Isozyme Variation and Linkage in Six Conifer Species[1]

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Abstract: Isozymes of female gametophyte tissue were analyzed for allelic variation in knobcone, lodgepole, loblolly, Jeffrey, and sugar pines and in Douglas-fir. Linkage was studied in the five pines. The average number of alleles and average herozygosity per enzyme locus were estimated. Knobcone pine ranked lowest among the six species in number of alleles and average heterozygosity; loblolly pine and Douglas-fir ranked highest. Numerous linkages were identified, and one chromosome segment contained between one-fourth and one-third of all loci examined. Linked loci had a consistent gene order in the different pine species. Linkage relationships support the concept that similar isozymes represent the same loci in each pine species. The consistency with which loci map in the same order and with similar map distances in species from different subsections of the genus, further suggests that evolution of the pines is not associated with major chromosome rearrangements.

The analysis of genetic variation in forest trees entered a new phase with the application of electrophoresis to detect allelic differences at isozyme loci. Gametophytes from seed of single trees provide excellent material for assessing isozyme variation. Enzymes are analyzed at the just-germinated stage of development, and diploid genotypes of parent trees are inferred from the segregation of allele phenotypes of the haploid female gametophytes. Seed from some of the heterozygous parent trees are analyzed in large numbers to determine if enzyme variants segregate according to simple Mendelian ratios. A one-to-one segregation ratio of phenotypic variants is evidence for allelism. Homozygous loci within a single tree are identified without error and the samples per tree are sufficient to minimize the misclassification of heterozygous loci.

This paper reports a two-stage analysis. In the first stage, the amount of genetic variation in several species of two conifer genera was estimated. Gametophytes from seed of individual trees were analyzed for segregation and data were obtained on the number of alleles and the average heterozygosity per locus. The six species compared were Douglas-fir and five North American pines. In the second stage, linkage from segregation in female gametophytes of heterozygous trees was estimated. Linkage data are provided for the five pine species.

ISOZYME VARIATION

The six conifers in this study were knobcone pine (*Pinus attenuata* Lemm.), lodgepole pine (*P. contorta* Dougl. ex Loud.), loblolly pine (*P. taeda* L.), Jeffrey pine (*P. jeffreyi* Grev. & Balf.), sugar pine (*P. lambertiana* Dougl.), and Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco). The geographic origins and numbers per species are listed in *table 1*. Sugar pine is classed within the subsection *Strobi* in the subgenus *Strobus* (the white pines). Within the subgenus *Pinus* (the hard pines), loblolly pine is within the subsection *Australes*, Jeffrey pine is in the subsection *Ponderosae*, lodgepole pine is within the subsection *Contortae*, and knobcone pine is in the subsection *Oocarpae*.

The minimum sample size was six gametophytes per tree and heterozygous loci were only misclassified when all six gametophytes carried one allele or its alternate, a probability of 2X $(1/2)^6$, or 0.03. Standard laboratory techniques for starch gel electrophoresis provided information for 21 enzyme systems *(table 2)* and as many as 43 loci per gametophyte.

While analyzing the loci of these species, some general observations concerning gene phenotypes became obvious:

- The number of zones that developed bands within a particular enzyme system was consistent for different species. That is, if an enzyme system resolved only one zone of activity for a particular species, it resolved a single zone of activity for the the other species.
- The relative mobilities of bands within enzyme systems were similar for different species. For example, if an enzyme system of one species had two zones of activity, two zones were also found in other species and the bands had identical or nearly identical mobilities.
- The characteristic isozyme banding phenotypes for different loci were consistent among different species. For example, band phenotypes for a particular locus were very dark staining and wide, and a second locus had band phenotypes that were light staining and narrow. These two loci had similar appearances in different species and genera. The number, mobility, and appearances of primary bands, and the characteristics of nearby secondary bands—whether leading, trailing, or in multimeric combinations—were constant for the conifer species I analyzed.

