

# Applying Isozyme Analyses in Tree-Breeding Programs<sup>1</sup>

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Abstract: Four examples illustrate the potential for practical use of isozyme analyses in applied breeding programs. These include identifying parent trees and clones, seed sources, and parentage of controlled crosses, and evaluating the effectiveness of different procedures involving open-pollination to produce seed of specific crosses. The improved ability to assess the true identity of parents, seed stocks, and controlled crosses justifies the added expense resulting from the use of isozyme analyses in applied tree-breeding programs.

Some papers in this symposium, as well as others previously published (Bergmann 1978, Guries and Ledig 1977, Lundkvist and Rudin 1977, Mitton and others 1977, Muller 1977, Rudin and others 1974), demonstrate the use of isozymes in studying genetic structure and variation patterns in populations of forest trees. Recently, isozyme investigations also have been started in seed orchards<sup>3</sup> (Rudin and Lindgren 1977). Information from studies in both natural and seed orchard populations no doubt will help to increase the efficiency of future tree-improvement programs.

In addition to their research value in forest genetics, isozymes have more immediate application as genetic markers in tree-breeding programs. By providing examples primarily from my own work, I illustrate several possible applications. These examples demonstrate that isozyme analyses can be used by tree breeders to determine and maintain the identity of individuals, crosses, and breeding populations with greater accuracy than was possible previously.

## IDENTIFYING PARENT TREES AND CLONES

If isozymes are to be effective genetic markers in breeding programs (Rudin and Lindgren 1977), isozyme variation among parent trees or orchard clones must be large. This is expected in most forest tree species because an effort is usually made to incorporate a broad base of genotypes in breeding populations. The large isozyme variation found among clones in one pitch pine (*Pinus rigida* Mill.) breeding orchard and three loblolly pine (*Pinus taeda* L.) seed orchards<sup>3</sup> (Hunter 1977) supports this expectation (table 1). As many as nine alleles were present

at any one isozyme locus, and the average percentage of heterozygous loci per clone ranged from 15 to 33 percent.

For clonal identification it is preferable to have many alleles per locus, each of about equal frequency. However, although isozyme loci in these four orchards (table 1) averaged about three alleles, one allele was usually in high frequency. Isozyme variation among these clones, therefore, was far from "ideal." Nevertheless, enough variation among clones existed so that most could be individually identified by using the sampled isozymes as genetic markers. On the basis of the 11 polymorphic loci scored in each clone, 28 unique genotypes were recognized among the 32 clones in the pitch pine orchard; similarly, 22 twelve-locus genotypes and 25 eleven-locus genotypes were recognized, respectively, among the 23 and 27 clones in the two South Carolina loblolly orchards. All 27 clones in the North Carolina loblolly orchard could be uniquely identified on the basis of their twelve-locus genotypes. Complete resolution of clones in the first three orchards could be achieved if additional loci were evaluated. These results indicate that isozyme gene markers can be useful for maintaining the identity of ortets and their ramets in a breeding program.

Hunter (1977), for example, has used isozymes to detect labeling errors in seed orchard trees. Six of the 22 clones with more than one ramet in the North Carolina loblolly orchard had at least one mislabeled ramet, discovered because the genotypes of the mislabeled ramets differed from those of the other ramets representing a clone.

The genotype of each orchard clone was determined from seed samples on the basis of the composition of allozymes (allelic isozymes) observed in megagametophytes. When this method is used, a genotype will be incorrectly identified only if all megagametophytes sampled from a heterozygote carry the same allozyme allele; with  $N$  megagametophytes, this probability is  $(1/2)^{N-1}$  for a single locus. For a sample of only 10 seeds, therefore, the probability of incorrectly identifying a clone's genotype at any one locus is less than 0.2 percent. Assuming all loci are independent, the probability of error in identifying a 12-locus genotype is less than 2.5 percent.

In general, extracting enzymes from seed tissues is easier than from vegetative tissues (for example, needles, buds, or bark) of seedlings or older trees. However, analyzing

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<sup>3</sup> Adams, W.T., and R.J. Joly. Allozyme studies in loblolly pine seed orchards: clonal variation and frequency of progeny due to self-fertilization. (In press.)

