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Abstract approved: Signature redacted for privacy.
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Genetic variability in natural populations of Douglas-fir was studied at molecular level by investigating the variations of some primary gene products, i.e. enzymes. Using the techniques of disc gel electrophoresis and biochemical staining, the isoenzyme patterns of leucine aminopeptidase, esterase and glutamate oxaloacetate transaminase are characterized in young seedlings at the stage of completely elongated cotyledons. Samples of 107 seedlots were collected from nine provenances located at Vancouver Island of Canada, Washington, Oregon and California.

The segregations of isoenzymic phenotypes or genotypes at four polymorphic loci are, in most cases, in accordance with the Hardy-Weinberg expectations except in those provenances from high elevations and from the northern or southern species margins.

Geographic variations of frequencies of most alleles are significant among those provenances examined, but appear to be rather complex. By contrast, the variation patterns in level of genic heterozygosity exhibited a certain degree of geographic uniformity or clinal change except that at EST-B locus. The combined effect of gene flow and some form of balancing selection was considered as the probable mechanism in maintaining uniformity or clinal variation of isoenzymic heterozygosity.

Regression analysis, principal component method and correlation approach using both the original variables and variables generated as principal component functions, were used to relate allelic variability of isoenzymes and environmental factors. The significance of adaptation through the selective importance of three enzyme systems was discussed.

Genetic differentiation in terms of genetic identity and genetic distance between provenances of Douglas-fir is more or less in parallel to the geographic distance of locations.

The parallelism between the level of genic heterozygosity and the growth potential or adaptability for survival of trees of different origins was noted. An approach through a multidimensional study involving functional properties and inheritance patterns of isoenzymes, and environmental modulations was suggested as a possibility to complement genetic improvement programs in Douglas-fir.

Isoenzyme Polymorphism in Provenances of Douglas-fir (*Pseudotsuga menziesii* [Mirb..] Franco var. *menziesii*)

by

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I. INTRODUCTION

In view of the wide-range distribution, multitude of variation in morphological, phenological, physiological as well as biochemical characters (Sziklai, 1967; Zavarin and Snajberk, 1972; Ching and Hermann, 1973; Hermann and Ching, 1973), it is likely that Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco) may have followed the mode of geographical speciation which runs through three levels of variation: polymorphism, local racial variation, and geographical variation. The diagram of the pathway may be shown below:

$$\text{Polymorphism} \rightarrow \begin{matrix}\text{Local}\\ \text{races}\end{matrix} \rightarrow \begin{matrix}\text{Geographical}\\ \text{races}\end{matrix} \rightarrow \text{Species}$$

Polymorphism or individual variability was defined as the existence of genetic differences between sympatric individuals, members of the same Mendelian population (Dobzhansky, 1955). Ford (1964) also defined the concept of genetic polymorphism as the occurrence of two or more discontinuous forms of a species in the same population. Therefore, polymorphism is the fundamental segregational variation within a population from which the advanced levels of phylogenetic architectures evolved, and is a characteristic feature of cross-fertilizing plant species (Grant, 1971).

The existence of genetic polymorphism (individual variation) and fairly high level of heterozygosity in the gene pool of Douglas-fir has been often noted in the last three decades, but it could not be effectively explored by using the traditional methods of analyzing the genetic diversity in Douglas-fir such as provenance trial, study of inheritable quantitative traits, investigation of rare recessive mutants and cytogenetic study.

The provenance trial and quantitative approach must employ statistical tools, and those quantitative parameters and indices are the summational estimates of polygenic expressions which were often strongly contaminated by environmental factors. Consequently, the genetic components of variance cannot be very effectively partitioned from the environmental ones, particularly when strong gene-environment interaction takes place. These studies have not given precise estimation of allelic variation at single loci.

The study on recessive mutants of chlorophyll-less and embryonic lethal genes showed that the genetic basis of mutant characters is simple and can be easily demonstrated (Orr-Ewing, 1957; Sorensen, 1967, 1969, 1971), but such characters constitute only a very small proportion of the genetic variation in natural populations of Douglas-fir, and, thus, cannot be effectively used as marker genes for the investigation of naturally occurring allelic polymorphism.

Although karyotype analysis of the genus Pseudotsuga has revealed significant genetic variability (Christiansen, 1963; Thomas and Ching, 1967; Owens, 1967; Ching and Doerksen, 1971; Doerksen and Ching, 1972), the cytogenetic information that we obtained so far is still not sufficient for the study of polymorphic variation at intra-specific level. This is probably due to the conservative nature of chromosomes and the lack of giant chromosomes, like those of Drosophila, in which precise gene-by-gene pairing can be easily observed and localized.

