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Review

Isozyme studies in provenance research of forest trees

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Summary. The nature and origin of the isozymes and the techniques for their detection in forest trees are briefly reviewed. The theories used to interpret the isozyme variation are summarized. Recent isozyme variation studies in provenance research are discussed in relation to known variation pattern detected by classical nursery and field tests. The populations of a tree species can be sometimes, but not always, distinguished by their isozyme patterns. For a number of species, relationships between environment of origin of the provenances and some isozyme frequencies have been statistically established. In one case (*Picea sitchensis*) where direct comparison between the variation pattern detected by isozymes and the genetic variation of silviculturally important traits was possible, no meaningful relationships between both patterns could be detected. Nei's genetic distances and indices of gene diversity do not appear to be useful in provenance research. The concept of genetic distance based on gene frequencies is probably not very useful in provenance research either.

Key words: Provenance research – Isozyme variation – Silvicultural traits – Genetic distances

Introduction

An isozyme, also called an isoenzyme, is one of the multiple forms of an enzyme, separable by electrophoretic procedure and having similar or identical catalytic activities (Feret and Bergmann 1976). Enzyme multiplicity may result from different amino acid sequences or from post-translational modifications (Rider and Taylor 1980). In the first case, the isozymes can be encoded by different alleles of the same locus or by different loci. Isozymes differ by the charge, size and shape of their protein and by their specific properties in metabolic regulation (Rider and Taylor 1980).

Needle and endosperm tissues have been mainly used for isozyme studies in provenance research. After tissue preparation, protein extraction and stabilization, the isozymes are separated by electrophoresis of the protein extracts in starch, polyacrylamide, cellulose acetate or agarose gels. Most authors have used simple zone starch gel electrophoresis. After electrophoresis, the gel can be stained for a particular enzyme by soaking it in a solution containing a substrate with a dye for the enzyme. Dark bands appear and their relative position is determined. These bands are then interpreted in terms of loci and alleles and their frequencies estimated. It is important to note that:

1) the nature and number of isozymes detectable depends on the separation technique used (Rider and

Taylor 1980). Polyacrylamide gel electrophoresis, isoelectric focusing or two dimensional electrophoresis can change the zymograms obtained by starch gel electrophoresis, generally increasing the number of bands or alleles detected. Even changes in temperature and pH during electrophoresis can break up homogeneous patterns (Rasmuson 1979);

2) the technique enables only a limited number of extracts to be run in parallel on the same gel; thus, to study many trees and provenances some standard protein should be run and migration rates relative to that protein band determined;

3) To avoid the possibilities that isozymes are artifacts resulting from laboratory manipulation of cells or cell extracts, different techniques are used – protective agents against precipitation by tannins, buffered extraction, low temperature extraction – to avoid protein denaturation (Feret and Bergmann 1976). Effects of different developmental and differentiation stages of the tissues studied on the zymograms are avoided by studying the same organs (needles, seed gametophyte (endosperm) in conifers, etc.) at the same age.

Once the isozymes bands have been interpreted in terms of loci and alleles, the frequency of the alleles are calculated using the frequency of heterozygotic and homozygotic individuals; then the expected frequencies of these individuals can be calculated according to the Hardy-Weinberg law and compared to the actual frequencies to assess possible departures from the conditions of that law.

For a discussion of the other problems encountered in isozyme studies, see Rider and Taylor (1980) and Feret and Bergmann (1976).

To explain the presence of multiple alleles at one locus in a population, two theories have been proposed: one based on the neo-darwinian theory of either transient or balanced polymorphisms maintained by natural selection, the other that these alleles are the result of mutations which are selectively neutral. The neutral mutation hypothesis has resulted in a theory of molecular evolution by neutral mutation and random drift (Nei 1975). Based on the theories of Nei (1975, 1976) most authors reviewed have calculated indices of gene diversity, degree of heterozygosity and genetic distances between provenances, as follows. Let X_{ik} be the frequency of the k-th allele at a locus in the i-th population (=provenance), the gene diversity in this population is defined as $H_i = 1 - \sum_k X_{ik}^2$. The average over all populations is H_S. The gene diversity in the total population is $H_T = 1 - \sum_k X_k^2$, where $X_k = \sum_i X_{ik}/s$, s being the number of populations. Then $H_T - H_S$, the gene diversity due to interpopulational gene differences, is denoted D_{ST} and the coefficient of gene differentiation is D_{ST}/H_T. H_S is called average degree of heterozygosity in a population.

Now if $J_i = 1 - H_i$, $J_{i'} = 1 - H_{i'}$ for populations i and i', $J_{ii'} = \sum_k X_{ik} X_{i'k}$, and J_i, $J_{i'}$, $J_{ii'}$ are all averaged over n loci, the normalized identity of genes I between populations i and i' is defined as $I = J_{ii'}/\sqrt{J_i J_{i'}}$.