Table 1—Variation at isozyme loci polymorphic among clones in one pitch pine breeding orchard and three loblolly pine seed orchards

Orchard <sup>1</sup>	Number		Alleles per locus		Most frequent allele at each locus		Heterozygous loci per clone		
	Clones	Loci	Most	Mean	Range	Mean	Range	Mean	Mean
								Number	Percent
Pitch pine	32	11	6	2.9	0.98 to 0.70	0.91	0 to 4	1.7	15
Loblolly pine <sup>2</sup>									
South Carolina-LSG	23	12	6	3.0	.98 to .46	.82	1 to 5	3.0	25
Loblolly pine <sup>2</sup>									
South Carolina-HSG	27	11	5	3.0	.98 to .44	.81	0 to 6	2.9	26
Loblolly pine <sup>3</sup>									
North Carolina	27	12	9	3.3	.96 to .26	.73	1 to 8	4.0	33

<sup>1</sup>The two South Carolina orchards are located at the same site. The low specific gravity (LSG) orchard consists of clones selected for wood of low specific gravity; the high specific gravity (HSG) orchard consists of clones selected for wood of high specific gravity.

<sup>2</sup>Data from Adams and Joly. (In press.)

<sup>3</sup>Data from Hunter (1977).

isozymes from vegetative tissues is necessary if genotypes of juvenile and nonflowering mature individuals are to be identified. A set of isozyme loci expressing clearly in both seed and vegetative tissues would be of most benefit; several examples of such loci have been reported (Bartels 1971, Lundkvist 1975, Mitton and others 1977, Rudin 1975).

## SEED CERTIFICATION

Interest in seed certification will intensify as demand for genetically improved seed increases (Rudolph 1974). Because considerable isozyme variation has been observed among orchard clones, the seed of individual clones or that from specific crosses can be distinguished from the seed of most other clones or crosses. Isozymes can be used, therefore, to certify the identity of specific seed lots.

The expected frequencies of isozymes in the seed of the two South Carolina loblolly pine orchards were determined by weighting each clonal genotype according to the number of its ramets and assuming random mating (table 2).<sup>3</sup> Genetic makeup of seed lots from these orchards was expected to be similar because the clones came from parent trees in the same breeding zone and, except for wood density, were selected for the same qualities. This was generally borne out—at most loci, the most frequent allele in one orchard was also the most frequent in the other. Differences between orchards were also observed, however; some alleles had dissimilar frequencies, and some occurred in one orchard but not in the other. Therefore, one could readily determine from which orchard a particular seed lot was derived.

In several species, evidence is accumulating to indicate that variation in isozyme frequencies between provenances can be expected (Bergmann 1978, Lundkvist and Rudin 1977, Mitton and others 1977, Rudin and others 1974). Thus, even greater differences in isozymes may occur among seed lots from orchards composed of clones from various geographical areas. Similarly, isozymes could certify the geographical origin of seed collected from

natural stands (Bergmann 1972). Changes in isozyme frequency may be strongly correlated with changes in environment (Bergmann 1978, Grant and Mitton 1977, Mitton and others 1977), in which case isozymes can be helpful in defining breeding and seed zones (Ferret and Bergmann 1976).

Table 2—Expected allelic frequencies in the seed crops of two South Carolina loblolly pine seed orchards

Locus	Allele <sup>2</sup>	Orchards		Locus	Allele	Orchard		
		LSG	HSG			LSG	HSG	
PGI1	1	0.015	0.015	LAP2	1	0.417	0.503	
	2	.985	.985		2	.583	.450	
					3	—	.047	
GOT1	1	.156	.007	PGI2	1	.047	—	
	1L <sup>3</sup>	—	.025		2	.822	.930	
	2	.184	.362		3	.108	.057	
AP2	3	.660	.606	GOT2	4	.023	.013	
	1	.015	.052		1	.168	.098	
	2	.889	.938		2	.007	—	
MDH2	3	.096	.010	3	3	.829	.902	
	1	.905	.993		6PGD	1	.025	—
	2	.095	.007			2	.397	.407
LAP1				3		.053	.015	
	1	.065	—	4	.015	.085		
	2	.836	.862	5	.503	.481		
	3	.048	.045	5L	.007	—		
GDH	N	.051	.093	6	—	.012		
	1	—	.038	PGM1	1	.944	.978	
	2	.939	.787		2	.056	.022	
	3	.013	—	PGM2	1	.028	—	
	4	—	.010		2	.972	1.00	
5	.048	.165						

<sup>1</sup> LSG is low specific gravity; HSG is high specific gravity. (From Adams and Joly. In press.)

<sup>2</sup> Alleles are numbered in ascending order from the fastest to the slowest migrating isozymes. N is a null allele; L is an allele coding a very lightly staining band.

<sup>3</sup> A dash (—) means no allele present.