The genetic variation of terpenes in stem cortex or needle tissues has been shown to provide an excellent aid to the biosystematic study of Douglas-fir (Zavarin and Snajberk, 1972; von Rudloff, 1972a, b, 1973). These chemical characteristics have solved the difficulty of distinguishing morphologically the intermediate forms between var. menziesii and var. glauca. However, the kinds of terpenes cannot serve, like protein and enzymes, as gene markers in the study of allelic variation and heterozygosity in Douglas-fir populations. This is due to the fact that a large number of structural genes acting collectively are required in order to direct the synthesis of the several enzymes necessary for the production of a particular terpenoid compound. Terpenes are, therefore, polygenic products in that they reflect the net result of the activity of multiple cistrons, but not single genic alleles.

It has been shown by a number of geneticists and molecular biologists that the technique of electrophoretic separation of enzymes, combined with biochemical staining, can provide information regarding the distribution of allelic variation in natural populations. This is because the electrophoretic variation in the banding pattern of enzymes can be directly equated to the variation on the gene structure or codon sequence. Variant enzymes or isoenzymes are produced by mutated genes. The isoenzymes were defined as the multimolecular forms of an enzyme, sharing a catalytic activity. Any mutation in a structural gene should result in the substitution, deletion or addition of at least one amino acid in the polypeptide chain produced by the genes. Such alterations in amino acid composition will result in changing the net electrostatic charge on the polypeptide chain, and finally cause the change of net charge on the enzyme molecule of which a polypeptide is the major constituent.

Since proteins and enzymes are composed of polypeptides synthesized by the action of one or more structural genes, it can be expected that the electrophoretic variation of enzymes and proteins always follows Mendelian segregation in ideal population. When one investigates a large number of enzymes by observing their electrophoretic mobility from single individuals, it should be possible to detect the allelic variability at single loci and to quantify the extent of heterozygosity in natural populations. In addition, electrophoretic

technique allows a sampling of genetic loci in a manner which at least approaches randomness.

It has been presumed that multimolecular forms of enzymes could strengthen the capacity of an individual to survive over a wide range of environmental conditions. If this were the case, it would be possible that the adaptability of individual trees may be associated with the occurrence and maintenance of isoenzyme variability. Some evidence which shows the adaptive nature of isoenzyme polymorphism in Avena, Triticum and Aegilops (Sing and Brewer, 1969, 1971; Marshall and Allard, 1970) has stimulated the interest in studying the relationship between the isoenzyme allelic variation and the adaptability of Douglas-fir to its natural habitats.

This study was initiated to reveal the existence and patterns of polymorphism in three enzymes, to assess the extent of genetic differentiation among Douglas-fir provenances in terms of allelic frequency changes, and to elucidate the probable effects of either natural selection or genetic drift on population variability at enzymic level.

II. LITERATURE REVIEW

Evidence for Polymorphic Variation in the Gene Pool of Douglas-fir

As noted in the previous chapter, the polymorphism or individual variation in Douglas-fir populations has long been documented. In this chapter, more details on this aspect will be reviewed and discussed in order to confirm the existence of polymorphic variation in this species.

Several factors are evident in promoting the formation of genetically heterogeneous gene pools of Douglas-fir, which, in turn, further evolve polymorphism and other advanced levels of variation in the natural stands. The first of them is the wide ranging distribution of the species over a variety of heterogeneous environments. Douglas-fir grows extensively through western North America from central British Columbia at latitude 55° N. south to middle Mexico at 19° N. The altitudinal distribution ranges from sea level to about 2624 feet in Vancouver Island, and from 7872 to 9512 feet in the southern Rocky Mountains (Fowells, 1965). In this huge area, not only are there found geographic gradients in respect to temperature, day length and precipitation, but rapid climatic changes in local niches often occur as well, such as the area from Seqium to Forks in Washington (a transect of a few miles, in which the precipitation changes from

20 inches per year to 100 inches per year); the mesic coastal fog belt to the adjacent xeric uplands in California; and significant difference in summer drought between southern slopes and northern slopes in southern Oregon. As regarding the realities of these circumstances, it has become clear that the occurrence and maintenance of sympatric divergence or polymorphism in terms of phenological or biochemical characters in natural populations of Douglas-fir are the usual phenomena rather than the exception, according to Antonovics' viewpoint (1971). Rehfeldt and Lester (1969) also suggested that for species occupying environments that vary widely in space and/or time either developmental homeostasis (individual buffering) or polymorphism (populational buffering) can be expected. Further, Levins (1963) postulated that ecological polymorphism is likely to be the optimal form of genetic structure where environmental extremes are lethal for different groups of genotypes. It seems to be applicable to Douglas-fir since most trees of non-native sources, except the local ones, died at Butte Falls plantations several years after outplanting because of drought (Ching, personal communication).