$J_{ii'}$, J_i and $J_{i'}$ are the probabilities of identity of two genes.

The genetic distance (D) of Nei is defined as $D = -\mathrm{Log}_E I$ (Nei 1975, 1976).

Bergmann (1974) has proposed as genetic distance

$$Do = \frac{1}{2} \sum_{k=1}^{p} |X_{ik} - X_{i'k}|$$

(using my notation), for p alleles at one locus. Do is averaged for n loci. Do has been recently shown by Gregorius (1984) to be a "metric" and, answering also three other conditions, to be unique in its kind.

Bergmann and Gregorius (1979) have used a new measure of genetic diversity $V_{(P)}$, which is defined as

$$V_{(P)} = \frac{1}{\sum_{k=1}^{p_1} X_{1k}^2} \cdots \frac{1}{\sum_{k=1}^{p_n} X_{nk}^2} - 1$$

for one population, with p_1 alleles for locus 1, etc.

Krzakowa and Szweykowski (1979), have used two types of genetic distances, one is based on geometric reasoning and defined as

$$\left[\sum_{l=1}^{n} (2 - 2\cos \alpha_l)\right]^{1/2} \quad \text{for n loci}$$

$$\text{with } \cos \alpha_l = \sum_{k=1}^{p} (X_{ik} X_{i'k})^{1/2}$$

(Sneath and Sokal 1973). According to Gregorius (1974), this distance is not a perfect genetic distance. The other is Hedrick's distance (Hedrick 1974):

$$D_H = 1 - \frac{\sum_{j=1}^{g} P_{ji} P_{ji'}}{\frac{1}{2}\left(\sum_{j=1}^{g} P_{ji}^2 + \sum_{j=1}^{g} P_{ji'}^2\right)}$$

where P_{ji} is the frequency of the j-th genotype in population i, and g is the number of genotypes.

Weber and Stettler (1981) have used Rogers' coefficient of similarity which, according to Ayala (1976), give similar results to Nei's similarity index I.

Rogers' Coefficient of similarity is defined as

$$D_R = \frac{1}{N} \sum_{l=1}^{n} \left[\frac{1}{2} \sum_{k=1}^{p} (X_{ilk} - X_{i'lk})^2\right]^{1/2}$$

(Sneath and Sokal 1973 and Hedrick 1974). A comparison of the genetic distances and diversity indices presented here is

beyond the scope of this paper. Some comparisons, from different points of views, have been done by Hedrick (1974) and Gregorius (1974). According to Hedrick (1974), Nei's and Rogers' indices of similarity give essentially the same results if the populations are under Hardy-Weinberg equilibrium.

Critical review of isozyme variation studies in provenance research

Table 1 summarizes the isozyme systems studied and their relationships with economically important traits or other characteristics of the provenances of the species studied in the articles reviewed which span approximately the last decade.

To indicate the enzyme systems studied the following abbreviations are used:

AAT = Aspartate aminotransferase
ACO = Aconitase
ADH = Alcohol dehydrogenase
AK = Adenylate kinase
ALD = Aldolase
DIA = Diaphorase
EST = Esterase
FE = Fluorescent esterase
GPI = Glucose phosphate isomerase
G6P = Glucose-6-phosphate dehydrogenase (= GCPD)
GDH = Glutamate dehydrogenase
GOT = Glutamate-oxalate transaminase
IDH = Isocitric dehydrogenase
LAP = Leucine amino peptidase
MDH = Malate dehydrogenase
ME = Malic enzyme
PDASE = Phosphodiesterase
PEP = Peptidase
PHAP = Peptidase Phenyl-Alanyl-Proline
PLA = Peptidase Leucyl-Alanine
PEROX = Peroxidase
PGI = Phosphoglucose isomerase
PGM = Phosphoglucomutase
PHI = Phosphohexose isomerase
PHOS = Acid phosphatase
6PG = 6 Phosphogluconate dehydrogenase (= CPGD)
SOD = Superoxide dismutase
TO = Tetrazolium oxidase

I have followed the advice of one reviewer and concentrated on the cases where new information was gained on population structure and differentiation. For clarity, I treat each tree species separately, but not all authors of Table 1 are mentioned again.

Picea abies

High polymorphism was detected for all loci studied by all the authors mentioned in Table 1. Minor differentiation in gene frequencies was found between populations separated by nearly 7 degrees of latitude (Bergmann 1973). Adjacent populations did not differ in heterozygosity (Tigerstedt 1974) and there was no clear relationship between heterozygosity and altitude of place of origin, contrary to expectations (Lundkvist 1979).

Hardy Weinberg equilibrium prevailed generally, when it was tested.

Provenances could be classified into groups using the rarest alleles of some isozyme loci and within each group, some provenances could be separated but others could not. Provenances of mixed origin and allochthonous ones could not be identified (Bergmann 1975 a). Lundkvist (1979) claims that the 4 populations of Norway spruce he studied could be identified by their allelic frequencies.

