ALLOZYMES OF LINKED LOCI SEGREGATE NORMALLY IN SEEDS OF AN AUSTRIAN X JAPANESE RED PINE HYBRID

M. Thompson Conkle and James J. Tobolski 1/

Abstract.--An F1 Pinus nigra and P. densiflora hybrid was polymorphic for seven enzyme loci. Data from haploid female gametophytes of the hybrid established that four of the seven loci were linked in one block. Three loci segregated independently: acid phosphatase 1, glutamic oxaloacetic transaminase 3, and glucose-6-phosphate dehydrogenase 1/2. The linked loci were alcohol dehydrogenase 1, alcohol dehydrogenase 2, glucose-6-phosphate dehydrogenase 2, and leucine aminopeptidase 2, arranged in that order. The recombination percentages between linked loci were 9, 12, and 14, respectively. The order and recombination percentages of the linked loci were similar to values reported for P. sylvestris, another pine in the subsection Sylvestres and other species in Pinus. The hybrid provides a means of enriching genetic variability by producing allelic combinations that do not exist in natural populations.

Additional keywords: Pinus nigra, Pinus densiflora.

Interracial and inter-specific hybrids are the principal means for enriching genetic variation in forest tree populations. Fertile hybrids with gametes containing numerous new allele combinations would be good materials for recurrent selection to develop trees with associations of traits that do not exist in natural populations.

The hybrid between Austrian pine (Pinus nigra Arn. var. austriaca (Hoess) Aschers. and Graebn.) and Japanese red pine (P. densiflora Sieb. and Zucc.) unites chromosomes from two species that are geographically separated by a distance of about 5,000 miles. Controlled crosses between these two species yielded the first pine hybrid ever made (Austin 1927).

Breeding barriers between these two species are difficult to assess. Sound seed set per cone from controlled pollinations of Pinus nigra seed parents crossed with P. densiflora pollen is only 25 percent of the seed set from P. nigra times P. nigra crosses (Wright and Gabriel 1958). But hybrids from wind pollination of P. nigra trees adjacent to a plantation of P. densiflora reaches proportions as high as 92 percent (Wright et al. 1969) Hybrid embryos are not favored in pollen-mix crosses. When P. nigra and P. densiflora pollen mixes in known proportions were applied to P. nigra parents, non-hybrid embryos were strongly favored over hybrid embryos (Tobolski and Conkle 1977).

Inter-species hybrids between Austrian and Japanese red pines have value in the Lake States Region. Hybrids, planted in the Lake States, differ in

1/ Geneticist, U. S. Department of Agriculture, Forest Service, Pacific Southwest Forest and Range Experiment Station, Box 245, Berkeley, California, 94701, and Associate Professor, Department of Biological Science, Indiana University - Purdue University, Fort Wayne, Indiana, 56805.

growth traits, tolerance to road salt, and susceptibility to pests (Wright et al. 1969). The F1 hybrids grow more rapidly than trees of either parental species.

The hybrid could be improved by combining rapid growth with tolerance and resistance factors. Selection for new combinations of desirable traits, by recurrent mass selection starting from a population of F1 hybrids or by backcrossing to a parental species, requires fertile hybrids with normal meiosis. Ongoing tests indicate that these F_1 hybrids are highly fertile. The current study examines meiosis by testing the recombination between enzyme loci in an F1 hybrid, P. nigra X P. densiflora.

LINKAGE TEST CONDITIONS

J. W. Wright found spontaneously produced hybrids between Austrian pines and Japanese red pines in a southern Michigan plantation (Wright et al. 1969). A previous analysis of enzymes determined genotypes of trees of the two parent species and hybrids (Tobolski and Conkle 1977) . During those investigations we discovered that one of the F1 hybrids from the southern Michigan plantation was polymorphic for seven allozyme loci.

Four of the seven polymorphic genes in the hybrid were genes that were linked in other species under investigation in Conkle's laboratory. One hundred and eight seed from this hybrid were analyzed to test patterns of segregation and linkage using starch gel electrophoresis (Conkle 1972) of extracts from the haploid tissue of female gametophytes. This tissue, which surrounds embryos within individual seeds, develops from a haploid nucleus that is a product of meiosis. The haploid nucleus repeatedly divides and at maturity the products of these divisions fill the ovule and produce eggs within archegonia.

