Allozyme Studies in Loblolly Pine Seed Orchards: Clonal Variation and Frequency of Progeny due to Self-Fertilization

By W. T. Adams[1,2]) and R. J. Joly[1])

Summary

As part of a study using allozymes to investigate factors associated with genetic efficiency in two adjacent loblolly pine (Pinus taeda L.) seed orchards, we wished to determine 1) the degree of allozyme variability among clones and 2) the proportion of orchard progeny resulting from self-fertilization. Considerable genetic variation was found among the 50 clones in the two orchards. Twelve of the 15 loci studied were polymorphic, averaging 3.4 alleles per locus; nearly 25% of the polymorphic loci were heterozygous in any one individual. The potential for using allozymes as clone markers in breeding programs is demonstrated by the fact that 47 of the 50 clones could be uniquely identified based on their allozyme genotypes.

Allozyme frequencies in the two orchards were similar in that alleles most frequent in one were generally most frequent in the other. This was not unexpected because wood specific gravity was the only trait by which the orchard clones were differentially selected. However, important allelic differences occurred, most notably the presence of alleles in one orchard not found in the other. Many of these alleles were also unique to individual clones and could be used to determine how specific clones contribute to the seed crop and how pollen is distributed within and between orchards.

By observing the frequency of unique alleles in the pollen of clones carrying them, we estimated the proportion of selfed progeny to be only 1.2%, based on 513 seeds from five clones. Apparently, selfing has only minor influence on the genetic makeup of the seed crops in these orchards.

Key words: Pinus taeda L., allozyme variation, seed orchards, genetic markers, self-fertilization.

Zusammenfassung

Als Teil einer Studie zur Untersuchung von Faktoren der genetischen Effizienz in zwei benachbarten loblolly pine (Pinus taeda L.)-Samenplantagen mit Hilfe von Allozymen, sollte (1) das Ausmaß der Allozymvariabilität zwischen verschiedenen Klonen geprüft und (2) der Anteil von Plantagen-Nachkommenschaften ermittelt werden, der aus Selbstung hervorgegangen war. Zwischen den 50 Klonen der zwei Plantagen bestand eine erhebliche genetische Variation. Zwölf der 15 untersuchten Loci waren polymorph mit durchschnittlich 3,4 Allelen pro Locus; in jedem Individuum waren fast 25% der polymorphen Loci heterozygot. Die Bedeutung des Gebrauchs der Allozyme zur Klonidentifizierung in Züchtungsprogrammen wird durch die Tatsache deutlich, daß 47 von 50 Klonen ausschließlich aufgrund ihrer Allozymgenotypen identifiziert werden konnten.

Die Allozymfrequenzen in den beiden Plantagen waren insofern ähnlich, als die häufigsten Allele in der einen Plantage auch in der anderen am häufigsten auftraten. Dies Ergebnis war auch zu erwarten, da das spezifische Gewicht

[1]) Assistant Professor of Forest Genetics and Research Forester, Institute of Natural and Environmental Resources, University of New Hampshire, Durham, New Hampshire 03824, U. S. A. Scientific Contribution No. 978 from the New Hampshire Agricultural Experiment Station.

[2]) Currently Assistant Professor of Forest Genetics, Department of Forest Science, Oregon State University, Corvallis, Oregon 97331, U. S. A.

Address reprint requests to Publications, Forest Research Laboratory, Oregon State University, Corvallis, Oregon 97331.

des Holzes als einziges Merkmal benutzt worden war, nach dem die Plantagenklone selektiert worden waren. Es traten jedoch auch wichtige Unterschiede zutage, wie z. B. das Auftreten von Allelen in lediglich einer der beiden Plantagen. Viele Allele waren nur auf bestimmte Klone beschränkt und konnten zur Lösung der Fragen benutzt werden, inwieweit diese zur Samenproduktion beitragen oder wie der Pollen innerhalb und zwischen Plantagen verteilt wird.