The variability of some loci was deemed to be selectively neutral but for others it was thought to be maintained by natural selection (EST, related to latitude of place of origin (Tigerstedt 1974). Conclusive evidence exists to indicate that PHOS varies according to some temperature-dependent factors of the environment (Bergmann 1975 b, 1978). Some PHOS isozymes might be adapted to operate at low temperatures, others at higher temperatures. Bergmann (1978) claims that the pattern of variation in the PHOS isozymes parallel the known variation pattern in bud set, shoot length : hypocotyl ratio of similar provenances. However, Tigerstedt (1974) could not detect any clinal variation at a PHOS locus active in spruce seed, but Lundkvist and Rudin (1977), were able to find some geographical variation in PHOS allelomorphs.

Bergmann and Gregorius (1979) have used several gene diversity indices to show that Norway spruce border populations have low gene diversity. Nei's genetic distances increased with geographic distances between provenances (Bergmann 1973; Lundkvist and Rudin 1977).

A new genetic distance used by Bergmann (1974) shows that closely situated provenances of Norway spruce are genetically more similar than provenances more widely separated.

Lundkvist (1979) has shown that 3 out of 11 loci studied are linked.

Pinus contorta

Yeh and Layton (1979) have studied isozyme variation at 25 loci in 9 widely separated populations of lodgepole pine in order to compare the genic variability of marginal populations with that of central populations in British Columbia.

At one locus, AAT-1, one allele frequency and the heterozygosity index increased with altitude of place of origin. A North South decrease in heterozygosity was also found for the 6PG locus.

Using Nei's index of gene diversity, Yeh and Layton (1979) claim that 4% of the genetic variability is due to inter-provenance effects. Based on 4 indices of variability, these authors claim that central populations

Table 1. Recent isozyme variation studied in provenance research and its relationships with economically important traits or other provenance traits

Species studied	Enzymes studied/ no. of Loci[a]	No. of alleles detected at each locus	Tissue used[b]	Relationships with place of origin reported	Relationships with economically important traits or other provenance traits reported	Reference
1) Pinaceae						
Abies sachalinensis	PEROX/–	–	Needles	NO	NO	Matsuura and Sakai (1972)
Larix gmelini	EST/– PEROX/–	–	Needles	YES	NO	Larionova and Milyutin (1981)
Picea abies	EST/2 LAP/2	2 and 3 4	Endosperm	YES	NO	Bergmann (1973)
	ADH/1 EST/2 LAP/2 PHOS/1	4 3 and 4 3 3	Endosperm	YES	NO	Tigerstedt (1974)
	EST/2 LAP/2	2 and 3 4	Endosperm	YES	NO	Bergmann (1974)
	EST/2 LAP/2 PHOS/2	2 and 3 4 3 and 4	Endosperm	YES	NO	Bergmann (1975 a)
	PHOS/1	5	Endosperm	YES	NO	Bergmann (1975 b)
	EST/1 LAP/2 PHOS/1	6 4 7	Needles and Endosperm	YES	NO	Lundkvist and Rudin (1977)
	PHOS/1	4	Endosperm	YES	YES	Bergmann (1978)
	GDH/1 GOT/2 LAP/2 MDH/2 PHOS/1	2 3 and 4 6 and 7 2 and 4 5	Endosperm	YES	NO	Lundkvist (1979)
	EST/2 LAP/2 PHOS/2 PHASE/1	1 to 5 2 to 5 2 to 5 1	Endosperm	YES	NO	Bergmann and Gregorius (1979)
Picea sitchensis	AAT/2 ACO/1 ALD/1 DIA/3 EST/1 GDH/1 G6P/1 IDH/1 MDH/3 ME/1 PEP/3 PGI/2 PGM/2 6PG/2	1 and 2 3 1 2 3 3 3 3 1, 3 and 2 2 1 2 and 3 3 and 2 2 and 4	Endosperm	YES	NO	Yeh and El Kassaby (1980)
Pinus contorta	AAT/2 ACO/1 ADH/1 AK/2 DIA/2 GDH/1 G6P/1 IDH/1	3 and 4 4 4 3 and 2 4 and 3 3 3 2	Endosperm	YES	NO	Yeh and Layton (1979)

Table 1 (continued)