The different allele products from gametophytes of a tree heterozygous for enzyme loci segregate with simple Mendelian ratios (Bergman 1974) . Linkage was tested using the frequencies of alleles in pairwise combinations. For heterozygous loci, we noted the faster allele by (1) and the slower allele by (2). For any two heterozygous genes the number of allele combinations is four: gene one - fast, gene two - fast (1,1); gene one - fast, gene two - slow (1,2) ; gene one - slow, gene two - fast (2,1) ; gene one - slow, gene two - slow (2,2). With free recombination between two loci, the allele pairs occur with equal frequency, f(1,1) = f(1,2) = f (2, 1) = f(2,2) . When genes are linked, two classes have frequencies lower than expected and two have frequencies above expectations (the combined frequency of 1,1 + 2,2 does not equal that of 1,2 + 2,1). When these two combined-classes are unequal, the smaller of the two is assumed to be the recombinant class. The number of gametes in the recombinant class is divided by the total number of gametes to arrive at the recombination fraction. Recombination values range from 0.0 to .50: 0.0 when two genes are totally linked and no recombinants are found, and .50 when there is free recombination between the two genes.

LINKAGE IN THE HYBRID

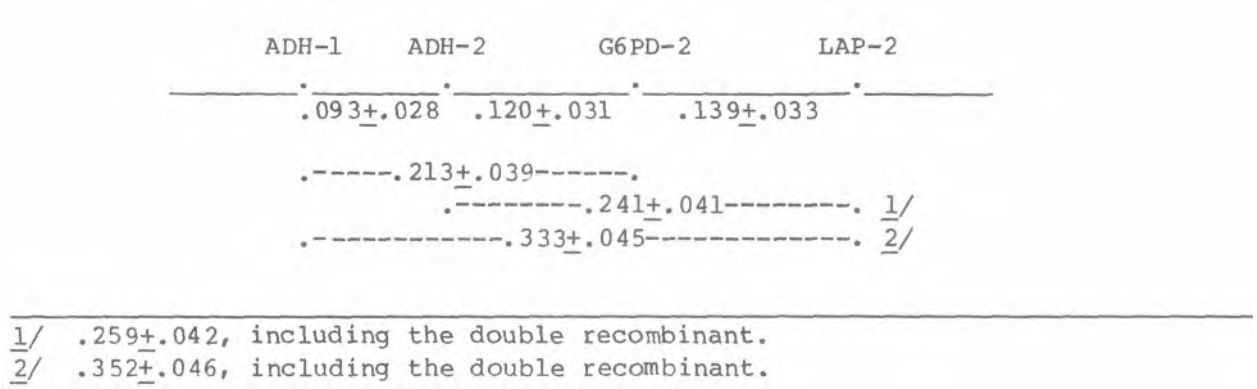
The seven polymorphic loci in the hybrid were acid phosphatase 1 (ACPH-1), alcohol dehydrogenase 1 and 2 (ADH-1, ADH-2), glucose-6-phosphate dehydrogenase 1/2 and 2 (G6PD-1/2, G6PD-2) , glutamic oxaloacetic transaminase

3 (GOT-3) , and leucine aminopeptidase 2 (LAP-2) . Our gene notation lists the most anodal band as the number 1 locus and successively numbers additional loci toward the cathodal edge of the gel. Most pines have only two loci for G6PD, but the hybrid in this test developed three sets of bands corresponding to three loci. We decided to show the similarity with other species notation by naming the more common zones, G6PD-1 and G6PD-2, and the faintly staining middle zone G6PD-1/2.

GOT-3 was the only gene in which segregation significantly deviated from a 1:1 ratio (chi-square, 1 degree of freedom = 7.26) . The observed ratio of fast to slow alleles was 40:68. This deviation did not preclude linkage tests for this locus, since recombination fractions are not disturbed when only one locus of a pair deviates from 1:1 segregation.

The seven loci provided 21 tests of linkage (table 1). Six of the 21 tests had values that significantly deviated from 1:1 segregation. The recombination values for pairs with significant deviations from free recombination are underlined in table 1. They involve four loci: ADH-1, ADH-2, G6PD-2, and LAP-2. GOT-3, ACPH-1, and G6PD-1/2 segregated independently.

The four genes with recombination values deviating significantly from expectations based on independent assortment form a single linkage block (fig. 1). The order of genes in the block was deduced from the recombination values in table 1. ADH-1 was .093 recombination units from ADH-2. ADH-2 was .120 from G6PD-2. The order of these three genes was established when the distance between ADH-1 and G6PD-2 was found to be .213. The position of LAP-2 in the block was determined in a similar manner.



1/ .259±.042, including the double recombinant.
2/ .352±.046, including the double recombinant.

Figure 1.--Linkage map for four loci in an F_1 hybrid of Pinus nigra X P. densiflora.