## DETERMINING VALIDITY OF CONTROLLED CROSSES

Tree-breeding programs usually require many controlled crosses, which generally are assumed to have been made without error. This assumption, however, has not been tested because no accurate method has been available. Using isozyme markers to test the validity of controlled crosses is relatively simple (if sizable isozyme variability exists among clones or parent trees): first, parental isozyme genotypes are determined, and second, their progeny are analyzed to see whether their isozyme makeup is as expected.

To demonstrate this application, we conducted the following study. Seeds were supplied from 30 two-parent loblolly pine crosses made by three different organizations (designated A, B, C) in the North Carolina State-Industry Cooperative Tree Improvement Program (*table 3*). Additional seed lots from all the clones involved as male parents and from clones of the female parents used in one-half of the 30 crosses also were obtained. Megagametophytes of at least 10 seeds from each parental seed lot were analyzed and the isozyme genotypes of the clones inferred at six loci. Embryo and megagametophyte tissues from 20 to 30 seeds of each of the 30 controlled crosses were then assayed at the same six loci. By analyzing both the haploid and diploid seed tissues, it was possible to determine the allelic composition of the ovule and pollen gametes forming the embryo. Because the allelic composition of the ovule is the same as that in the megagametophyte, the remaining allele (after accounting for the allele contributed by the ovule) must be from the pollen gamete. The pollen pools (that is, pollen gametes effective in fertilizing viable seeds) of the crosses, therefore, can be compared with those expected on the basis of each pollen parent's isozyme genotype; similar comparisons can be made for the ovule pools of the 15

crosses for which independent information on the genotypes of the female parents is available.

Evidence of error in female identity was found in only one (cross 18 X 19) of the 30 crosses. Seed from clone 18 had apparently been mixed with seed from one or more other clones, because five of the 20 seeds sampled contained alleles in megagametophytes that could not have come from clone 18. At least some of the contaminated seed could have come from clone 19; this clone has alleles at two loci not found in any of the other sampled clones, and both of these alleles were found in the contaminated seed. The allelic composition of megagametophytes was as expected in the remaining 14 crosses for which the isozyme genotypes of the maternal parents were known, and no more than two alleles per locus were observed among the megagametophytes of the other crosses sampled.

Pollen contamination seemed to be a real problem in organization C (*table 3*). Although the allelic compositions of the pollen pools were as expected in all but one of the 20 crosses made by organizations A and B, 8 out of 10 crosses for organization C contained alleles that could not have come from the supposed pollen parent. Apparently, procedural problems in pollen handling, bagging, or making the crosses resulted in considerable pollen contamination.

Caution is needed in comparing the validity of crosses made by different organizations when using the technique of isozyme markers. The ability to detect errors is somewhat dependent upon parents carrying alleles at one or more marker loci that are relatively infrequent among other sources of pollen. If the variation among clones is generally large, however, the results from different organizations should be roughly comparable. The extent of contamination is underestimated with this technique because contaminants having the same allelic compositions as those expected would remain undetected. Nevertheless, isozyme gene markers will probably uncover most errors in seed-handling and control-crossing procedures.

Table 3—Number of detected pollen contaminants (d.c.)<sup>1</sup> observed in the pollen pools of seeds from 10 controlled two-parent crosses of loblolly pine from each of three organizations

Organization A			Organization B			Organization C		
Cross	Observed pollen gametes		Cross	Observed pollen gametes		Cross	Observed pollen gametes	
	♀	♂		♀	♂		♀	♂
1 X 2	0	29	15 X 16	0	29	<sup>2</sup> 31 X 32	5	30
<sup>2</sup> 3 X 2	0	30	17 X 16	0	28	<sup>2</sup> 33 X 32	7	30
<sup>2</sup> 4 X 5	0	30	<sup>2</sup> 18 X 19	0	20	<sup>2</sup> 34 X 31	0	30
6 X 5	0	27	20 X 21	0	22	<sup>2</sup> 35 X 31	1	30
7 X 8	0	28	15 X 22	0	30	<sup>2</sup> 36 X 35	2	30
9 X 8	0	28	23 X 18	0	30	<sup>2</sup> 3 X 35	0	30
10 X 11	0	28	24 X 25	0	29	<sup>2</sup> 3 X 37	2	30
12 X 11	0	26	<sup>2</sup> 26 X 27	0	30	<sup>2</sup> 38 X 39	23	30
13 X 14	0	30	28 X 27	12	30	<sup>2</sup> 40 X 41	16	30
7 X 14	0	30	<sup>2</sup> 29 X 30	0	21	<sup>2</sup> 30 X 39	14	30

<sup>1</sup> Detected pollen contaminants (d.c.) are pollen gametes with an allele at one or more of six isozyme loci *not* carried by the supposed pollen parent involved in the cross.