Species studied	Enzymes studied/ no. of Loci [a]	No. of alleles detected at each locus	Tissue used [b]	Relationships with place of origin reported	Relationships with economically important traits or other provenance traits reported	Reference
	MDH/4 ME/2 PEP/1 PGI/2 PGM/1 6PG/2 PHOS/1 glucosidase/1	2, 3, 3 and 4 1 and 5 2 3 and 3 4 4 5 2				
	ADH/1 EST/1 PEROX/1 PHI/1	3 3 3 4	Needles	NO	YES	Knowles and Mitton (1980)
Pinus nigra	LAP/2	5	Endosperm	NO	NO	Nikolic and Bergmann (1974)
	GOT/4	2, 3, 6 and 3	Endosperm	YES	YES	Bonnet-Masimbert (1979)
Pinus ponderosa	PEROX/1	3	Needles and seedling needles	YES	NO	Beckman et al. (1976)
	PEROX/1	3	Needles and seedling needles	YES	NO	Mitton et al. (1977)
Pinus pungens	EST PEROX	up to 6 up to 9	Needles, seedling needles and endosperm	NO	YES	Feret (1974)
Pinus sylvestris	EST/–	–	Needles	NO	NO	Rasmuson and Rudin (1971)
	6PG/3	3, 3 and 2	Endosperm	YES	NO	Krzakowa and Szweykowski (1979)
Pseudotsuga menziesii	PEROX/–	11	Seedling needles	NO	NO	Muhs (1974)
	PHOS/1	4	Endosperm	YES	NO	Bergmann (1975 b)
	EST/2 GOT/1 LAP/1	3 3 3	Seedling needles	YES	NO	Yang (1976)
	EST/3 GOT/2 LAP/2	1, 3 and 3 1 and 3 1 and 3	Germinant tissue	YES	NO	Yang et al. (1977)
	LAP/2	3 and 3	Endosperm	NO	YES	Mejnartowicz (1976)
	AAT/2 ACO/1 ALD/1 DIA/1 EST/1 GDH/1 G6P/1 IDH/1 MDH/4 ME/2 PEP/2 PGI/1	1 and 3 2 3 3 4 2 3 4 4, 3, 3 and 2 2 and 3 1 4	Endosperm	YES	NO	Yeh and O'Malley (1980)

(continued overleaf)

Table 1 (continued)

Species studied	Enzymes studied/ no. of Loci[a]	No. of alleles detected at each locus	Tissue used[b]	Relationships with place of origin reported	Relationships with economically important traits or other provenance traits reported	Reference
	PGM/1	3				
	SOD/1	3				
	6PG/1	3				
2) Taxodiaceae						
Taiwania cryptomerioides	PEROX/–	–	Needles	NO	NO	Wang et al. (1976)
3) Cupressaceae						
Thuja plicata	EST/–		Seedling needles	NO	YES	Copes (1981)
	FE/–					
	GOT/–					
	GPI/–					
	GDH/–					
	PEROX/–					
	PHOS/–					
	TO/–					
4) Salicaceae						
Populus trichocarpa	AAT/2	2 and 1	Root tips of cuttings	YES	YES	Weber and Stettler (1981)
	DIA/1	2				
	EST/2	1 and 2				
	G6P/1	2				
	LAP/1	2				
	MDH/2	1 and 2				
	PEROX/1	2				
	PHAP/1	1				
	PHOS/2	1 and 2				
	PGI/2	1 and 2				
	PGM/2	1 and 2				

[a] For explanation of abbreviations used: see text
[b] Unless otherwise mentioned needles refer to needle tissue of adult trees

show increased genetic variation. Outcrossing rates are also estimated.

Knowles and Mitton (1980) show that lodgepole pine trees with high degrees of overall enzymic heterozygosity have less growth variability as measured by coefficient of variation (CV) of the ring widths; however, taken individually, the heterozygosity of each locus was not related to CV. Thus, the relationships between the loci studied and growth variability seem tenuous (average CV for homozygous trees: 79.2%, for heterozygous trees: 70.2%).

Pinus nigra

Bonnet-Masimbert (1979) has studied the isozyme polymorphism of 4 GOT loci of 40 provenances of *P. nigra* and has been able to distinguish between two subspecies but not between the other subspecies nor between the provenances. Other morphological or physiological traits (terpenes) would have been successful in separating the other sub-species, however.

Pinus ponderosa

Beckman et al. (1976) have shown substantial population differentiation over short distances (20 km) in isozyme polymorphism of a PEROX locus active in needles while Mitton et al. (1977) have shown some striking differences in allelic frequencies of a PEROX locus, between populations separated by 100 m, on contrasting slopes. The kinetic properties of the isozymes would be temperature dependent and would fit the variation pattern observed in the field.

Pinus sylvestris

Rasmuson and Rudin (1971), using grafts, have shown that individual trees can be distinguished by their zymogram. Palozova and Dukharev (1978) have shown that the coefficient of variation of annual ring width decreased with increasing number of isozymes of the EST system in trees of a stand.

Krzakowa and Szweykowski (1979) have studied isozyme variation of 3 loci of the 6PG in 8 populations

and shown differentiation maps based on 2 genetic distances (see "Introduction").