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Table 1—*Species, geographic origin, and number of trees in the enzyme analyses*

Species	Stands	Geographic origin	Trees
Knobcone pine (*Pinus attenuata* Lemm.)	10	Range wide	49
Lodgepole pine (*P. contorta* Dougl. ex Loud.)	1	Sierra Nevada, California	40
Loblolly pine (*P. taeda* L.)			
Natural stand	1	Schenck Forest, Raleigh, North Carolina	146
Superior trees	(Sample)	Throughout Southeastern U.S. sample of trees from the North Carolina State Tree Improvement Program	90
Jeffrey pine (*P. jeffreyi* Grev. & Balf.)	4	Central Sierra Nevada, Sierra Nevada, California	75
Sugar pine (*P. lambertiana* Dougl.)	(Sample)	Rangewide in California	58
Douglas-fir (*Pseudotsuga menziesii* [Mirb]Franco)	1	Cascades, west-central Oregon	152

Table 2—*Enzyme systems analyzed from conifer gametophytes*

Isozyme	Abbrev.	Commission Enzyme Code	Reference
Acid phosphatase	ACPH	3.1.3.2	Scandalios 1969
Aconitase	ACO	4.2.1.3	Yeh and O'Malley[1]
Alcohol dehydrogenase	ADH	1.1.1.1	Scandalios 1969
Aldolase	ALD	4.1.2.13	Yeh and O'Malley[1]
Catalase	CAT	1.11.1.6	Scandalios 1969
Diaphorase	DIA	1.6.4.3	Yeh and O'Malley[1]
Esterase (colorimetric)	EST	3.1.1.2	Scandalios 1969
Esterase (florescent)	FLEST	3.1.1.2	Mitton and others 1979
Glutamate dehydrogenase	GDH	1.4.1.2	Shaw and Koehn 1968
Glutamate oxalacetic transaminase	GOT	2.6.1.1	Brewbaker and others 1968
Glucose-6-phosphate dehydrogenase	G6PD	1.1.149	Shaw and Prasad 1970
Isocitrate dehydrogenase	IDH	1.1.1.42	Nichols and Ruddle 1973
Malic dehydrogenase	MDH	1.1.1.37	Nichols and Ruddle 1973
Peptidases			
Leucine-amino peptidase	LAP	3.4.11.1	Scandalios 1969
Alanine-amino peptidase	ALAP	3.4.11.1	Ott and Scandalios 1978
Peptidase	PEP	3.4.13.11	Nichols and Ruddle 1973
Peroxidase	PER	1.11.1.7	Shaw and Prasad 1970
6-Phospho-gluconate dehydrogenase	6PGD	1.1.144	Brewer 1970
Phosphoglucose isomerase	PGI	5.3.1.9	Brewer 1970
Phosphoglucomutase	PGM	2.7.5.1	Brewer 1970
Superoxide dismutase	SOD	1.15.1.1	

[1] Yeh, F. C., and D. M. O'Malley.1980.Enzyme variation in natural populations of Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco from British Columbia. I. Genetic variation patterns in coastal populations. (In press).

It seemed reasonable to assume, therefore, that the same genes were being expressed in different species.

Two measures of isozyme variation were evaluated. The number of alleles per locus (A), was a count of the different allele phenotypes; heterozygosity per locus (H), was computed from allele frequencies. The H values, derived from Hardy-Weinberg expectations, were computed by subtracting the sum of the squared allele frequencies for each locus from 1.00. This measure was chosen instead of the direct count of heterozygotes because it is the value most widely reported in the literature. Admittedly, it is a first approximation that is accurate only when the trees within a species are randomly interbreeding, and when geographic differentiation within a species is lacking. Although supporting data will not be provided here, the observed proportions of heterozygotes per gene per species closely approximated the computed values.