Durch die Beobachtung der Frequenzen einziger Allele in den Pollenpools von Klonen schätzten wir den Anteil von Selbstungs-Nachkommenschaften auf nur 1,2%. Dieses Ergebnis basiert auf 513 Samen von 5 Klonen. Die Selbstbefruchtung hat folglich nur einen geringen Einfluß auf die genetische Zusammensetzung von Samenernten in diesen Plantagen.

Introduction

Conventional seed orchard designs emphasize using large numbers of clones with wide separation between ramets of the same clone to promote cross-fertilization. The resulting seed crop is expected to reflect both the genetic superiority and broad genetic base present among the orchard clones. The degree to which a seed orchard achieves this expectation is its genetic efficiency. Yet despite the economic and silvicultural importance of seed orchards, very little is known of their genetic efficiencies.

Woessner and Franklin (1973) have listed five conditions about wind-pollinated seed orchards that must be met to maximize genetic efficiency: 1) orchard ramets are essentially isolated from non-orchard pollen sources; 2) natural self-fertilization occurs at insignificant rates; 3) ramets produce equal numbers of flowers; 4) ramets flower synchronously; and 5) no cross-incompatibilities exist among clones. Those working with seed orchards know from experience that these conditions are not fully met in practice. To ensure sound orchard design and management, researchers must learn the degree to which these assumptions have been violated and then assess how the violations will ultimately affect genetic efficiency.

Genetic efficiency of seed orchards has received little attention due to the lack of suitable genetic markers. Simply inherited traits that could be used to determine the genetic makeup of orchard clones and their seedling progeny have not been readily available. However, electrophoretic analysis of isozymes has now made it possible to directly quantify genetic (i. e., allozyme) variation in conifers. Conifers are particularly suitable for allozyme analysis because of the two tissue types [embryo (2N) and megagametophyte (1N)] in their seed. If we know the genetic makeup of both these tissues, the pollen contribution to the embryo can be inferred (Müller, 1976). Thus, by sampling many progeny of the same clone, the genetic makeup of the pollen pool (i. e., the pollen effective in producing viable seeds) of that clone can be determined.

We have been using allozymes to investigate factors associated with genetic efficiency in two loblolly pine (Pinus taeda L.) seed orchards. In this article we will focus on two of the questions addressed in that study:

1) How much allozyme variability exists among clones in loblolly pine seed orchards?

2) What proportion of the progeny in those orchards results from self-fertilization?

Allozyme variation among orchard clones must be large if allozymes are to be valuable genetic markers in breeding programs (RUDIN and LINDGREN, 1977). This is quite likely in loblolly pine because parent trees used for most first-generation production orchards have come from many widely separated stands (ZOBEL and McELWEE, 1964). HUNTER (1977) found considerable allozyme variability among the 27 clones in a loblolly pine seed orchard in North Carolina. In addition, the 12 allozyme loci studied were found to be useful for identifying clones because all the orchard clones were uniquely recognizable based on their 12-locus genotypes.

Particularly valuable for studying genetic efficiency in seed orchards are alleles unique to single clones. Such alleles can be used to indicate whether individual clones are contributing to the seed crop or pollen pool in expected proportions. Using techniques similar to those described by MÜLLER (1976, 1977), alleles unique to individual clones could also be used to determine how pollen has been distributed within orchards or to estimate the proportion of a clone's progeny resulting from self-fertilization.

Selfing in wind-pollinated seed orchards is of particular concern because selfing in conifers often results in reduced seed yields and seedling vigor (FRANKLIN, 1970). The percentage of filled seed following selfing in loblolly pine is only 19% of that of outcrossed seed (FRANKLIN, 1969). In addition, selfed seedlings have twice the mortality of outcrossed seedlings and generally grow at much slower rates. Since improved nursery practices and the trend to wider spacing may permit inherently weak seedlings to become established in plantations, knowing the proportion of selfed seedlings is important (HADDERS and KOSKI, 1975). FRANKLIN (1968), using morphological markers to study selfing in an old field stand of loblolly pine, found that the frequency of progeny due to self-fertilization averaged 1.75% over two years, and varied from 0—13.5% among progenies of the 25 trees sampled. Although the proportion of selfed progeny might be low in natural loblolly pine stands, selfing in clonal orchards could be substantial because selfs can result not only from selfing of individual ramets but also from cross-pollination of ramets of the same clone.