Rudin and Ekberg (1978) have studied the occurrence of linkage among 12 isozyme loci identified in the endosperm of 30 trees originating mostly from a 120–130 year old stand. The ADH system showed 2 loci with three alleles each; the EST system 1 locus with two alleles; the GOT system 3 loci with 3, 5 and 2 alleles respectively; the LAP 2 loci with 4 and 7 alleles (including silent alleles); the MDH 2 loci with 3 and 4 alleles; and the PHOS showed 2 loci with 3 and 2 alleles. The two loci of ADH, one of LAP and one of GOT were associated in a strong linkage group. Other linkage groups probably existed which were less significant or found in fewer trees. Recombination data and map distances between the loci involved, according to different principles, were estimated for 14 couples. Gamete selection and air temperature during meiosis might have influenced recombination frequency.

Pseudotsuga menziesii

Fourteen provenances were shown to differ in PEROX zymograms but no relationship was found between geographical proximity and identity of the zymograms (Muhs 1974). However, Bergmann (1975) showed that isozyme frequencies of a PHOS locus were dependent on place of origin, probably on some temperature dependent factor. Yang (1976) has used principal component analysis (PCA) to relate the genetic variation of 4 polymorphic loci to factors of environment. Three correlations between PCA scores could indicate some role for natural selection processes.

Mejnartowicz (1976) has shown that some alleles of a LAP locus were significantly correlated with provenance origin and that a poor growing provenance was characterized by the predominance of a inactive LAP isozyme.

Yang et al. (1977) have studied 3 enzyme systems (LAP, EST and GOT) in germinants and found that in most provenances most loci were under the Hardy Weinberg equilibrium. Heterozygosities did not vary between provenances, except for one locus (EST-B), but decreased with either latitude or altitude of place of origin. Nei's genetic distances varied from 0.002 to 0.053 and generally increased with geographic distances.

On the basis of 15 enzyme systems and 21 loci, Yeh and O'Malley (1980) showed that up to 69% of the loci (depending on criteria) were polymorphic in eleven provenances of Douglas-fir from British Columbia. The average proportion of heterozygous loci per population was 2.19% and per individual 15.5%. Ninety-seven per cent of the total gene diversity, using Nei's index of gene diversity, was attributed to within population contribution. The genetic distances between pairs of provenances varied between 0.0007 and 0.0082. Allelic frequencies and heterozygosities for some loci and genetic distances were related to latitude, longitude and elevation of place of origin.

Populus trichocarpa

Twelve isozyme systems active in root tips of cuttings were studied for 10 populations of black cottonwood. Populations were very similar genetically, as measured by Rogers' coefficient of similarity. Six percent of the total gene diversity (according to Nei's indices) was attributable to gene differences between populations. Thirteen percent of all loci deviated from the Hardy-Weinberg equilibrium with deficiency in heterozygotes. Some allele frequencies varied with latitude or longitude of place of origin (Weber and Stettler 1981).

Discussion

Isozyme polymorphism is universal for all the enzymes and the forest tree species studied. Many of the loci were under the Hardy-Weinberg equilibrium. Interestingly, deficiencies in heterozygotes have been noticed for *Populus trichocarpa.* However, Marshall and Allard (1970) have shown that in two natural populations of *Avena barbata,* one was more polymorphic and had higher level of heterozygosis than the other population, using 4 loci of a EST system, one of PHOS and one locus of anodal PEROX systems. Excess heterozygotes in the former population indicated that natural selection favoring heterozygotes was a major factor in maintaining polymorphisms in that population. They also claim that the patterns of morphological and isozyme polymorphism were positively correlated in that species as well as in a closely related species of *Avena.* Note that *Avena* species are mainly autogamous, contrary to most tree species.

Generally, few differences existed between the provenances either in terms of allelic frequencies or in terms of genetic distances. One exception seems to be *P. ponderosa* where populations separated horizontally by 100 m, on two slopes, showed marked allelic differentiation. On the contrary, *Tawainia cryptomerioides* (Wang et al. 1976) and *Thuya plicata* (Copes 1981) did not show any provenance differences. Statistically significant correlations were found between some allelic frequencies and genetic distances and latitude or altitude of place of origin of the provenances, for some isozyme system but not by all the authors who studied the same system (it is the case of PHOS loci). Thus, empirical evidence exist that at least some structural genes and their isozymes might be submitted to natural selection of the environment of origin, either directly or

indirectly via other genetically controlled traits. But many other loci and alleles were deemed selectively neutral.

Provenance identification has been claimed for *Picea abies*. Individual trees could be identified in *Pinus sylvestris*.

Genic diversity, when calculated, was overwhelmingly confined to intra-provenance variation, although Muhs (1974) using simple analyses of variance of PEROX bands frequencies has found that the component of variance between provenances of Douglas-fir was much larger than the component within provenances.

Nei's genetic distances were small, for all forest trees studied and consistent with isozymes based estimation of genetic distances between races of animal species (see Table 5 of Nei 1976).