The estimates of isozyme variation for the six species studied resulted in few general statements regarding variability (*table 3*). The values represented contrasts; for example, the number of alleles per locus (A) for ACPH-2 were low (2 or 3) in knobcone pine, sugar pine, and Douglas-fir, intermediate (5) in lodgepole and Jeffrey pines, and high (8) in loblolly pine. Heterozygosity (H) of ACPH-2 was low for knobcone pine and sugar pine, 0.06 and 0.02, but high for Douglas-fir (0.49). ADH-2 had values of A ranging from 2 to 6 with H values from 0.02 to 0.68. A and H are sometimes widely divergent: Jeffrey pine had 3 alleles for ADH-2 and an H value of 0.60; the superior trees in the loblolly sample had 5 alleles and a low H estimate (0.06). GDH was homozygous in knobcone, Jeffrey, and sugar pines, but was highly variable within lodgepole and loblolly pines. GOT-1 was nearly homozygous in Douglas-fir but variable in the five pine species. Characteristically, EST-1 had numerous alleles per locus and high values for H, and LAP-1 and LAP-2 were variable in all six species.

Values for A and H averaged for all loci show differences for the six species in the study. In general, the A and H values have similar trends. The average number of alleles per locus grouped into three general classes, with values near 2, 3, and 4 (*table 3*). Knobcone pine had the lowest value for A with an average of 2. Lodgepole, Jeffrey, and sugar pines had values approaching 3 alleles; loblolly pine and Douglas-fir had high average values approaching 4 alleles per locus. Average heterozygosity values ranged from a low of 0.13 for knobcone pine to a high of 0.36 for the natural stand of loblolly pine; Jeffrey and sugar pines approached intermediate values; and, loblolly pine and Douglas-fir averaged high H values.

I believe the two values for loblolly pine may be similar. Slightly different H values for these resulted from inclusion of the additional loci in the superior tree sample. The additional loci did not modify the A mean values, but usually one allele was in high frequency. The result was the addition of several loci with low values for H. The data from the two sources of loblolly pine should not be used to argue that a higher average heterozygosity exists in natural stands than in a broad geographic sample of superior trees. When comparisons were restricted to the nine loci analyzed within both the natural stand and the select trees, the superior trees averaged more alleles per locus than the natural stand (4.78 compared with 3.89) and H values were similar for both samples.

Estimates of A and H for numerous species were compared by Brown and Moran (1981) and Hamrick and others (1981), but two points need mention. The lowest estimate of A and H for conifers has been found in red pine (Fowler and Morris 1977). In a sample of nine isozyme loci, no variation could be attributed to allelic differences; therefore, the estimates of genetic variation are A = 1.00 and H = 0.00. This finding is remarkable when compared with other conifers. It is, however, consistent with findings that red pine is highly self-fertile, has low variability in growth traits, and does not exchange genes with any other species. Some researchers would argue that significant genetic variance in phenotypic traits has yet to be demonstrated in red pine.

The estimates of nearly four alleles per locus and heterozygosities near one-third for loblolly pine and Douglas-fir are among the highest values obtained for any species of higher organism thus far reported (Hamrick and others 1981). As data become available for other conifers, a continuum of values for genetic variation in isozymes is expected. The six species reported here probably will rank from below average to among most variable.

LINKAGE

Isozyme analyses of female gametophytes tested deviations from independent assortment between gene loci. Data on genetic linkage were obtained on the five pines, but not on Douglas-fir. Highly heterozygous trees, identified in the species surveys for allele frequencies, were efficient items for estimating linkages. The number of pair-wise gene combinations increased exponentially with increasing numbers of heterozygous loci.

Pair-wise linkage of loci was established when the sum of the recombinant classes had depressed frequencies and the parental classes had inflated frequencies in comparison with random expectations. Species recombination values were computed from pooled single-tree estimates and all recombination values were adjusted to provide estimates of map distances in centiMorgans (cM) (Kasambi 1944). Complete data on sample size and segregation ratios will be published elsewhere; however, each distance was estimated with seed from a minimum of two different trees within each species. The minimum number of gametophytes used to estimate distances was 144 and the maximum exceeded 1000. Gene order was established by comparing distances in multiple heterozygotes. Because data were limited by the availability of appropriate multiple heterozygotes, not all gene combinations could be examined in each species.