Materials and Methods

Two five-acre loblolly pine orchards owned by Champion International Corporation, Newberry, South Carolina, were selected for study. One orchard contains clones selected for their high specific gravity (HSG) wood; the other, clones selected for their low specific gravity (LSG) wood. The two orchards, located on the same site, are separated from each other by a 100-m-wide strip containing a Virginia pine (*Pinus virginiana* MILL.) orchard. The three-orchard complex is surrounded by a 122-m-wide isolation strip comprising a cleared area, a slash pine (*Pinus elliottii* ENGELM.) plantation, and a mixed loblolly-hardwood stand from which all flowering-age loblolly pines are periodically removed. Orchard grafts averaged 15 years old when sampled and had been producing large quantities of pollen and seed for several years.

The seeds used in this study resulted from wind-pollinations occurring during spring 1974. At that time, the LSG orchard had 301 ramets of 23 clones, the HSG orchard, 300 ramets of 27 clones. Ramets had an average spacing of 8.2 m² but were not spaced uniformly because the orchards had been rogued three times since establishment. Roguing had also caused uneven representation of clones in both orchards, the number of ramets per clone ranging from 1—39. A fourth roguing occurred during summer 1974, leaving at least one ramet of each clone that existed during the 1974 pollination season. The positions of all ramets existing prior to this operation had been mapped.

Cone samples from at least one ramet of each of the 50 clones in the two orchards were collected during fall 1975. In total, 79 and 38 ramets were sampled from the LSG and HSG orchards, respectively. Sampled ramets were flagged

prior to cone maturity; as each was commercially harvested, 3—5 cones were placed into labeled bags. The collection crew was not told from which portion of the crown to collect cones. Seed lots for all ramets were kept separate throughout extraction and storage.

Megagametophytes from a minimum of 10 seeds from each clone were assayed electrophoretically. Based on the array of allozymes observed in their megagametophytes, the genotype of each clone was inferred at 15 loci. Using this method, error in correctly identifying the genotype of a clone will occur only if all megagametophytes sampled from a heterozygote carry the same allozyme allele; with N megagametophytes, this probability is $(\frac{1}{2})^{N-1}$ for a single locus. Thus, for 10 megagametophytes, the probability of incorrectly identifying a clone's genotype at any one locus is less than 0.002.

The 15 loci studied coded for allozyme variants in 10 enzyme systems: glutamate dehydrogenase (GDH); leucine aminopeptidase (LAP); phosphoglucose isomerase (PGI); aconitase (ACO); glutamate-oxaloacetate transaminase (GOT); 6-phosphogluconate dehydrogenase (6PGD); phosphoglucomutase (PGM); acid phosphatase (AP); malate dehydrogenase (MDH); and isocitrate dehydrogenase (IDH). Respective loci were labeled, GDH, LAP1, LAP2, PGI1, PGI2, ACO, GOT1, GOT2, 6PGD, PGM1, PGM2, AP2, MDH1, MDH2, and IDH. Details of the electrophoretic procedures for detecting these allozymes, their banding patterns, and analyses of their Mendelian genetics are reported elsewhere. (ADAMS and JOLY, 1980).

Clearest banding of allozymes in both megagametophyte and embryo tissues occurred when the radicle of a germinating seed had emerged 3—5 mm beyond the seed coat. In general, embryo band patterns did not resolve as well as those in megagametophytes. However, in eight of the 15 loci studied, distinct allozyme expression was found in embryos for many of the alleles present in megagametophytes. Five of the 50 clones possessed an allele at one of these eight loci which was unique to a single clone and could be clearly identified in embryos. Whenever an offspring of such a clone has been fertilized by a pollen gamete containing the unique allele of that clone, either selfing or cross-pollination with another ramet of the same clone must have occurred. This situation can be used to estimate the frequency of selfs in the progeny of these five clones.