Out of 29 papers reviewed in this article only 6 have attempted to relate the isozyme variability studied to other traits of the provenances of the species studied. Of these 6 papers, only one (Feret 1974) compares directly the zymograms of parent trees with those of their progeny and with growth traits of these progenies. Isozyme variation in the PEROX and EST systems and morphological variation in parent trees and their progeny of three stands separated by up to 42 km were studied for *Pinus pungens*. Some isozyme and morphological variation was detected in parent trees and individual progenies but there were no differences whatsoever among stands on the basis of their progeny (Feret 1974). The relationship between isozyme variation and other traits, both parental and progeny, is thus debatable, in that pine.

All the other papers refer indirectly to some variability detected in tests using other provenances. It is difficult to relate the isozyme variation detected in most species of forest trees studied with the large ecotypic and/or ecoclinal variation detected in many morphological and physiological traits assessed in greenhouse, growth chamber, nursery and field provenance trials, although other gene diversity indices and other genetic distances might change the picture. Yeh and Layton's findings (1979) that 96% of total "genic" variability in isozyme is confined to within population variability contrasts with the large ecotypic and clinal variation detected by Critchfield (1957) and other authors in lodgepole pine (see proceedings of 1978 IUFRO joint meeting of different working parties held in Vancouver, Canada). It is also doubtful if 97% of the genetic variation in Douglas-fir is confined to intra-population variability as Yeh and O'Malley (1980) claim, on the basis of their study of 15 enzyme systems. Large clinal and ecotypic variation in many traits is well known in Douglas-fir and need not be reviewed here. Yeh and O'Malley's idea that the pattern of variation detected by isozyme studies "should influence selection methods in an applied tree improvement programme" (Yeh and O'Malley 1980) or that isolation by distance may be an important factor in population differentiation in Coastal Douglas-fir (Yeh and O'Malley 1980) cannot be accepted. Genetic distances averaging 0.0037 between Douglas-fir provenances do not seem to represent the known genetic differentiation of that tree. An average distance of 0.0037 means, according to Nei (1975), that the mean number of net codon differences per locus (1 locus = $\pm$ 300 codons?) is 0.0037 or that provenances of Douglas-fir spanning four degree of latitude differ by 1.11 codon per locus, on average. This is highly unlikely on the basis of what is known about the ecotypic and ecoclinal variation in that species.

Value of isozyme studies in predicting genotypic variation: the case of the Sitka spruce

There is a dire need to compare the variation pattern shown by isozyme studies and the genetically based variation shown by nursery, growth chamber or field test studies of the same provenances used for zymogram research.

In the case of Sitka spruce, however, the same provenances, or nearly so, have been studied by the use of isozyme electrophoretic studies and by nursery tests, although by different authors.

Yeh and El-Kassaby (1980) have studied 14 enzymes at 24 loci of 10 IUFRO Sitka spruce provenances and present, for each provenance, allelic frequencies, heterozygosities, differentiation indices and genetic distances between provenances according to Nei's methods. Implications of their study for breeding Sitka spruce are also proposed.

The 10 provenances studied by Yeh and El-Kassaby (1980) are part of a large IUFRO collection of Sitka spruce provenances. The phenotypic and genotypic variation of 41 provenances of that collection has been extensively studied by Falkenhagen (1976, 1977, 1978, 1984) and Falkenhagen and Nash 1978. Of the 10 provenances studied by Yeh and El-Kassaby, 7 provenances are part of Falkenhagen's studies, but the three American provenances not studied by Falkenhagen should not change the overall picture of genetic variation in Sitka spruce.

Yeh and El-Kassaby show the observed proportion of polymorphic loci per population and the average proportion of heterozygous loci per individual tree active in endosperm of seeds. These two proportions do not vary from provenance to provenance. The authors' interpretation of an average proportion of heterozygous loci per individual of 15% as indicating a high level of gene diversity is doubtful because this would indicate that 85% of the loci are homozygous which is improbable for wild tree species.

Table 2. Maximum ratio of the values of the nursery traits studied for Sitka spruce growing near Vancouver, Canada. Legend: see Falkenhagen 1977

	Traits					
	Budset 1	Budset 2	Bud Burst	Budset 72	Epicotyl length (cm)	Total height (cm)
Ratio (max./min.)	5.49	8.76	1.54	6.43	1.55	1.84

Falkenhagen's papers show the magnitude of the geographic, genetically based variation of Sitka spruce and its probable narrow adaptation to its local environment of origin as expressed in a nursery environment, thus the probable importance of natural selection in shaping the populations of Sitka spruce, contrary to what the two authors claim.

Table 2 shows the ratio between the maximum average provenance value and the minimum average provenance value for the traits studied in the nursery (after Falkenhagen 1974, 1977).

