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USE OF ISOENZYME TECHNIQUES IN FOREST GENETICS RESEARCH

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Abstract.--Genetic variation among loblolly pine (Pinus taeda L.) samples from a natural stand and among clones in seed orchards was analyzed using simply inherited isozyme markers. Alleles for eleven enzyme loci were found useful for genotyping trees in a natural stand in North Carolina. The pines were highly variable with as many as seven alleles per isozyme gene. On the average, close to 30 percent of the loci per tree were heterozygous. Similar levels of variability and heterozygosity were found among the clones in two seed orchards. Such variability makes it possible to uniquely identify most, if not all, of the clones in a seed orchard. Additional genes and other southern pine species are suited for similar analyses. A study with 15 different enzyme systems and samples from six species; loblolly, shortleaf, slash, pond, longleaf, and Virginia pines, suggested that as many as 26 different loci may be available for analysis and most loci appear to be similar for these species.

Additional keywords: Single genes, seed enzymes, conifers, seed orchards, Pinus taeda, P. echinata, P. elliottii, P. serotia, P. palustris, P. virginiana.

Ask a forest geneticist what could be accomplished if a variety of single gene markers were available and you are likely to hear research proposed that would keep armies of scientists busy for many years. The analysis of conifer enzymes yields data for numerous single gene markers.

Enzymes are primary gene products. Their molecular structure is determined by the DNA code of nuclear genes. Different DNA codes of a gene can alter the form of the specific enzyme produced. A technique called gel electrophoresis separates different forms of similar enzymes. The forms segregate and recombine as expected for Mendelian hypotheses.

The research results in this paper will be drawn from studies of southern pines with the principal emphasis on loblolly pine. In independent investigations, the authors analyzed seed from trees growing in a natural stand in the Schenck Forest, North Carolina State University, Raleigh, North Carolina (M. T. Conkle) and also from trees in two clonal-seed-orchards belonging to Champion International, Newberry, South Carolina (W. T. Adams). Our studies show that this technique can be extended to include more enzyme systems and other species of southern pines. The results will be presented after a short description of electrophoresis and seed sampling techniques.

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METHODS

Electrophoresis technique

Starch gel slabs (13% starch) are loaded with a row of paper wicks. Each wick carries a liquid sample from a seed. The samples contain a mixture of enzymes. Direct electric current is applied to gels, and enzymes with negative electric charges migrate toward the anode. Positively charged enzymes migrate toward the cathode. The rate of migration of any particular enzyme depends upon the size of the net electric charge on the molecule. The current is applied for a specific length of time or until marker dyes reach a specific distance. Thick gels are cut into several slices and each slice is stained for a different enzyme system. The bands that appear on the stained slices mark the relative migration distances of specific enzymes. In this manner, numerous seed samples can be analyzed for genes within several enzyme systems. For a more complete description of techniques, see Brewer (1970) and Conkle (1972).

Seed samples

Conifer seeds have excellent tissues for isoenzyme analyses. The female gametophyte, which is the nutrient tissue surrounding the embryo, is haploid (1N) and has the same genetic constitution as the female gamete, the egg. The analysis of several gametophytes from different seeds of a tree reveals the genotype of that tree. The enzyme bands for a particular allele consistently migrate to the same location on gels. A tree that is homozygous for a gene will have bands from gametophytes in only one location on the gel. A tree heterozygous for alleles of a gene will have bands in two locations. Heterozygotes are tested to determine whether the two different forms segregate with a 1:1 ratio.

The genotype of the embryo (2N) also can be determined for several enzymes. Knowing the genotype of an embryo and its adjacent gametophyte, the pollen contribution to the embryo can be inferred. Thus, the parent tree genotype, and the genotypes of individual eggs and pollens can be determined by the analysis of seed from a single tree.

Dry seed-samples can be used, but pine seeds that are just beginning to germinate give darker staining bands (Conkle, 1971) and are easier to prepare. The data presented in the remainder of this paper are based on the analysis of six gametophytes for the determination of each tree's genotype. Where several species were compared, 24 seeds from general seed collections for each species were analyzed.