Thirty to 80 wind-pollinated seeds from each of nine ramets of the five clones were assayed electrophoretically to determine the allelic composition of their pollen pools. Since all the unique alleles were carried by their clones in the heterozygous state and segregated according to the expected 1:1 ratio in megagametophytes, the probability of observing a unique allele in the pollen pool of any of these clones is $\frac{1}{2} \varrho$, where ϱ is the proportion of selfs. Therefore, the maximum likelihood estimator of ϱ was $\hat{\varrho} = 2x/N$ [Var $\hat{\varrho} = \hat{\varrho}(2-\hat{\varrho})N$], where X is the number of pollen-pool gametes with the unique allele observed in a progeny sample of N seed.

Results and Discussion

Allozyme Variability among Clones

Considerable genetic variation among clones was evident; 12 of the 15 loci were polymorphic, averaging 3.4 alleles per locus, and on the average, nearly 25% of the polymorphic loci were heterozygous in any one individual (*Table 1*). Because of this large amount of variation, orchard clones can be individually identified using allozymes as genetic markers. Twenty-two and 25 unique 15-locus genotypes were recognized for the 23 clones in the LSG orchard and the 27 clones in the HSG orchard, respectively. Similarly, 47 unique genotypes were recognized among the 50 clones of the two orchards considered together. The allozyme variation observed here and that seen by HUNTER (1977) are

2

Table 1. — Variation at allozyme loci polymorphic among the 50 clones in two loblolly pine seed orchards.

Orchard	No. of clones	No. of polymorphic loci	Alleles/locus		Heterozygous loci/clone (%)	
			Range	Mean	Range	Mean
LSG	23	12	2–6	3.0	8–42	25.0
HSG	27	11	1–5	2.8	0–50	24.4
Combined	50	12	2–7	3.4	0–50	24.7

consistent with the broad genetic base expected in loblolly pine seed orchards.

Allelic frequencies were roughly comparable in the two orchards, at least in that the alleles most frequent in one were generally the most frequent in the other (Table 2). This similarity might have been expected because wood specific gravity was the only trait by which orchard clones were differentially selected. But the orchards also manifested important allelic differences, most notably the presence of alleles in one orchard which were not present in the other. These alleles would be useful for indicating from which orchard a particular seed lot was derived. In addition, alleles unique to single clones (Table 2) are important for determining the relative contribution of individual clones to the seed crop.

Note that alleles unique to single clones are not necessarily rare. For example, LAP1—1 is unique to one clone in the LSG orchard but occurs at a frequency of 0.065

Table 2. — Allelic frequencies among orchard ramets for 12 polymorphic loci.[1]

Locus	Allele[3]	Orchard		Locus	Allele	Orchard	
		LSG	HSG			LSG	HSG
PGI1[2]	1	0.015	0.015	LAP2	1	0.417	0.503
	2	0.985	0.985		2	0.583	0.450
					3	--	0.047[5]
GOT1[2]	1	0.156	0.007	PGI2	1	0.047[5]	--
	1L	--[4]	0.025[5]		2	0.822	0.930
	2	0.184	0.362		3	0.108	0.057
	3	0.660	0.606		4	0.023	0.013
AP2[2]	1	0.015	0.052	GOT2	1	0.164	0.098
	2	0.889	0.938		2	0.007[5]	--
	3	0.096	0.010		3	0.829	0.902
MDH2[2]	1	0.905	0.993	6PGD	1	0.025	--
	2	0.095	0.007		2	0.397	0.407
LAP1	1	0.065[5]	--		3	0.053	0.015
	2	0.836	0.862		4	0.015	0.085
	3	0.048	0.045		5	0.503	0.481
	N	0.051	0.093		5L	0.007[5]	--
					6	--	0.012[5]
GDH	1	--	0.038	PGM1	1	0.944	0.978
	2	0.939	0.787		2	0.056	0.022
	3	0.013[5]	--				
	4	--	0.010[5]	PGM2	1	0.028[5]	--
	5	0.048	0.165		2	0.972	1.00