The ratio for potassium dry needle content is 1.26 although only 10 provenances were studied (Falkenhagen 1976). Simple, multiple and canonical correlation analyses show that, on average, the Sitka spruce provenances are narrowly adapted to or at least correlated strongly with their environment of origin (Falkenhagen 1976, 1977, 1978 and in press; Falkenhagen and Nash 1978), at the nursery stage. In fact, there is a large congruence between the space of the provenance traits studied and the space of the geographical co-ordinates of the place of origin of these provenances.

In terms of proportions of total variability attributable to the different levels of variation (tree/population), the contentions of the authors are incompatible with Falkenhagen (1977) estimations for epicotyl length (EPL) and second year height (HT). On an ecological region basis – in which variation is more limited – the proportion of the total variation attributable to the provenance effect varied for EPL from 3 to 39% and for HT from 3 to 20% (Falkenhagen 1977).

Analysis of Nei's genetic distances between Sitka spruce provenances

If we compare Nei's genetic distances with the Mahalanobis' generalised distances – which enable each trait studied to carry its own amount of information about the separation of the provenances and eliminates the effects of correlation between these traits – that I have calculated for a selected group of Sitka spruce provenances (Falkenhagen, in press) using 7 seedling traits, we can see that the Mahalanobis' distances increase with increasing geographic separation and lead to useful biological interpretation. Nei's distances vary haphazardly and would indicate a far too small genetic differentiation (from 0.004 to 0.030 codon differences per locus). Nei's distances are not related with the morphological and/or physiological, genetically based, variations of Sitka spruce as detected by my studies and those of Lines (1978) and other authors.

Gregorius (1974) has shown that Nei's distance does not have the most important properties of a distance and has proposed another one (see "Introduction"). However, any genetic distance based only on gene frequencies is bound to be an oversimplification, especially if they are structural genes. The genetic distances based on allele frequencies might be irrelevant to the problem of calculating some distance between the genotypic structure of organisms or populations because

1) genes do not act isolately but their actions depend on inter and intra loci interactions (group linkage, epistasis, dominance effect, regulatory genes, etc.) i.e., their actions depend on their genetic environment;

2) genes act in interaction with their non genetic environment be it inter or extra cellular (tissue relationships, ecological conditions).

Nei's distance can be interpreted in terms of evolutionary time. Nei (1975, 1976) has proposed the formula:

$$t = 5 \times 10^6 D$$

as a crude approximation to estimate the time of divergence between two taxa, where

t = time in years
D = genetic distance in codon per locus.

Thus, for Sitka spruce, an average distance of 0.014 would correspond to an average divergent time of 70,000 years, the range being from 20,000 to 150,000 years. Are these divergence times compatible with the known paleohistory of Sitka spruce? First, Yeh and El-Kassaby's genetic distances indicate that there is no relationship between divergence time of two popula-

tions and their geographic proximity or separation although we may hypothesize that two geographically close populations diverged more recently than distant ones. Secondly, Sitka spruce is thought to have survived pleistocene glaciations on nunataks well scattered along the coast from Northern Washington state to Juneau (Alaska) (Daubenmire 1968). According to Harris (1978), Sitka spruce was able to reoccupy its previous distribution area 8,500 years ago in Washington State (USA), 8,000 years ago in South Coastal British Columbia and 3,000 years ago on Kodiak Island (Alaska). However the nunatak populations must be considered as considerably older than 8,000 years, they must have diverged from a common population, before the start of Pleistocene or at least 2,500,000 years ago. According to recent data, the Pleistocene might have started 2.5 million years ago (West 1979; Berggren et al. 1980). During the many glacial and interglacial periods of Pleistocene, Sitka spruce suffered population destructions, with drastic range restrictions and several migrations in opposing directions (Martin and Mehringer 1965; Heusser 1965). However, the fact is that the remaining populations on nunataks or south of the limit of the maximum glaciation must be very old, much older than 150,000 years, older than 2.5 million years of pleistocene period. Stebbins (1950) indeed believes that, by the end of the Mesozoic era, the coniferous flora of the world was not materially different from that of modern time. Thus modern conifer genera are mostly 150 million years old. Mirov (1967) thinks that it is not unreasonable to conclude that the genus *Pinus* originated from some ancestral stock during Permian-triassic time. It possibly differentiated during the upper Triassic from the closely related genera *Cedrus* and *Larix*. Some species of *Pinus* of the section Oocarpaceae may be as old as 50 millions of years, in California (Axelrod 1980) and Sitka spruce is most probably as old or older.

Thus, time of divergence between the populations of Sitka spruce estimated from Nei's genetic distances based on isozyme studies do not fit with the known time length of the evolutionary history of Sitka spruce.

Yeh and O'Malley (1980) average genetic distances between Douglas-fir provenances of 0.0037 lead to a even more disputable evolutionary divergence time (on average) of 18,500 years.