Linked genes were distributed among several linkage blocks in the five pine species. Linkage maps were

Table 3—*Number of alleles per locus (A) and heterozygosity per locus (H) for five pine species and Douglas-fir*

Locus	Knobcone pine		Lodgepole pine		Loblolly pine (natural stand)		Loblolly pine (superior trees)		Jeffrey pine		Sugar pine		Douglas-fir	
	A	H	A	H	A	H	A	H	A	H	A	H	A	H
ACPH 1	2	0.06	2	0.04			4	0.33	4	0.27				
ACPH 2	2	0.06	5	0.41	7	0.32	8	0.20	5	0.40	2	0.02	3	0.49
ACPH 3			1	0.00										
ACPH 4	2	0.06	2	0.04			2	0.46						
ACO			2	0.21					3	0.40				
ADH 2	3	0.45	5	0.53	2	0.02	5	0.06	3	0.60	6	0.68		
ALD 1			2	0.04					2	0.33				
ALD 2			2	0.04					1	0.00				
CAT 1			2	0.04					3	0.35				
DIA 1			3	0.08					5	0.55				
DIA 2			3	0.08					2	0.25				
DIA 3									2	0.01				
DIA 4			3	0.26					2	0.32				
EST 1	6	0.46	7	0.52	6	0.72	9	0.51	6	0.63	3	0.34	8	0.69
EST 2			3	0.65					6	0.61				
EST 4			3	0.37	4	0.47	4	0.53	3	0.25				
FLEST			2	0.08					3	0.43				
GDH	1	0.00	3	0.30			3	0.37	1	0.00	1	0.00		
GOT 1	2	0.23	4	0.32	3	0.37	3	0.41	4	0.41	4	0.37	2	0.01
GOT 2	2	0.04			3	0.54			3	0.08	2	0.04	4	0.21
GOT 3	2	0.02	2	0.02	2	0.33	3	0.27	2	0.13	5	0.30	3	0.16
G6PD 1	2	0.04	2	0.08			3	0.22	3	0.16	3	0.08	4	0.54
G6DP 2	1	0.00	2	0.08			3	0.29	2	0.64	2	0.02	2	0.40
IDH	2	0.06	2	0.02			2	0.04	2	0.01	1	0.00	5	0.39
MDH 1	1	0.00	2	0.08			4	0.10	2	0.01	2	0.48	4	0.05
MDH 3	2	0.08	5	0.76			4	0.52	3	0.24	2	0.45	2	0.32
MDH 4	1	0.00	3	0.18			3	0.04	2	0.51	6	0.70	5	0.44
LAP 1	3	0.46	4	0.32	4	0.24	4	0.24	6	0.55	3	0.20	8	0.72
LAP 2	2	0.20	5	0.20	4	0.51	4	0.52	4	0.12	6	0.68	4	0.18
ALAP 1			2	0.25			3	0.08	3	0.31				
ALAP 2			2	0.10			3	0.13	4	0.32				
PEP 1			1	0.00					1	0.00				
PEP 2									1	0.00				
PER 1			2	0.21			4	0.51	3	0.45				
PER 2			7	0.65			7	0.40	5	0.64				
6PGD 1	2	0.43	2	0.04			8	0.65	3	0.28	2	0.48	3	0.14
6PGD 2	2	0.02	2	0.08			2	0.08	3	0.07			3	0.44
PGI 1			2	0.04					2	0.01				
PGI 2			2	0.04					3	0.24				
PGM	2	0.08	1	0.00	3	0.10	3	0.10	2	0.20	2	0.38	3	0.31
SOD 1	1	0.00	1	0.00			1	0.00	2	0.03	1	0.00	4	0.14
SOD 2	1	0.00	2	0.04					1	0.00	1	0.00		
SOD 3									1	0.00				
SOD 4									2	0.03				
SOD 5									3	0.39				
TOTAL	22		39		10		25		43		19		17	
A														
Mean	2.00		2.74		3.80		3.96		2.86		2.84		3.94	
S.E.	±0.22		±0.23		±0.49		±0.39		±0.21		±0.39		±0.42	
H														
Mean	0.125		0.185		0.362		0.282		0.261		0.275		0.331	
S.E.	±0.035		±0.032		±0.063		±0.038		±0.032		±0.057		±0.049	

[1]Construction of the table implies that a particular gene is common to all six species. No importance should be attached to the blanks within the table; species with the greater numbers of loci reflect laboratory advances over earlier analyses.