The two single values for the middle and right segments (ADH-2 to G6PD-2 and G6PD-2 to LAP-2) sum to a value greater than the recombination value for the region ADH-2 to LAP-2. This difference is due to a double recombinant in the ADH-2 to LAP-2 region. We expect double and triple recombinants in proportion to the products of the smaller recombination fractions only when recombination in one chromosome segment is independent of events in other segments. The expectation for double recombinants in the left and middle segments is 1.2 (.093 X .120 X 108 seeds) . For the left and right segments the expectation is 1.4, and for the middle and right segments 1.8. In all, 4.4

Table 1.--Polymorphic loci grouped in pairs, the frequencies of gametophytes in different allele classes (1 = fast allele, 2 = slow) , and the recombination proportions for 21 pairs of loci from a Pinus nigra X P. densiflora hybrid. Recombination fractions deviating significantly from .50 are underlined.

Gene 1 - Gene 2	Gametophytes in each allele pair class				Recombination fraction
	allele classes (gene 1, gene 2)				
	1,1	1,2	2,1	2,2	
	----------Number----------				
GOT-3 - ACPH-1	18	22	34	34	.481
GOT-3 - LAP-2	23	17	36	32	.491
GOT-3 - ADH-2	15	25	30	38	.491
GOT-3 - G6PD-1/2	23	17	34	34	.472
GOT-3 - G6PD-2	15	25	31	37	.481
GOT-3 - ADH-1	19	21	30	38	.472
ACPH-1 - LAP-2	28	24	31	25	.491
ACPH-1 - ADH-2	22	30	23	33	.491
ACPH-1 - G6PD-1/2	24	28	33	23	.435
ACPH-1 - G6PD-2	22	30	24	32	.500
ACPH-1 - ADH-1	23	29	26	30	.491
LAP-2 - ADH-2	11	48	34	15	.241
LAP-2 - G6PD-1/2	27	32	30	19	.426
LAP-2 - G6PD-2	6	53	40	9	.139
LAP-2 - ADH-1	18	41	31	18	.333
ADH-2 - G6PD-1/2	24	21	33	30	.500
ADH-2 - G6PD-2	39	6	7	56	.120
ADH-2 - ADH-1	42	3	7	56	.093
G6PD-1/2 - G6PD-2	27	30	19	32	.454
G6PD-1/2 - ADH-1	29	28	20	31	.444
G6PD-2 - ADH-1	36	10	13	49	.213

double recombinants were expected and only one was recovered. Fewer double recombinants are observed than are theoretically expected because of chromosome interference which restricts multiple crossovers.

The alleles at each locus along the linkage block can be assigned to a parental chromosome strand and the chromosome can be assigned to a parental species. Recombination between ADH-2 and ADH-1 shows 42 gametophytes in the 1,1 class and 56 in the 2, 2 class (table 1) . The sum of the gametophytes in these two classes (98 gametophytes) indicates that these are the parental classes and 1,2 and 2,1 with a sum of 10 gametophytes are the recombinants. Therefore, the allele arrangements on the parental chromosomes are: ADH-1(1)--ADH-2(1) for one strand and ADH-1(2)--ADH-2(2) for the other. Further note that 1,1 and 2,2 are the parental classes for all combinations of ADH-1, ADH-2, and G6PD-2. The 1,2 and 2,1 classes are parental combinations for linkages involving LAP-2. The arrangements of alleles for ADH-1, ADH-2, G6PD-2, and LAP-2 on the two parental strands are therefore : 1--1--1--2 and 2--2--2--1 (fig. 2).

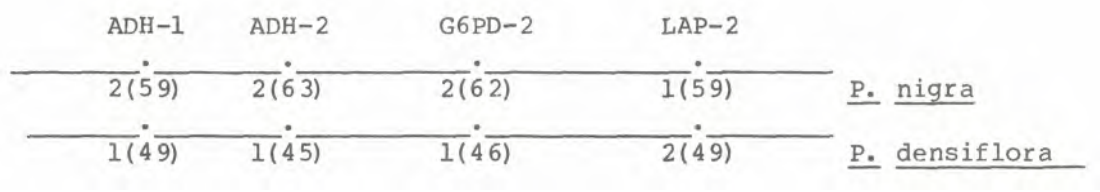


Figure 2.--Allele arrangement (1 = fast allele, 2 = slow) and segregants recovered (in brackets) for linked loci in the F1 hybrid.

