>PGI</u> 1
669										
426	100n	.	84	.	.	110	.	.	<u>94</u>	.
427	.	.	.	<u>90</u>	.	.	.	.	.	.
428	120	.	.	.	.	.	.	.	.	.
429	.	.	84	90	.	110	.	120	94	.
430	.	.	.	90	.	110	.	.	94	.
679										
486	120	120	.	90	.	110	.	.	94	.
488	<u>120</u>	.	.	.	.	110	.	.	94	120
489	<u>120</u>	.	.	.	.	110	75	.	.	.
490	<u>120</u>	.	76	90	.	.	.	.	94	.
680										
501	.	.	76	.	.	.	.	.	.	.
502	.	120	76	.	.	.	.	.	94	.
503	.	.	76	.	.	.	.	.	94	.
504	120	.	.	.	.	.	75	.	94	.
505 ^c	<u>120</u>	.	.	90	.	.	.	.	94	.
689										
1196	100n	.	.	<u>90</u>	.	.	75	.	94	.
1198	.	.	.	.	.	110	.	.	.	.
1199	120	.	.	90	.	110	.	.	94	.
1200	120	.	76	90	.	110	.	.	<u>94</u>	.

^a homozygotes for common allele (100) symbolized with a dot, heterozygotes with the common allele by a number and homozygotes for other alleles by an underlined number

^b family 113 heterozygous for AAT-1

^c family 505 heterozygous for G6P-1 and MDH-4

APPENDIX

B. JOINT SEGREGATION RATIOS FOR PAIRS OF LOCI IN
FOURTEEN PONDEROSA PINE FAMILIES

LOCI	FAMILY	SEGREGATION ^a	χ^2_1 d.f.
<u>ADH-1</u> : <u>AAT-1</u>	113	12: 6:12: 9	0.23
<u>ADH-1</u> : <u>AAT-2</u>	8	12:11: 5:23	7.08**
	9	19: 4:11:16	8.00**
	113	11: 4: 4:20	13.56***
<u>ADH-1</u> : <u>AAT-3</u>	111	8:11: 8:13	0.10
<u>ADH-1</u> : <u>IDH</u>	8	6:12: 7:15	0.10
	10	7:14:13: 6	4.90*
	111	13: 6: 9:12	2.50
	113	8:10:13: 9	0.90
	351	8:12: 5:10	0.03
	388	6:11:14: 9	2.50
<u>ADH-1</u> : <u>LAP-2</u>	10	12: 9: 7:12	1.60
	388	7:10:13:10	0.90
<u>ADH-1</u> : <u>MDH-2</u>	10	10:10: 9:10	0.03
	111	6:13:14: 7	4.90*
	113	11: 6: 3:18	10.53**
	351	8:12: 8: 7	0.71
	390	12: 9: 8: 9	0.42
<u>ADH-1</u> : <u>MDH-3</u>	8	5: 9:10: 7	1.58
<u>ADH-1</u> : <u>6PG-1</u>	351	12: 8: 5:10	2.31
<u>ADH-1</u> : <u>6PG-2</u>	8	10: 8:13: 9	0.10
	113	10: 8:10:12	0.40
	351	7:13: 9: 6	2.31
	388	13: 4:16: 7	0.00
<u>ADH-1</u> : <u>PGI-1</u>	22	7:17:14: 8	5.57*
	388	18: 4: 2:23	26.06***
	390	16:10: 4:14	5.82*
<u>AAT-1</u> : <u>AAT-2</u>	113	10:14: 5:10	0.03
<u>AAT-1</u> : <u>IDH</u>	113	15: 9: 5:10	3.10
<u>AAT-1</u> : <u>MDH-2</u>	113	8:15: 6: 8	0.68

APPENDIX B. (continued)

LOCI	FAMILY	SEGREGATION ^a	χ^2_1 d.f.
<u>AAT-1</u> : <u>6PG-2</u>	113	12:12: 7: 8	0.03
<u>AAT-2</u> : <u>AAT-3</u>	502	7:10:12:11	0.40
<u>AAT-2</u> : <u>IDH</u>	8	3:10:10:17	0.00
	113	9: 6:11:13	0.64
<u>AAT-2</u> : <u>MDH-2</u>	113	7: 7: 7:16	2.19
<u>AAT-2</u> : <u>MDH-3</u>	8	6: 4: 9:12	0.81
<u>AAT-2</u> : <u>6PG-2</u>	8	6: 7:17:10	1.60
	113	7: 8:12:12	0.03
	502	7:10:10:13	0.00
<u>AAT-3</u> : <u>IDH</u>	111	8: 8:14:10	0.40
	429	10: 9: 9:12	0.40
<u>AAT-3</u> : <u>MDH-2</u>	111	7: 9:13:11	0.40
<u>AAT-3</u> : <u>6PG-1</u>	429	10: 9:12: 9	0.10
<u>AAT-3</u> : <u>6PG-2</u>	429	10: 9:13: 8	0.40
	502	8:11: 9:12	0.00
<u>G6P-1</u> : <u>IDH</u>	505	9:11:12: 8	0.90
<u>G6P-1</u> : <u>MDH-4</u>	505	7:12:13: 7	3.10
<u>G6P-1</u> : <u>6PG-2</u>	505	12: 7: 6:13	3.79
<u>IDH</u> : <u>LAP-2</u>	10	7:13:12: 8	2.50
	388	10:10:10:10	0.00
<u>IDH</u> : <u>MDH-2</u>	10	8:11:11: 9	0.64
	113	6:13: 8:11	0.42
	111	12:10: 8:10	0.40
	351	5: 8:11:11	0.26
	430	12:13: 6: 9	0.10
<u>IDH</u> : <u>MDH-3</u>	8	6: 5: 9:11	0.29
<u>IDH</u> : <u>MDH-4</u>	505	10:10:10: 9	0.03
<u>IDH</u> : <u>6PG-1</u>	351	7: 6:10:12	0.26
	429	12: 7:10:11	0.90