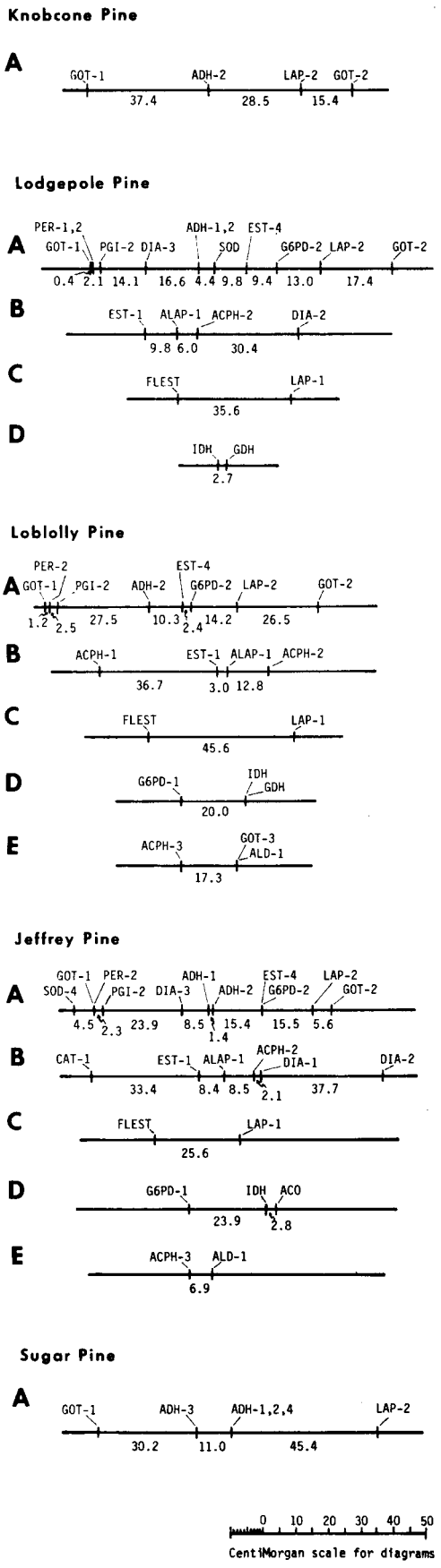

Figure 1—Linked genes detected in five species of pine with map distances in centiMorgan units.

constructed with a common base using cM distances and linkage groups were indicated by capital letters (*fig. 1*).

A high proportion of the genes sampled were linked. Some were closely associated, others were separated by distances that required large sample sizes for detection.

The presence and arrangement of particular genes within chromosome segments for different species were not random; linkage blocks had common relationships for all species. Segment A, for example, was the longest linkage group found. For lodgepole, loblolly, and Jeffrey pines, the number of genes in linkage block A was large; for example, 12 for lodgepole pine. And the gene order within the block was almost identical for all five species. Three closely associated genes, GOT-1, PER-2, and PGI-2 anchored the left-hand portion of the segment, ADH-2 was 30 to 35 map units to the right, and EST-4 and G6PD-2 were to the right of ADH-2. Because LAP-2 was a substantial distance from ADH-2, the estimates were subject to large sampling errors. Nevertheless, ADH-2 and LAP-2 linkage estimates were statistically significant and map distances ranged from 25.4 in Jeffrey pine to 45.4 in sugar pine. In most species GOT-2 was difficult to resolve but some families provided estimates that located it to the right of LAP-2. Weak evidence suggests that linkage group A was linked with group B. If so, this means that a larger proportion of the genes in the total sample of loci were on a single chromosome. The evidence for linkage between blocks A and B needs further analysis; it is premature to consider them linked at this time.