<sup>2</sup> Those clones used as female parents whose genotypes at six isozyme loci were determined from megagametophytes of an independent seed sample (see text for details).

## EFFECTIVENESS OF OPEN-POLLINATION TO PRODUCE SEED OF SPECIFIC CROSSES

To avoid costly control-pollinations, alternate methods for producing hybrid seed or seed of outstanding specific combinations have been sought. Among the possibilities are wind-pollinated two-clone (or two-species) seed orchards (Hyun 1976, Wright 1976) or supplemental mass pollination (Little and Trew 1977, Wakeley and others 1965, Woessner and Franklin 1973). If the isozyme makeup of parents is sufficiently different, crosses between and within parents can be distinguished. Isozymes, therefore, can help to determine the effectiveness of such alternatives.

The isozyme approach was used to estimate the proportion of hybrid seed obtained when unbagged female flowers of pitch pine were mass pollinated with pollen mixes from loblolly pine parents.<sup>4</sup> An isozyme gene marker was found in high frequency in the pollen mix but was rare in any pitch pine source. On the basis of the proportion of pollen pool gametes of mass-pollinated clones carrying this marker, the proportion of hybrid seed was estimated to range from 2 to 42 percent (averaging 16 percent) for six clones. A nearly identical mean percentage (17 percent) of hybrids was found for the progeny of these clones by scoring 1-year-old seedlings in the nursery into hybrid and nonhybrid classes on the basis of their stem and foliage characteristics.<sup>5</sup> Because the isozyme technique determines the extent of hybrid seed set before nursery establishment, only the seed of clones with high hybrid seed percentages need be sown.

## DISCUSSION AND CONCLUSIONS

The preceding examples are but a few of the many possible uses of isozymes in applied tree-breeding programs. Although these applications can be invaluable to the tree breeder, their practical implementation will depend on a cost-benefit analysis. The ability to better determine and maintain the true identity of breeding materials can only be assessed relative to the probability of errors under current breeding technology. As seen in the controlled crosses, this may vary widely among organizations. Even if error in the handling of breeding materials is now relatively small, its effect on reduction of genetic gains is likely to be magnified in advanced generation breeding. Maintaining the identity of breeding materials, therefore, will become increasingly important in the future.

Although costs of isozyme analyses will vary substantially among laboratories, it is helpful to provide some

rough estimates. For instance, I have found that processing from 100 to 150 seed tissue extracts by using horizontal starch gel electrophoresis (Conkle 1972) on 1 working day is about maximum for a full-time technician with a half-time assistant to help prepare plants, wash dishes, and do other required tasks. A laboratory with this capability can be equipped for less than \$15,000 (including large glass door refrigerator, power supplies, incubator, balance, pH meter, plexiglas gel forms and buffer trays, and miscellaneous glassware and supplies). This estimate assumes a standard laboratory with piped-in distilled water.

For the enzymes normally analyzed, our daily cost of chemicals and supplies averages about \$40. With 1 day's run, we can assess the genetic makeup of 9 to 14 parent trees at 10 to 15 loci or analyze from 25 to 30 progeny each from 1.5 to 2.5 controlled crosses for the same number of loci. We can genetically identify a clone for \$3 to \$5 and determine the validity of a controlled cross for \$15 to \$25. Although these figures do not include labor, utilities, or depreciation on equipment, at even double the costs, they seem fairly reasonable compared with other expenses normally incurred in a tree-improvement program (Porterfield 1974).

I am not suggesting that it is economically feasible or prudent for most individual organizations to establish their own electrophoresis laboratories at this time. The potential benefits of isozyme analyses in breeding programs, however, seem great enough for research units of larger organizations and tree-improvement cooperatives to consider establishing such laboratories soon. Because of the long generation times faced by most tree breeders, we can hardly afford not to seriously consider isozyme analyses for increasing breeding efficiency.

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1981. **Proceedings of the symposium on isozymes of North American forest trees and forest insects, July 27, 1979, Berkeley, California.** Gen. Tech. Rep. PSW-48, 64 p., illus. Pacific Southwest Forest and Range Exp. Stn., Forest Serv., U.S. Dep. Agric., Berkeley, Calif.

These 10 symposium papers discuss gene resource management, basic genetics, genetic variation between and within tree species, genetic variability and growth, comparisons of tree life history characteristics, genetic variation in forest insects, breeding systems, and applied uses of isozymes in breeding programs.

*Retrieval Terms:* forest tree breeding, isozymes, genetic variation, North America, symposia

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