[1] Allelic frequencies have been derived from the genotypic frequencies determined by weighting each clonal genotype by the number of ramets with that genotype present during pollination in 1974.
[2] PGI1, GOT1, AP2, and MDH2 coded allozymes which expressed clearly in megagametophytes only. The remaining loci carried genes that expressed clearly in both embryo and megagametophyte tissues.
[3] Alleles are numbered in ascending order from the fastest to the slowest migrating allozymes. N is a null allele; L is an allele coding a very lightly staining band.
[4] Dashes (— —) mean no allele present.
[5] Alleles unique to individual clones.

among the orchard ramets (Table 2). This unique allele has a relatively high frequency because a disproportionately high number of ramets of this clone (13% of total ramets) have remained after roguing.

Eight of the 12 polymorphic loci (LAP1, LAP2, GDH, PGI2, GOT2, 6PGD, PGM1, and PGM2) have two or more allozymes which resolved clearly in both megagametophyte and embryo tissues. However, the 30 alleles found at these loci in megagametophytes must be reduced to 21 in embryos because it is impossible to distinguish certain allozymes in GDH and GOT2; consequently, the alleles coding these allozymes were grouped with neighboring alleles. Despite this reduction in allozyme resolution, there is still substantial variability available for pollen pool analyses. Many alleles or allelic combinations unique to individual clones resolve clearly in embryos and could be used to measure the relative contributions of different clones to the pollen pools of other clones or to the seed crop, to measure pollen distribution within one orchard or between orchards, and to estimate rates of selfing.

Considering the sizable allozyme variation observed in these orchards, we believe that the potential for using allozymes as clone markers in breeding programs is great. In addition to certifying special seed lots, the markers could verify the identities of particular clones or the parents of control-pollinated seed lots if doubt arose. Allozymes could also help determine whether the male parents of a pollen-mix cross were equally represented in the seed and measure the effectiveness of control-pollination procedures. For example, RUDIN and LINDGREN (1977) were able to confirm suspected contamination in artificial selfing of Scots pine (Pinus sylvestris L.) with the aid of a GOT locus; seven of 32 progeny of a selfed clone were classified as resulting from illegitimate pollination.

Proportion of Progeny due to Selfing (ϱ)

No more than a single unique allele was observed in each of the pollen pools of the five clones sampled (Table 3). Because selfed progeny seem to be rare, we have calculated 95 % confidence limits for the $\hat{\varrho}$ of each clone, assuming a Poisson distribution (PEARSON and HARTLEY, 1966). These confidence limits are relatively large, despite an average sample size of over 100 seeds per clone. Nevertheless, the observed numbers of unique alleles appear to be uniformly low among the clones sampled; a chi-square test (SNEDECOR and COCHRAN, 1967, Section 9.3) indicates that the data are homogeneous ($\chi^2_4 = 2.00$, $0.50 < P < 0.75$). Pooling the data of the five clones, a combined estimate of $\hat{\varrho}$ is only 1.2 %. Thus, the overall influence of selfing on the genetic makeup of the seed crops in these orchards is probably minor. The possibility that unique markers may also have been carried by contaminating pollen cannot be ignored because we have not yet determined the allozyme makeup of trees in the surrounding stands. However, if contamination had occurred, the already low $\hat{\varrho}$ would, in fact, overestimate the true proportion of selfs in the orchard progeny.

Our estimated frequency of selfs (1.2%) approximates the average estimate of 1.75% found for progeny of the natural loblolly pine stand sampled by FRANKLIN (1968). Thus, there is no evidence that selfing rates in these orchards increased due to cross-pollinations among ramets of the same clone; present orchard designs stressing large numbers of clones and wide separation between ramets of the same clone are probably sufficient in keeping this source of selfs at a negligible level. But because our sample of clones was rela-

Table 3. — Estimated proportion of selfed progeny ($\hat{q}$) of five loblolly pine clones with unique alleles.