RESULTS

Isozymes from loblolly pines in a natural stand

The locations and ages of the 146 cone bearing trees sampled were recorded and open-pollinated cones of each tree were collected. The seeds of each tree were analyzed for seven different enzymes systems (ACPH, ADH,

BANA, EST, GOT, LAP, PGM; see [2/] for the full names of enzymes, these abbreviations will be used throughout the paper). One gene with two or more alleles was identified in the ACPH, ADH, BANA, and PGM enzyme systems. Two genes were active in LAP and EST systems (EST-1, EST-2, LAP-1, and LAP-2). Three genes were scored in the GOT system (GOT-1, GOT-2, and GOT-3). In all, each tree was analyzed for genotypes at 11 different loci.

All 11 loci segregated for two or more alleles and the segregation of alleles from the gametophytes of heterozygous trees did not differ significantly from the expected 1:1 ratio. Nine of the 11 loci had 3 or more alleles within the stand (table 1). The gene with the greatest number of alleles was ACPH with 7 distinctly different allelic forms.

The genotypes of all 146 trees were used to estimate the allele frequencies for the stand (table 1). Each tree has two alleles per gene and the frequency estimates are based on a total of 292 observations.

Table 1.--Allele frequencies for eleven genes from trees of a natural stand of loblolly pine near Raleigh, North Carolina

Gene	Allele number 1	2	3	4	5	6	7
	Frequency						
ACPH	.82	.05	.05	.03	.03	.01	.01
ADH	.99	.01					
BANA	.92	.04	.04				
EST-1	.40	.31	.11	.08	.07	.03	
EST-2	.64	.34	.01	.01			
GOT-1	.77	.20	.03				
GOT-2	.51	.44	.05				
GOT-3	.79	.21					
LAP-1	.87	.05	.04	.04			
LAP-2	.53	.46	.01	a/			
PGM	.95	.03	.02				

a/ Rare allele found in only one tree.

The trees of the stand are genetically highly variable. Some genes have one allele in high frequency. For example, ACPH, ADH, BANA, LAP-1, and PGM have one allele that occurs greater than 80 percent of the time.

2/ ACPH = acid phosphatase, ADH = alcohol dehydrogenase, BANA = endopeptidase, CAT = catalase, EST = esterase, GDH = glutamate dehydrogenase, GOT = glutamate oxaloacetate transaminase, G-6-PDH = glucose-6-phosphate dehydrogenase, IDH = isocitrate dehydrogenase, LAP = leucine amino peptidase, MDH = malate dehydrogenase, PER = peroxidase, 6-PGDH = 6-phosphogluconate dehydrogenase, PGI = phosphoglucoisomerase, PGM = phosphoglucomutase, TO = tetrazolium oxidase (indophenol oxidase).

Other genes, such as EST-1, EST-2, GOT-1, GOT-2, GOT-3, and LAP-2, have two or more alleles with intermediate frequencies. All the genes, except GOT-3, have rare alleles that occur at frequencies of 5 percent or less.

This natural stand was chosen because two age classes could be identified. A group of 48 older trees are the suspected parents of 98 younger trees that sample the regeneration of an old field. The gene frequencies for the two age classes and the geographic distribution of genes over the stand are the subjects of future analyses. Preliminary observations are that the allele frequencies are approximately equal in the older and younger trees, and that genotypes are randomly distributed within the stand.

The isozyme data was used to estimate the degree of heterozygosity present in the stand. The number of heterozygous loci per tree was counted. On the average, trees were heterozygous for 3.5 of the 11 genes; --heterozygosity is estimated to be 32 percent. A large number of trees were heterozygous at 2 to 5 loci. Only one tree was homozygous for all 11 loci. On the other end of the scale, one tree was heterozygous for 7 loci and another was heterozygous for 8. These estimates are comparable to those reported for other pine species (Feret 1974, and Rudin et al. 1974).