The second factor is the conservative genetic system of Douglas-fir. Douglas-fir is one of the forest tree species which show an extremely conservative genetic system leading to the formation of cosmopolitan species adapted to very different niches in various parts of the distribution range (Stern, 1970). This indicates that

developmental homeostasis may be the property of most individual trees and, hence, high individual levels of heterozygosity exist in every population.

The third one is the system of wind-pollination and outbreeding. Like most conifers, Douglas-fir is a wind-pollinated tree. As a result, its neighborhood size is relatively large. Although Douglas-fir is a monoecious species, it is usually considered as an obligate outcrosser due to the differential phenological development of pollen-cone and seed-cone buds on the same tree. Therefore, a high proportion of heterozygotes exists in natural stands of Douglas-fir.

Bursting dates of male and female buds do often overlap somewhat, so that self-fertilization is not entirely excluded and, in some instances, it is a frequent occurrence (Orr-Ewing, 1957). However, most seeds are unable to develop to maturity following self-fertilization, probably due to certain gene systems which control the slow growth or collapse of the developing embryo. Other studies indicated that weak deviant germinants often occur from self-pollinated seeds although in various amounts, indicating that such gene systems may have embryonic lethal factors that are carried in the parents as heterozygous recessives, and the lethal effect comes to function as the recessive homozygote is produced (Sorensen, 1967).

Ford (1964) suggested that the occurrence of recessive lethals and semilethals can also contribute to the unequal viability of the three

genotypes, favoring the heterozygotes. Such recessives are a common type of mutants, although individually rare because they are usually eliminated by selection. However, heterozygotes for the unit controlling a polymorphism must be relatively frequent, and in them disadvantageous recessives which are harmful only to the corresponding homozygote can accumulate. Thus it seems likely that polymorphic heterozygotes are favored by selective pressures and gain a relatively heterozygous advantage over either homozygotes in natural populations of Douglas-fir.

As early as 1937, Larsen pointed out the individual differences in a stand of Douglas-fir (Sziklai, 1967). He stated that one has to travel very widely throughout the natural range of Douglas-fir in order to get an impression of differences in geographical type, but standing in one place one can, without moving a foot, see many individuals differing widely in their structure; everywhere one is bound to be impressed by the great individual variation of this tree species. The genetic diversity within a single seedlot in terms of height growth, as reported by Silen (1964) also indicates the existence of individual variability within a restricted source of gene pool of Douglas-fir.

Stern (1968) took two illustrative examples of polymorphism existing in Douglas-fir and suggested that the polymorphic phenomena were controlled by supergenes. One is the polymorphism of strobilus

color ranging from green through yellowish green, pink-red to deep red, based on the biochemical study of Ching, Aft and Highley (1965). He further pointed out the existence of a relatively simple polymorphism of flavonoid compounds in Douglas-fir strobili from the same report. The other example is the polymorphism of needle color represented by the green, grey and blue forms of Douglas-fir which had once been considered as separate subspecies. However, Stern (1968) postulated that the change of needle color in this case really represents an instance of polymorphism caused by genetic factors, which have different selective values.

In the findings mentioned above, it is apparent that there is significant polymorphic variation of morphological, phenological, physiological and biochemical characters existing in natural populations of Douglas-fir, indicating a fairly high degree of heterozygosity in the germ plasm of such a species.

The Application of Isoenzyme Variation in Forest Genetic and Genetically-oriented Physiological Researches

Isoenzyme Methodology Applicable to Forest Trees

Isoenzyme techniques have been successfully used in various biological studies in the last decade, including clinical studies of

serum proteins, genetics and physiology of tissue culture and its differentiation and development, biosystematic investigations, population genetics and evolution. The animal and plant organisms which were extensively subjected to isoenzyme studies are human beings, fruit flies, mice, ants, frogs, fishes, maizes, wheats, barleys, oats, cottons, tobaccos and legumes, to name a few. The accomplishments of such studies have excited the interest of forest geneticists in recent years.

Stern (1968) discussed the existence of genetic polymorphism in forest trees and suggested biochemical research on such inheritable polymorphism for better understanding of the adaptive strategies of tree populations to their respective environments.