Linkage group B was characterized by a block of three genes: EST-l, ALAP-1, and ACPH-2. They were found in the same order in lodgepole, loblolly, and Jeffrey pines with ALAP-1 in the center.

Linkage block C represented a long segment including FLEST and LAP-1. The main features of the D linkage block, investigated in lodgepole, loblolly, and Jeffrey pines, was a close linkage between IDH and GDH, with G6PD-1 located about 20 map units from IDH and GDH. In loblolly pine, GOT-3 was linked with ALD-l with no recombination, and both genes were linked to ACPH-3 (linkage block E). One final linkage, block F, was observed between MDH-2 and TO-5 in families of Jeffrey pine.

Two reports of linkage groups in pines compare favorably with these findings. Rudin and Ekberg (1978) report a linkage block in Scots pine (*P. sylvestris* L.) consisting of GOT-1, ADH-1 and 2, LAP-2, and GOT-2 (*fig. 2*). Map distances in Scots pine are comparable to those reported for the species in this paper, and the arrangement is identical. Similar linkage in Scots pine is important because it samples the subsection *Sylvestres* which includes most pines of the Eastern Hemisphere. Guries and others (1978) found recombination frequencies of 0.03 and 0.04 between GOT-1 and PGI-2 (their GPI-2) for two trees of pitch pine (*P. rigida* Mill.) and these genes are tightly linked in lodgepole, loblolly, and Jeffrey pines.

Isozyme phenotypes led me to assume that many genes were common to different species and the linkage data supports this assumption. Genes, with common pheno-

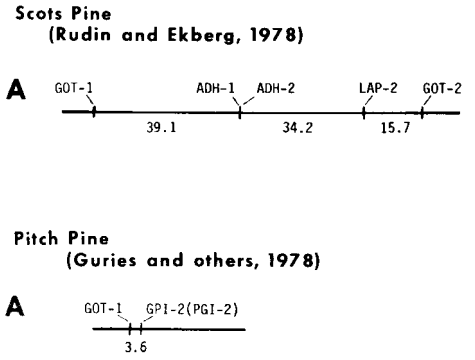

Figure 2—Linkage in two pine species, previously reported, compare well with the findings of this study.

types, map in the same order and with about the same distances in the different species.

Gene arrangement is highly conservative in the pines studied. These data indicate that evolution within the genus has not required large structural rearrangements within or between the gene blocks thus far examined. The linkage results invite comparison with other work on the arrangement of the genetic material in conifers. Specifically, the cytological studies of Sax (1960) and Saylor (1972) on pine chromosomes indicate that the karyotypes of conifers are uniform and almost indistinguishable. Major structural differentiation in the form of inversions and translocations is lacking, according to evidence from interspecific hybrids.

The interpretation of isozyme studies substantiate the conclusions of cytologists. Heterozygous inversions would suppress recombination, and inversion homozygotes would produce recombinants. If the trees differed for inversions, then linkage estimates could be expected to show wide divergence from tree to tree. Although more data are desirable, variances in linkage estimates from different trees in this study were within limits of random error and did not suggest the presence of inversion polymorphisms.

Saylor and Smith (1966) found no evidence of heterozygous translocations and the isozyme data support this observation. Translocations would disturb linkage relationships but linkage block A thus far demonstrates a consistent relationship between major genes in the block, not only within but also between the pine species thus far studied.