The uniqueness of each tree's genotype is related to the number of alleles per locus and their frequency in the population. Each gene with multiple alleles has $n(n+1)/2$ possible genotypic classes, where n = the number of alleles. The ACPH gene with 7 different alleles could produce 28 different classes of diploid genotypes. If this line of reasoning is extended to include all 11 loci, there could be 10 genotypic classes for LAP-1, 10 classes for LAP-2, and so on. The total number of different diploid genotypes that are possible for these genes, is the product of the number of genotypic classes for each locus ($28 \times 10 \times 10 \times \ldots$). For these 11 genes 6,858,432,000 different genotypes are possible. If the proportions found for the most frequent genotypic class of each locus are multiplied together, the most frequently duplicated 11-locus genotype would be expected to be found in only 3 out of 1,000 individuals. Various factors such as the breeding system, family relationships, linkage, selection, and genetic drift can alter this expectation, but the point is that each tree is virtually a unique genotype.

Genetic identity of clones in loblolly pine seed orchards

The large amount of genetic variability found in the natural stand leads to the expectation that many of the twenty to thirty clones of a seed orchard should be uniquely identifiable on the basis of their isozyme genotypes. The analysis of seed from trees in two orchards shows that most clones have unique genotypes (table 2).

The uniqueness of clone genotypes in orchards (table 2) is based on the analysis of 10 loci from six enzyme systems (GDH, GOT, LAP, 6-PGDH, PGI, and PGM). Twenty six of the 27 clones in the HIGH SPECIFIC GRAVITY orchard have unique 10-locus genotypes. A similar result was found for the clones in the LOW SPECIFIC GRAVITY orchard (22 of 23 clones have unique genotypes). When the genotypes of clones from both orchards are considered together, 47 of the total of 50 clones are unique. Thus, not only are most clones within an

orchard unique; --virtually all clones in the two orchards have unique genotypes. Only one genotype is common to a clone in each orchard.

Table 2.--Average heterozygosity and genotypic uniqueness of clones in two loblolly pine seed orchards

Seed orchard	Number of clones	Average heterozygosity per clone (Percent)	Number of unique genotypes
HIGH SPECIFIC GRAVITY CLONES	27	27	26
LOW SPECIFIC GRAVITY CLONES	23	29	22

The high degree of variability among clones in these loblolly orchards agrees with data supplied by Ms. Serena Hunter, North Carolina State University, Raleigh (personal communication, May, 1977). She finds all 27 clones in a Weyerhaeuser Company orchard, Washington, North Carolina, to be uniquely identifiable using twelve genes from six enzyme systems (ACPH, EST, GOT, LAP, MDH, and 6-PGDH).

The estimates of average heterozygosity per clone for the two orchards (table 2) are close to the 32 percent value found for the native stand and Ms. Hunter's estimate of 32 percent for clones in the North Carolina seed orchard. Although these individual estimates are based on different loblolly pine samples and different enzyme loci, they cluster around a common value of 30 percent.

Isozymes from six southern pine species

Loblolly, shortleaf, slash, pond, longleaf, and Virginia pines were analyzed using the same techniques but including more enzyme systems. The goal of this study was to identify more enzyme systems and extend the analysis to other southern pines. Our interest centers upon estimating the number of potentially useful loci.

Each of the species was represented by a small number of seeds (24 seeds per species). The seeds were samples from large open-pollinated collections. The female gametophyte and the embryo of each seed were analyzed separately. In all, each tissue was examined for isozymes in 15 different enzyme systems (ACPH, ADH, CAT, EST, GDH, GOT, G-6-PDH, IDH, LAP, MDH, PER, 6-PGDH, PGI, PGM, and TO).

All 15 enzyme systems resolve some bands for gametophytes and embryos of the six southern pine species that were included. The first noteworthy observation is that there is a striking similarity between the patterns of enzyme bands for all species within each enzyme system. The number of bands, their migration distances, their characteristic sizes and stain intensities all indicate that similar if not identical genes are acting within the six

species. Similar findings were reported for a comparison of pitch and loblolly pines (Adams and Coutinho, 1977). It seems safe to predict that differences between these species will be a function of the allele frequencies that are found for a set of genes common to all southern pines.