Clone	No. of ramets		Unique allele	Observed pollen-pool gametes		$\hat{p}$	SE($\hat{p}$)	95% Poisson confidence limits	
	Sample	Orchard		With unique allele	Total			Lower	Upper
				LSG Orchard					
10	2	12	PGM2-1	1	136	0.015	0.015	0.000	0.082
17	2	4	GDH-3	1	78	0.026	0.025	0.001	0.143
				HSG Orchard					
33	1	2	GDH-4	1	80	0.025	0.025	0.001	0.139
36	3	14	LAP2-3	0	148	--	--	0.000	0.050
46	1	6	6PGD-6	0	71	--	--	0.000	0.104
				Combined					
				3	513	0.012	0.007	0.002	0.034

tively small, we cannot rule out the possibility of occasional orchard clones with much higher proportions of selfed progeny than we observed. FRANKLIN (1968) found a wide range in selfing rates among the 25 trees he sampled, but only three of the 25 produced more than 1.5% selfs (2.1, 3.6, and 9.1%) in their progeny when averaged over the two seed years. A few ramets with higher-than-normal selfing would have little impact on the overall genetic efficiency of orchards. In addition, because selfing generally causes a large proportion of aborted seed, clones with unusually high selfing rates are probably low seed producers and would likely be rogued out of seed orchards for this reason.

Acknowledgments

Literature Cited

ADAMS, W. T., and JOLY, R. J.: Genetics of allozyme variants in loblolly pine. J. Hered.: in press (1980). — FRANKLIN, E. C.: Artificial self-pollination and natural inbreeding in Pinus taeda L. Ph. D. thesis, North Carolina State Univ., Raleigh, N. C., U. S. A. 127 p. (1968). — FRANKLIN, E. C.: Inbreeding depression in metrical traits of loblolly pine (Pinus taeda L.) as a result of self-pollination. Tech. Rep. No. 40, North Carolina State Univ., Raleigh, N. C., U. S. A., 19 p. (1969). — FRANKLIN, E. C.: Survey of mutant forms and inbreeding depression in species of the family Pinaceae. USDA Forest Serv. Res. Pap. SE-61., 21 p. (1970). — HADDERS, G., and KOSKI, V.: Probability of inbreeding in seed orchards. p. 108 to 117. In: R. FAULKNER (ed.): Seed orchards. Brit For. Comm. Bull. 54. (1975). — HUNTER, S. C.: An electrophoretic analysis of isoenzyme variation in a Piedmont loblolly pine seed orchard. M. S. thesis, North Carolina State Univ., Raleigh, N. C., U. S. A. 48 p. (1977). — MÜLLER, G.: A simple method of estimating rates of self-fertilization by analysing isozymes in tree seeds. Silvae Genet. 25: 15—17 (1976). — MÜLLER, G.: Short note: Cross-fertilization in a conifer stand inferred from enzyme gene-markers in seeds. Silvae Genet. 26: 223—226 (1977). — PEARSON, E. S., and HARTLEY, H. O.: Biometrika tables for statisticians. Vol. I (Third ed.). University Press, Cambridge, Mass., U. S. A. 235 p. (1966). — RUDIN, D., and LINDGREN, D.: Isozyme studies in seed orchards. Studia Forestalia Suecica Nr. 139. Swedish Coll. Forestry, Stockholm. 23 p. (1977). — SNEDECOR, G. W., and COCHRAN, W. G.: Statistical methods (Sixth ed.) Iowa State Univ. Press, Ames, Iowa, U. S. A. 593 p. (1967). — WOESSNER, R. A., and FRANKLIN, E. C.: Continued reliance on wind-pollinated southern pine seed orchards, is it reasonable? Proc. 12th Southern Forest Tree Improve. Conf., p. 64—73 (1973). — ZOBEL, B., and McELWEE, R. L.: Seed orchards for the production of genetically improved seed. Silvae Genet. 13: 4—11 (1964).