Three years later, the technique of isoenzyme electrophoresis in polyacrylamide gel was recommended by Feret and Stairs (1971a) as a promising tool for the investigation of allelic variation in forest trees. The polyacrylamide gel medium which contains many pores after polymerization and has sieving as well as supporting effects is pipetted into small glass tubes with two open ends, and then inserted in buffer solution of separated reservoirs. Isoenzymes are separated through polarized gel media by their ionic charge at a specific pH, and by molecular size and shape. However, several factors which may restrict the broad use of the technique should be considered.

They are allelic interactions, genic regulators, environmental alteration of enzyme expression and extraction artifacts.

Conkle (1972) illustrated the procedures of starch gel electrophoresis which is specifically adapted to conifers. The support of gel medium is starch, and is prepared as a slab in a glass mold of 16.5 cm by 25 cm. About 20 paper wicks each containing a crude extract of macerated plant material can be inserted across a single gel slab, while only the extract of a single individual can be pipetted into an acrylamide gel cylinder. Subsequent processes of electrophoresis, slicing and staining are similar to acrylamide gel technique.

McMullan and Ebell (1970) developed a technique for the protein extraction and separation from phenolic-protein complexes of conifer foliage by the use of 8 M urea. This refined method permits a better resolution of protein bands on polyacrylamide gels after the disc electrophoresis. By using the same technique of polyacrylamide gel electrophoresis, Hamaker and Snyder (1973) screened 53 enzymes existing in the needles of longleaf (Pinus palustris Mill.) and Sonderegger pines (P. palustris x P. taeda), and listed the appropriate formulations of electrophoretic buffers, substrates, cofactors as well as staining dye-couplers for different enzymes. Nine of the 53 enzymes, namely, peroxidase, esterase, catalase, acid phosphatase, ascorbic acid oxidase, cytochrome oxidase, phenoloxidase, polyphenoloxidase, and malate dehydrogenase, were shown to have potential in genetic studies of longleaf pine and its hybrids.

Analysis of Isoenzyme Inheritance Patterns

Using acrylamide gel disc electrophoresis, Bartels (1971a) examined the multiple esterases in diploid needles and haploid macrogametophytes from several individual trees, clonal grafts and their hybrid offspring, and cultivated seedlings from a native population of Norway spruce (Picea abies). The esterase patterns were in agreement with Hardy-Weinberg expectations in native populations; followed the Mendelian segregations in the offspring of crosses between clonal grafts; and segregated in a 1:1 ratio in the haploid megagametophytes of individual trees. These findings led to a conclusion that one locus with two alleles was involved in the inheritance of esterase patterns in Norway spruce and suggested the usefulness of these multiple esterases as genetic markers in the study of genetic structure of tree populations without long-period progeny test. In another report, the same conclusion and suggestion were given due to the similar esterase patterns of progeny and parents in Picea abies needles (Bartels, 1971b).

By using starch gel electrophoresis, Bergmann (1973a) also found significant variation on esterase and leucine aminopeptidase zymograms in the haploid endosperm of dormant seeds of Norway spruce. The esterase was under the control of three loci (A, B, C) with three codominant alleles at the first two loci and two at the third one. The leucine aminopeptidase on the other hand was controlled

by one monomorphic locus (A) and a polymorphic locus (B) with four codominant alleles. Feret (1971) also detected peroxidase and esterase isoenzymes from needle tissue of white spruce (Picea glauca [Moench] Voss) seedlings and found that both enzyme systems are under genetic control but no specific control system could be formulated.

The peroxidase phenotypes and genotypes in the S_1 and F_1 families of Siberian elm (Ulmus pumila L.) undergo the expected segregational patterns, indicating a system of one locus with three alleles operating in the peroxidase of Siberian elm leaves (Feret and Stairs, 1971a, b). But, a disturbance of segregation was frequently observed in the hybrids of U. pumila with U. rubra and U. japonica. The proposed mechanisms to account for such an abnormal phenomenon consist of crossing over between peroxidase loci, accumulation of lethal deficiencies, and dominance of some peroxidase genes (Feret, 1972).

One alcohol dehydrogenase locus (ADH) and two leucine aminopeptidase loci (LAP) were identified in related families of knobcone pine (Pinus attenuata Lemm.) by Conkle (1971a). The three polymorphic loci were found to follow the expected diploid segregational patterns by assuming two alleles per locus. One ADH locus and one LAP locus exhibited no linkage between them. Long (1972) used both the techniques of starch gel and acrylamide gel electrophoresis to

study three enzymes in dry loblolly pine (Pinus taeda L.) seed. Only two peroxidase bands can easily be scored and are assumed to be under the control of two genes.