The enzyme bands of the various species are so similar that inferences from genetic studies of loblolly pine might apply to the other species. First, using data from gametophytes only, at least one locus may be available from the ACPH, CAT, GDH, G-6-PDH, and PGM enzyme systems. Two loci may be available from each of the following systems; ADH, EST, LAP, 6-PGDH, PGI, and TO. It may be possible to obtain information on as many as three loci from the GOT, MDH, and PER enzyme systems. Thus, current techniques for isozyme analysis of southern pines may be capable of providing genotypic information for as many as 26 loci. This is more than twice the number of loci that were used for the current analyses of trees in the natural stand and clones in seed orchards.

In general, the enzyme bands from embryos of all six species were not as well resolved as were the corresponding bands for gametophytes. Despite this fact, embryos yielded some bands in most enzyme systems for all species. However, PER enzymes from embryos, were poorly resolved. Embryos stained for ADH were best resolved for loblolly and longleaf pine samples. The IDH bands from longleaf and slash pine embryos were resolved, other species stained poorly. With these exceptions, it appears likely that embryo genotypes will be available for loci in several enzyme systems. Many of the bands in embryos appear to be expressing genes that are common to gametophytes.

DISCUSSION

Much work in the area of population genetics of forest trees has been based upon the statistical partitioning of the total variation in phenotypic traits into causes that are related to genetics and causes that are related to test environments. In the majority of such studies, the observed response cannot be attributed to the action of a specific gene and the response is often related to the specific test conditions. In contrast, isozyme techniques identify alleles for specific loci and the identification of the tree genotype does not depend upon the test environment.

Trees may have as many as 100,000 different kinds of protein and the effect of a single enzyme variant is likely to be unmeasurable. But enzymes can serve to estimate the genetic variability within and between groups of trees.

A concern that forest researchers have voiced is whether sufficient genetic variability is present to characterize individual forest trees, forest tree populations, and species. Another concern has been whether the number of enzyme loci analyzed is sufficiently large to be considered an adequate sample. Finally, forest researchers are asking whether the enzyme loci that can be analyzed are independent samples of a tree's genes.

Loblolly pines have large amounts of genetic variability judging from these enzyme studies. On the average, we find approximately 3.75 alleles per locus for the genes reported in this paper. Loblolly pines are heterozygous

at nearly 30 percent of the loci that were sampled. As a consequence, an enormous number of different tree genotypes is possible and each tree is virtually a unique genotype. This genotypic uniqueness was shown useful for identifying individual clones within seed orchards. In the future, the technique may be used to determine how variability is distributed among samples from natural populations of southern pines and it may prove useful for seed source identification. The technique may also be used to compare trees in tree improvement programs and to follow the genetic variability of these selected populations through future cycles of selection.

The current study also attempted to expand the number of enzyme systems that are appropriate for the analysis of southern pines. There are preliminary indications that at least 15 enzyme systems are capable of resolving bands and each enzyme system may resolve between one and three loci. It appears that current technology could provide analyses for about 26 loci. Since this aspect of the work has not been thoroughly researched, we conclude that this is a minimum estimate and future analyses should increase the number of usable loci.

The technique is appropriate for many southern pines, namely; loblolly, shortleaf, slash, pond, longleaf, and Virginia pines. Seeds from these various species resolved enzyme bands with similar characteristics. The loci of the various species appear to be comparable. Future studies of speciation, and introgression will depend upon estimating the allele frequencies for genes common to the different species.

Though no linkage studies for southern pines were reported in this paper, it should be pointed out that they are planned. The haploid gametophyte of conifer seeds may promote conifers as the best possible materials for such studies. The test of independent assortment of alleles for various loci can be done using the female gametophytes of a tree heterozygous for several loci. Since pollen genotypes can be determined, linkage studies are also possible using data from male gametes but selfed-families or full-sib families are required.

Other studies are possible using the information from alleles in pollen. Investigations of outcrossing and selfing (i.e.; Muller, 1976), studies of hybridization (i.e.; Adams and Coutinho, 1977, and Tobolski and Conkle, 1977) and studies of pollen dispersion, migration, and effectiveness are a few examples. Recall, also, that numerous loci in the loblolly pine natural stand had alleles occurring with low frequencies. These rare alleles may prove very useful as markers in pollen studies.

The research and thoughts concerning future research presented in this paper are but a sampling of the potential this technique holds for investigating genetic variability in forest trees.

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