Data showed that about one-fourth to one-third of the sampled loci were associated with one linkage block; pines have 12 chromosomes, all but one or two with median centromeres (Saylor 1972). Twelve or 24 linkage groups might be expected, because whether considering the number of chromosomes or the number of chromosome arms, a random distribution throughout the chromosome complement of the 10 to 43 genes analyzed would lead one to expect a moderate number of linkages with low numbers of genes per linkage block. The likelihood of various numbers of genes being linked by chance on pine chromosomes, or on arms of chromosomes, can be approximated by the binomial distribution function. Linkage analyses evaluated about 35 genes and the expected distributions were computed on this basis (*fig. 3*). For 12 chromosomes, about 5 percent would not receive a gene, 15 percent would get one, 23 percent would have 2, and so on. This study indicated that as many as 12 genes were linked in the A block. The probability of a linkage block this large or larger on a chromosome is 1.5×10^{-5}. When chromosome arms are considered, the proportions of blocks with numerous genes are reduced. The probability of a linkage block with 10 or more genes on a chromosome arm is 1.0×10^{-7}

If we are to maintain that the large number of genes in the A linkage block results from some causal association of loci, the obvious observation is that the genes are all active during a specific developmental stage, namely, the growth of the developing embryo. The function of ADH, which is in the center of this segment, is the conversion of the end products of anaerobic respiration. Esterases are involved in the metabolism of fats and peroxidases are known to serve as IAA oxidases. These functions would implicate them in processes of embryo growth.

Close linkages suggest the possibility that selection operates upon specific allelic combinations. Fitness values for tightly linked loci depend on the multilocus combination of alleles rather than the fitness of individual loci (Lewontin 1974). Although pine species vary in the number of alleles per locus for the three tightly linked loci on the A block, GOT-1, PER-2, and PGI-2, three alleles per locus would produce 27 gametic classes and a total of 378 zygotic classes. The problem of estimating fitness values for such a complex system is overwhelming.

Lewontin (1974) cited Fisher's proposal that natural selection favors closer linkage of genes influencing the same polymorphism. Tight linkage could slow the process

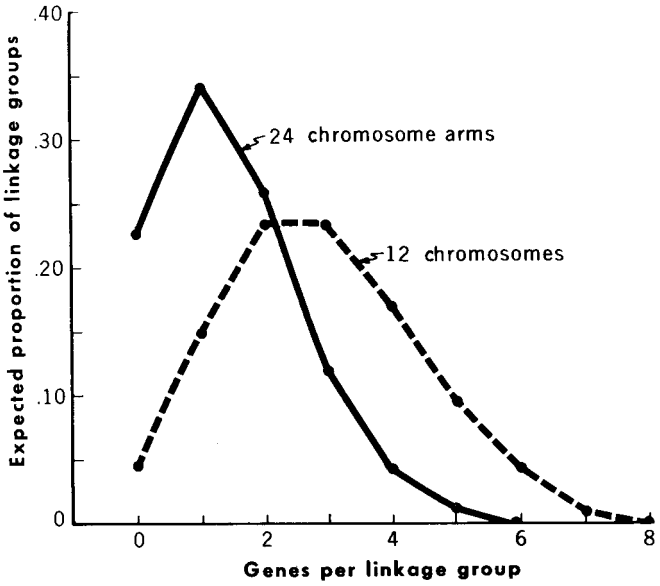

Figure 3—Expected proportion of linkage groups with different numbers of genes per group when 35 genes are randomly allotted to chromosomes and chromosome arms.

of allele fixation at a locus by operating upon complexes of loci and the effect could extend to include genes at greater map distances (Lewontin 1974). The analysis of pine loci points to a significantly large block of linked genes. One could question if this linkage block represents genes that are related in function but sufficiently separated to accommodate recombination.

Conifers stand, almost unchallenged, as higher organisms that readily yield information on isozyme linkage. Data in pines strongly suggest nonrandom association of numerous loci and provide new evidence on conifer evolution. Allelic variation within these linkage blocks may provide for precise genetic evaluation of species relationships. Linkages may also serve as the basis for mapping and manipulating genetically controlled phenotypic traits. These possibilities bring closer the goal of relating genetic variation in enzymes to variation in phenotypic responses; and conifers, once thought to be poor organisms for precise genetic analyses, offer advantages over many, if not most, other higher organisms.

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