One-year-old needles from progeny of selfings and crosses among eight mother trees of Pinus sylvestris L. were assessed for genetic variation of esterase by acrylamide gel electrophoresis, and the results revealed the presence of six independent two-allele systems among which three could be genetically verified by available crosses (Rudin and Rasmuson, 1973). In a later study Rudin et al. (1974) examined the homogeneity of three Scots pine (P. sylvestris) populations based on allele frequencies of three isozyme loci (EST-B, GOT-B and LAP-B). A significant excess of homozygotes at EST-B locus from Gårdstjärn population was found, indicating the probable effect of inbreeding. This is because trees in Gårdstjärn population have been previously selected for fast growth, trunk straightness, etc. and, hence, the heterozygosity level has probably been lowered in the gene pool.

Population Genetics and Geographic Variation

In an attempt to study the relations of electrophoretic variations of general protein and selected enzymes of seed from various geographic origins, Lewis and Cech (1969) found a high degree of uniformity in acid phosphatase, leucine aminopeptidase and peroxidase

among trees of black cherry (Prunus serotima Ehrh.) within geographic area. But the general protein and esterase patterns exhibited much more variation among trees. Work by Feret and Stairs (1971a) with Ulmus pumila L. peroxidase indicated that, of the eight electrophoretic variants, three were seed-source specific and five exhibited varying frequencies in most seed sources.

Durzan and Chalupa (1968) showed that jack pine (Pinus banksiana Lamb.) embryos contained nearly twice as much soluble protein as does the female gametophyte, and found, in both tissues, an electrophoretic difference in quantity and quality of soluble protein among the seed sources, implicating that climate at the seed source has a more or less preconditioning effect on seeds.

Hare and Switzer (1969) noted that the electrophoretic patterns of seed proteins from western sources of loblolly pine (P. taeda L.) are more similar to those of shortleaf pine (P. echinata Mill.) than are the eastern sources. Such biochemical evidence further supported the previous findings of introgression between the western source of loblolly pine and shortleaf pine based on morphological characteristics.

Esterase zymograms of needles of clonal grafts representing 16 mother trees of Scots pine (P. sylvestris L.) from different provenances exhibited considerable variation between trees (Rasmuson and Rudin, 1971). Work by Feret (1974) on peroxidase and esterase of needles and macrogametophytes plus a number of needle

morphological characters of mother trees and their half-sib offsprings in Pinus pungens indicated that significant differentiation among three small stands were observed in measured characters from parent trees but not from progenies. The author could not define the proper causal mechanisms to account for the genetic differences among stands.

Significant differences in the occurrence of 42 peroxidase isoenzyme bands and some leaf characters led Sakai, Miyazaki and Matsuura (1971) to conclude that there is distinct genetic differentiation between two natural forests of Thujopsis dolabrata, which are separated only by 10-km wide straits. They considered the possibility of using electrophoretic techniques to study genetic variation between subpopulations within a limited area owing to the fact that little was previously known about the genetic differences between adjacent populations of forest trees.

Within the same two stands of Thujopsis dolabrata, Sakai and Miyazaki (1972) further noted that trees growing within a 20 or 25 meter radius are more similar not only in isoenzyme patterns of peroxidase but also in some vegetative characters than are trees standing in remote places, and they may probably be genetically related members within a family. Therefore, the authors concluded that one can identify families in a natural forest and find the genetic nature of quantitative characters in trees with the aid of the

electrophoretic variation of peroxidase. In other words, on the basis of families detected by peroxidase variation, a new genetic parameter, designated "degree of family likeness (M)" can be estimated in some quantitative characters. This estimate may have some advantage over the heritability estimate usually used in animal breeding, due to the fact that the genetic interpretation of the variance components usually put forth by animal geneticists is not suitable for Thujopsis dolabrata forests possibly because of the occurrence of inbreeding in natural stands (Sakai et al., 1972; Sakai and Miyazaki, 1972).

In another report, Sakai and Park (1971) also detected significant variations of peroxidase isoenzymes among three subpopulations of Cryptomeria japonica, and thus, a natural forest of such a species is not genetically homogeneous, but may evolve several subpopulations which are genetically somewhat different from one another. The authors concluded that the distance of pollen dispersal is relatively small in the populations of Thujopsis dolabrata and Cryptomeria japonica based on the comparative studies of isoenzyme variation of peroxidase among individual trees.

Working with peroxidase isoenzymes, Matsuura and Sakai (1972) compared the average number of isoenzyme bands per tree and its intrapopulation variability, the number of lost or fixed bands, the incidences of each band and the disagreement count among individual trees in six populations of Saghalian fir (Abies sachalinensis [Fr.