Nei's genetic distance based on isozyme frequencies is probably not relevant to the genetic differentiation between populations of Sitka spruce.

According to Nei (1976), the evolutionary interpretation of his genetic distance between two populations is based on the following hypotheses:

1) the parent population is split into two subpopulations of same effective size as that of the parental population;
2) the evolutionary factors at play are random drift and mutation; selection and migration are thus considered as negligible;
3) mutations are selectively neutral, mutation rate is the same for all loci considered and the mutation is irreversible;
4) gene substitution is the most basic process of evolution and the best measure of genetic distance is that distance which uses the accumulated number of gene substitutions per locus;
5) rate of gene substitutions per unit of length of time is constant.

All these hypotheses are disputable as far as forest trees are concerned. Intra-genic mutation is a random process, reversible and which can affect at random all the mutons of a gene (Watson 1970). The exact number and effects of codon substitutions in the past evolutionary history of a species cannot then be ascertained (Rider and Taylor 1980).

The data published by Bergmann (1973) and Lundkvist and Rudin (1977) show clearly that Nei's distance is not a metric, as already noted by Gregorius (1974), but also cannot be interpreted in the light of what is known of provenance variation. For instance, the genetic distances between provenances 1 and the other provenances (excluding provenances 7 and 8) studied by Lundkvist and Rudin (1977) increase with latitude of place of origin, although not in a statistically significant way, but the genetic distances between provenance 2 and the others do not increase (see Fig. 2 of these authors).

The genetic distances presented by Yeh and El-Kassaby (1980); Yeh and O'Malley (1980), and Bergmann (1974) also vary haphazardly and inconsistently. However the distances presented by Yang et al. (1977) are more consistent, for some reasons difficult to ascertain.

Conclusions

By studying isozyme systems, the forest geneticist has tried to move closer to the gene. Zymograms are phenotypic expressions whose interpretation is delicate because of complex gene interactions controlling isozyme expressions as exemplified by Korochkin (1981), although, isozymes seem to be inherited in a Mendelian way. Progress in isozyme research would be enhanced by estimating the quantity and activity in time and in different tissue of the isozymes studied and by using refined electrophoretic techniques.

Lewontin (1977), claims, mentioning the isozyme studies, that it is not known whether the "structural genes" for which exists ample information, represent

the genetic variation in shape, size, metabolic rate, etc., in which plant or animal breeders are interested.

Allozymes or isozymes are enzymes with the same catalytic function, but different physical properties, although tissue – specific isozyme patterns in a developing organism as well as the same metabolic pathway controlled by different isozymes are known (Rider and Taylor 1980). Some evidence that some isozyme systems in forest trees might be selected by some temperature dependent factor of the environment of origin of the populations has been presented in this article. However, the problem of relating morphological and physiological trait differences between populations to differences in intragenic codon substitutions detectable by electrophoresis is overwhelming.

Muhs (1981) has reviewed briefly the progress in isozyme studies achieved in Europe in the last decade.

It seems to me that isozyme studies can be used:

1) to characterize individual trees, stands and provenances;
2) using rare alleles as markers, to study gene flows in stands (Lundkvist 1979) and seed orchards (Rudin and Lindgren 1977);
3) to study linkage groups (Rudin and Ekberg 1978; Lundkvist 1979) and linkage disequilibrium for testing some theoretical problems of population genetics (Rasmuson 1979).

The indices of gene diversity proposed by Nei (1975, 1976), G_{ST} and H_T need to be cautiously used. Lundkvist (1979) has shown that H, the expected proportion of heterozygotes, is less informative than other indices when the number of alleles per loci is larger than 4. Gregorius (1978) in Bergmann and Gregorius (1978) has proposed a better genic diversity index (see "Introduction").

Nei's genetic distance seems defective theoretically and practically at least as far as its application to forest tree species is concerned. Nei (1975) has himself discussed some limitations of this distance.

Much isozyme variation seems to be due to random drift. It is possible that organismal and molecular evolution are to a large extent independent from one another; this independence should be expected when one considers that the resemblance between man and chimpanzee macro-molecules measured by protein sequencing, immunology, electrophoresis and nucleic acid hybridization is striking, yet in anatomy and way of life chimpanzees and humans differ widely (King and Wilson 1975). Differences between species and populations might result from genetic changes in regulatory systems (point mutation of regulatory genes or change in the order of the genes). Internal gene organizations along chromosome, gene communication and creation mechanisms in relation with chromosome numbers would explain how a living organism is controlled genetically (Lima de Faria 1980 a, b).

While routine electrophoretic surveys may underestimate the amount of alleles per loci, they may overestimate the amount of polymorphism present in a organism because the enzymes typically surveyed are those found in relatively high concentrations in tissue or body fluids and exclude the products of regulatory loci and such loci that code for ribosomal proteins and transfer RNA (Hartl 1980).

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