APPENDIX B. (continued)

LOCI	FAMILY	SEGREGATION ^a	χ^2_1 d.f.
<u>IDH</u> : <u>6PG-2</u>	8	6: 7:17:10	1.60
	113	9:12:11: 8	0.90
	351	4: 9:12:10	1.40
	388	16: 4:13: 7	0.90
	429	13: 6:10:11	1.60
	430	15:10: 7: 8	0.90
	505	10:10: 8:10	0.11
<u>IDH</u> : <u>PGI-1</u>	388	7:13: 9:11	0.40
<u>LAP-2</u> : <u>MDH-2</u>	10	8:10:11:10	0.23
<u>LAP-2</u> : <u>MDH-3</u>	114	12:10:11: 7	0.10
<u>LAP-2</u> : <u>6PG-1</u>	114	7:17:23:13	6.67**
<u>LAP-2</u> : <u>6PG-2</u>	388	14: 6:15: 5	0.10
<u>LAP-2</u> : <u>PGI-1</u>	388	9:11: 7:13	0.40
<u>MDH-2</u> : <u>MDH-3</u>	489	11:10:10: 9	0.00
<u>MDH-2</u> : <u>6PG-1</u>	351	8: 8: 9:10	0.03
<u>MDH-2</u> : <u>6PG-2</u>	113	8: 6:10:14	0.95
	351	8: 8: 8:11	0.26
	430	4:14:18: 4	14.40***
<u>MDH-2</u> : <u>PGI-1</u>	390	11: 9: 4:14	3.79
<u>MDH-3</u> : <u>6PG-1</u>	114	11:12: 6:11	0.40
<u>MDH-3</u> : <u>6PG-2</u>	8	9: 6: 9: 7	0.03
<u>MDH-4</u> : <u>6PG-2</u>	505	10:10: 8: 9	0.03
<u>6PG-1</u> : <u>6PG-2</u>	351	7:10: 9: 9	0.26
	429	13: 9:10: 8	0.10
<u>6PG-2</u> : <u>PGI-1</u>	388	12:17: 4: 7	0.10

^a where A and B are two loci, each with two alleles, 1 and 2, the segregation ratio is represented by

$$A_1B_1:A_1B_2:A_2B_1:A_2B_2$$

APPENDIX

C. STATISTICAL METHODS USED FOR SEGREGATION AND
LINKAGE ANALYSIS (from Robinson 1971)

Considering two heterozygous loci where a, b, c, and d represent the frequencies of the four possible gamete genotypes and n is the number of individuals examined:

A. Test for one-to-one segregation at each locus:

$$\text{locus 1} \quad x_1^2 = \frac{[(a+b)-(c+d)]^2}{n} \quad 1 \text{ d.f.}$$

$$\text{locus 2} \quad x_2^2 = \frac{[(a+c)-(b+d)]^2}{n} \quad 1 \text{ d.f.}$$

B. Test for linkage:

$$x_{\text{linkage}}^2 = \frac{(a-b-c+d)^2}{n} \quad 1 \text{ d.f.}$$

C. When segregation is not one-to-one, test for linkage:

$$\text{criss-cross formula} \quad x^2 = \frac{(ad-bc)^2 n}{(a+b)(c+d)(a+c)(b+d)} \quad 1 \text{ d.f.}$$

or

$$\text{ratio formula} \quad x^2 = \frac{(a-R_2b-R_1c+R_1R_2d)^2}{R_1R_2n} \quad 1 \text{ d.f.}$$

$$\text{where } R_1 = (a+b)/(c+d)$$

$$R_2 = (a+c)/(b+d)$$

APPENDIX

D. PHOTOGRAPHS OF GELS

Segregation of ADH-1 Electrophoretic Variant

Segregation of ADH-1 Reduced Activity Variant

Segregation of AAT-3 Electrophoretic Variant

Joint Segregation of AAT-3 and AAT-2 Variants

Joint Segregation at AAT-2 and AAT-1 Variants

Segregation of G6P-1 Electrophoretic Variant

Segregation of IDH Electrophoretic Variant

Segregation of LAP-2 Null Variant

Typical MDH Pattern, No Variation

Segregation of MDH-4 Electrophoretic Variant

Segregation of MDH-3 Electrophoretic Variant, Showing the Triple-Banded Pattern of MDH-2 and MDH-3, and Variation But Inconsistent Staining for MDH-1

Segregation of MDH-2 Variant, Showing the Shifted Position of the MDH-2/3 Hybrid Band (MDH-1 did not stain on this gel)

Segregation of 6PG-1 Electrophoretic Variant

Joint Segregation of 6PG-1 and 6PG-2 Variants

Segregation of PGI-1 Electrophoretic Variant