Schm.] Mast.). The variation pattern was found to fit Vavilov's theory of distribution centers because one proposed central population maintained the highest variability and bore the largest number of isoenzyme bands in common with other populations, assuming that peroxidase variation is selectively neutral.

In a study on geographical variation of Norway spruce (Picea abies) in Sweden with regard to esterase and leucine aminopeptidase, Bergmann (1973b) observed north-south clinal variations of allele frequencies in some cases and found that genetic identity and genetic distance values imply an increasing degree of genetic differentiation along with increasing distance between provenances. The author concluded that the results obtained are not in agreement with the neutrality hypothesis. Similar results were obtained from another study on geographic variation of two esterase and two leucine aminopeptidase loci among six provenances of the Finnish populations of Picea abies (Bergmann, 1973c). However, a marked genetic divergence between closely located Finnish and Swedish provenances was also noted probably due to the isolating barriers (Bergmann, 1974).

With the aid of frequencies of peroxidase isoenzyme bands and the proper statistical methods (measure of distinctiveness [D^2] and analysis of variance), Muhs (1974) found that the component of variance "between provenances" of Douglas-fir was much higher than

"within provenances" and concluded that isoenzyme frequencies are best qualified for distinguishing between provenances.

Biosystematic Studies

With the aid of specific allele frequency changes of various isoenzymes in seed samples from provenances of Norway spruce, Bergmann (1971, 1972) showed a possibility for genetic certification of seed materials and postulated that it might be possible to differentiate even between closely related seed sources, or between provenances with similar ecological conditions if more gene markers at enzymic level can be revealed.

In an attempt to determine if the protein composition in seeds is related to the taxonomic architecture of conifers, Juo and Stotzky (1970) examined globulins and albumins from seeds of nine conifers by disc electrophoresis. They found that the similarities and differences with respect to zymograms between species of a genus and between genera were not as large as those detected in seeds of some angiosperms. Ziegenfus and Clarkson (1971) used serological techniques and polyacrylamide disc electrophoresis to compare seed soluble proteins of seven taxa of the genus Acer. The protein data obtained support the taxonomic classifications based on the morphological and genetic studies.

Bartels (1971b) reported that different clones of Scotch pine exhibited different banding patterns of esterase. Miyazaki and Sakai (1969) identified a Kumotoosi clone of Cryptomeria japonica D. Don. by examining the peroxidase zymographic as well as morphological characters.

Proteins, Isoenzymes, Pollen Physiology and Tree Physiology

Work by Hagman (1964, 1967) with soluble proteins and enzymes from pollen and female flower of certain Betula species indicated the possibility of solving the problem of crossing incompatibility, particularly when the combined technique of disc electrophoresis and serological reactions is used. This is because incompatibility reaction depends upon a highly specific interaction system between the enzyme in a pollen tube and the substrate in which it is growing. Bingham, Krugman and Estermann (1964) attempted to relate pollen protein differences and incompatibility in Pinus; however, they found only little differences in banding patterns of pollen proteins between certain pine species, and the differences may have resulted from aging effects during pollen storage.

Durzan (1966) noted that the separation of soluble proteins by disc electrophoresis may reveal the protein metabolism in conifer seed, as evidenced by Conkle's recent work (1971b) that considerable

changes of isoenzyme patterns of five enzyme systems occurred at various stages of development and early growth of knobcone pine (P. attenuata Lemm). germinants; some isoenzymes appeared only in dry seed embryo while others occurred only in roots or in cotyledons and epicotyl.

Qualitative and quantitative changes of oxidative isoenzymes in tissues of forest trees grown under different temperature or nutritional stress have also been reported. Perry (1971) found racial differences in dynamic changes of banding patterns and staining intensity of soluble proteins and polyphenol oxidase in response to the genetic differences in dormancy patterns, cold tolerance, and winter chilling requirements of red maple (Acer rubrum) races. In slash pine (Pinus elliottii var. elliottii Engelm.) shoots, significant changes of soluble proteins and mineral-containing enzymes such as polyphenol oxidase, peroxidase, and ascorbic acid oxidase, occurred on acrylamide gels under different substrate nutritional regimes. These changes would help tissue nutrient analysis to solve some diagnostic problems in tree physiology (Van Lear and Smith, 1970).

Hamlin (1974) revealed intraspecific variation, tissue specificity and seasonal variation in isoenzymes of peroxidase, acid phosphatase, and catalase in Douglas-fir. The greatest number of isoenzyme bands occurred immediately after budburst, indicating that increase of enzyme activity parallels onset of active metabolism and tissue

development. In addition, Saito (1971) found that high intensity of certain bands of soluble protein extracted from needle tissue occurs in Cryptomeria japonica clones with low rooting capacity. The exact mechanism which may account for this relationship was not ascertained.