P. densiflora and P. nigra differ in alleles at the ADH-2 locus (Tobolski and Conkle 1977), and this difference is useful in assigning the two chromosome strands of the hybrid to the respective parental species. All the P. densiflora tested from the southern Michigan plantation were monomorphic for allele 1 of the ADH-2 locus. The three alleles of P. nigra trees were all slower migrating than allele 1. Thus, the parental strand with ADH-2(1) in the hybrid came from P. densiflora (fig. 2).

Pinus densiflora genes in the linkage block are underrepresented in the progeny of the hybrid (fig. 2). Segregation values for alleles from the four loci were pooled by species to determine if the alleles from each species were equally represented in the female gametes of the F1 hybrid. The range of proportions for P. densiflora alleles is from .417 for ADH-2 to .454 for ADH-1 and LAP-2. Though the single locus segregation values did not significantly vary from 1:1 expected ratios, the combined value for the four linked loci has a mean value of .437 ± .019 for P. densiflora alleles. This deviation from equal representation, though significant, does not preclude the recovery of numerous new allele combinations.

SIGNIFICANCE OF THE RESULTS

There are similarities between the block of genes in the F1 hybrid and genes in Pinus sylvestris (Rudin and Ekberg 1978), which is in the same subsection of the genus (Sylvestres) as P. nigra and P. densiflora. Three loci in the hybrid, ADH-1, ADH-2, and LAP-2, are also linked in P. sylvestris (G6PD-2 has not been reported for P. sylvestris). In P. sylvestris, ADH-1 and ADH-2 are tightly linked with no recombination in a sample of 120 gametophytes and the ADH loci have an average recombination fraction of .309 ± .025 with LAP-2. Thus, of the loci that can be compared between the three species: all are in the same linkage block; both ADH loci are closely linked, and are located at a similar distance from LAP-2 in each species; and GOT-3 and ACPH-1 are not linked with ADH-2 in the hybrid or in P. sylvestris.

The comparison can be extended to include species from throughout Pinus, all in other subsections of the genus: P. attenuata, P. contorta, P. jeffreyi, P. lambertiana, and P. taeda (Conkle 1981). Recombination fractions between ADH-2 and LAP-2 range from .258 to .362 for these five species. The value for the hybrid is .259, within the range of values for the other species. ADH-1 is closely linked with ADH-2 when recombination has been tested in three of the five species. G6PD-2 is located in the middle of the segment between ADH-2 and LAP-2 in at least three of these species. Thus, the order and the recombination fractions between loci are similar for the hybrid and other species in the genus.

This study precisely determines that alleles from two parental species recombine in the gametes of an F_1 hybrid to produce combinations of alleles that are not possible in pure species populations. The regularity of recombination of alleles at linked loci of this hybrid supports the value of breeding efforts that are underway using a population of F1 hybrids from which to start recurrent selection for rapid growth, salt tolerance, and pest resistance.

LITERATURE CITED

Austin, Lloyd. 1927. A new enterprise in forest tree breeding. J. Forestry 25:928-953.

Bergmann, F. 1974. The genetics of some isozyme systems in spruce endosperm (*Picea abies*). Genetics 6:353-360.

Conkle, M. Thompson. 1972. Analyzing genetic diversity in conifers... isozyme resolution by starch gel electrophoresis. USDA Forest Service Research Note PSW-264. 5 p. Pacific Southwest Forest and Range Exp. Stn., Forest Serv., U.S. Dep. Agric., Berkeley, Calif.

Conkle, M. Thompson. 1981. Isozyme variation and linkage in six conifer species. In M. T. Conkle (technical coordinator) Proceedings of the symposium on isozymes of North American forest trees and forest insects, July 27, 1979, Berkeley, California. Gen. Tech. Rep. PSW-48, p. 11-17. Pacific Southwest Forest and Range Exp. Stn., Forest Serv., U.S. Dep. Agric., Berkeley, Calif.

Rudin, D. and I. Ekberg. 1978. Linkage studies in *Pinus sylvestris* L. — using macro gametophyte allozymes. Silvae Genetica 27:1-12.

Tobolski, James J. and M. Thompson Conkle. 1977. Enzyme identification of Austrian X Japanese red pine hybrids in seed from mixed pollen controlled crosses. In W. F. Beineke (ed.), Proc. of the Tenth Central States Forest Tree Improvement Conference, Sept. 22-23, 1976, p. 35-41. Purdue Univ., West Lafayette, Indiana.

Wright, Jonathan W. and W. J. Gabriel. 1959. Species hybridization in the hard pines, series *Sylvestres*. Silvae Genetica 7:109-115.

Wright, J. W., W. A. Lemmien, and D. S. Canavera. 1969. Abundant natural hybridization between Austrian and Japanese red pines in southern Michigan. For. Sci. 15:269-274.