Isoenzymes and Genetics of Disease Resistance in Forest Trees

Hare (1966) has pointed out the interrelationships among growth substances, phenolics, and oxidative enzymes in a variety of plant diseases in a number of reviews. He also found that certain isoenzyme patterns, particularly IAA oxidase and polyphenol oxidase, are associated with rust resistance in loblolly pine, and considered that these patterns may be used as gene markers in rust-resistance breeding programs (Hare, 1969). In another report, Hare and Switzer (1969) suggested introgression with shortleaf pine as a source of loblolly pine resistance on the basis of protein and morphological similarities between these two species.

III. MATERIALS AND METHODS

Cone Collection

Since the detection of geographical variation at the isoenzyme level is one of the purposes in this study, sampling was made to cover the distributional range of coastal Douglas-fir in terms of latitude and elevation.

The number of trees chosen for cone collection and the location data are shown in Table 1. Cones were collected from at least ten dominant or codominant trees in each of the seven provenances (D, E, F, G, H, I and J) in August and September of 1971 after the ovuliferous scales turned brown but were still closed. Seeds of provenance C were obtained from the commercial bulked seedlot of the 1971 crop through the courtesy of Simpson Timber Company, Albany, Oregon. Cones from provenance A were supplied through the courtesy of Dr. Josef Racz of West Germany.

The appropriate time for cone collection depends on the latitude and elevation of provenance locations. Cones from provenances A and C were harvested at the end of September to the first part of October. (Racz and Visminas, personal communications). Cones were collected from provenances F, E and D in the second half of August and the beginning of September respectively. Cones from the four Californian

Table 1. Geographic Locations of Douglas-fir Provenances in Isoenzyme Study.

Region	Provenance	Latitude (°N)		Longitude (°W)		Elevation (ft.)	Elevation Range Sampled (ft.)	Location	No. Trees Sampled
		°	'	°	'				
Vancouver Island, B. C.	A	50	15	125	43	900	800-1000	White River Valley, Big Tree Creek	13
Washington	C	48	29	121	32	1750	1500-2000	Rockport area	*
Oregon	D	45	10	122	19	3600	3400-3800	Clackamas Tree Farm, Molalla	10
Oregon	E	44	30	123	12	1900	1800-2000	McDonald Forest, Corvallis	10
Oregon	F	42	28	122	25	3000	2700-3300	Butte Falls, Medford	20
California	G	41	56	122	47	2693	1700-3800	Mt. Ashland	14
California	H	40	14	124	00	996	850-1540	Humboldt Redwoods State Park	13
California	I	40	23	123	23	3581	2400-4650	Forest Glen	13
California	J	37	08	122	11	568	100-830	Santa Cruz	14

* Bulked seedlot

provenances were collected by Griffin (1974) in August, 1971.

The range of age of the trees chosen for cone collections from Vancouver Island, Washington, and Oregon was from 17 to 30 years. However, the trees from California were not restricted to any particular age or crown class for the reasons of safety and accessibility. This procedure may partially have caused a negative selection for form, but it is reasonable to assume that this will not have affected the progeny seedling characteristics studied (Griffin, 1974).

Seed Processing

Cones were spread out to dry soon after they were transported back to Corvallis. When the cone scales fully opened, a hand extraction of the seed was performed in order to minimize the mechanical damage. Seeds were then dewinged by hand-rubbing and cleaned by using an air column. A cutting test was used to ascertain that there was at least 90% of full seeds in each seedlot. All seedlots were placed in glass jars and stored in the cold room (0^{o} to 4^{o} C) prior to use. A total of 107 seedlots or individual tree collections was obtained for this isoenzyme study.

Culture of Plant Material

Dry seeds were immersed in water for four hours and then the soaked seeds were stratified at 3^{o} C for four weeks prior to

germination. Each seedlot was germinated separately on spongerok in a plastic box which was placed in the germination chamber with a temperature of 86° F during eight-hour photoperiod and 68° F in the dark.

When the cotyledons fully extend and elongate after the seed coat was shed, the young germinants were removed from the plastic box for protein extraction. The average time of germination needed to attain such a developmental stage was two weeks. This developmental stage was selected for the subsequent analysis because of that young diploid seedlings have the full complement of enzymes at a very active stage, and hence they are sufficient in enzyme kinds and quantity for detection. This was evidenced by the facts that esterase was found to exhibit greater activity in terms of stainability at this stage than any other stages and produce clear and well-defined banding patterns; and glutamate oxaloacetate transaminase likewise showed the monomorphic zymograms among individuals at the early stages of growth, but exhibited the variable banding patterns at later stages. Leucine aminopeptidase, however, consistently showed the same strongly stained bands throughout all the developmental stages.

Extraction of Soluble Protein

In most cases, fresh plant material was used in protein extraction soon after it was removed from the germination box.

Occasionally, when not enough germinants were available for one electrophoretic run, they were wrapped in saran wrap and refrigerated overnight. The resolution patterns and staining intensity of overnight-refrigerated materials did not show any difference from those of fresh seedlings.

A single germinant was homogenized in cold 1.3 ml. Tris-HCl buffer, pH 7.4 (Table 2). The homogenate was then centrifuged at 18,000 R. P. M. for ten minutes at 0° C. Finally, supernatant containing soluble proteins was dialyzed against Tris-HCl buffer, pH 7.5 (Table 2) overnight at 0° C. The dialysis of the protein extract is necessary in order to remove soluble metabolites which may cause complex aggregates of proteins or enzymes. The dialyzed extracts showed better resolution patterns on the gels than do the original extracts.

Electrophoretic Apparatus

The disc electrophoretic apparatus was designed, with slight modifications, based on the model adopted by Davis (1964). The upper buffer reservoir contains twenty holes fitted with rubber grommets for tubular glass gel containers of 5/16 inch in outer diameter. The two electrodes were sealed in the center of the bottom of the lower buffer reservoir and the plate cap of the upper reservoir. The upper reservoir was stacked onto the lower one during the

Table 2. Formulation of Extraction, Dialysis and Reservoir Buffers.

Extraction Buffer	
TRIS	0.6057 gm. (0.05 M)
EDTA	0.10026 gm. (3 mM)
Mercaptoethanol	0.078 ml. (10 mM)
Add glass-distilled water to 50 ml. Mix and titrate to pH 7.4 with 1 N HCl. Add glass-distilled water to 100 ml.	
Dialysis Buffer	
TRIS	3.0285 gm. (0.05 M)
Mercaptoethanol	0.23 ml. (1.0 mM)
Add glass-distilled water to 100 ml. Mix and titrate to pH 7.5 with 1 N HCl. Add glass-distilled water to 500 ml.	
Reservoir Buffer*	
TRIS	6.0 gm. (0.05 M)
Glycine	28.8 gm. (0.38 M)
Add glass-distilled water to 500 ml. Mix and titrate to pH 8.3 with 1 N HCl. Add glass-distilled water to 1000 ml.	

* The buffer is diluted to one tenth strength prior to use.

electrophoresis, and the tubular glass gel containers bridged the two buffer reservoirs. A direct current power supply of 250-milliampere with 1000 volt capacity made by Buchler Instruments Co. N. J. was used as the electrophoretic power source.

Gel Formulation and Electrophoresis

The gel which the protein sample first encounters is known as spacer gel or large pole gel. The electrophoretic concentration of the protein sample ions is initiated and then completed in the spacer gel. The second gel which the sample encounters is defined as the separation gel or small pole gel. The electrophoretic fractionation of isoenzymes takes place in the separation gel.

The procedure for the preparation of stock solutions and gel formulations is essentially that of Davis (1964) with some modifications as shown in Table 3. An important modification is the addition of 0.5% electrostarch to the separation gel, which will improve the resolution of LAP and GOT isoenzymes. It was found that the electrophoretic reproducibility was significantly enhanced with these modifications.

The glass tubes were cleaned, using double distilled water and then a mixed solution of 1 part Kodak Photo-Flo : 200 parts water. The tubes were drained and allowed to dry prior to use. Gels were prepared by first pipetting the mixture of A, C and G stock solutions

Table 3. Stock Solutions, and Formulations of Acrylamide and Starch Gels.

Separation Gel

A.	1 N HCl	48 ml.	C.	Acrylamide	30.0 gm.
	TRIS	36.6 gm.		Electrostarch*1	2.0 gm.
	TEMED	0.23 ml.		BIS	0.8 gm.
	Water to	100 ml.		Water to	100 ml.
	pH 6.7				
G.	*2 Ammonium persulfate	0.14 gm.			
	Water to	100 ml.			

Formulation: Mix 1 part A, 1 part C, and 2 parts G.

*1. The electrostarch is added only when gel medium is prepared for the separation of isoenzymes of leucine aminopeptidase and glutamate oxaloacetate transaminase.

*2. Stock solution G should be used within 7 days of preparation.

Spacer Gel

B.	1 N HCl	48 ml.	D.	Acrylamide	10.0 gm.
	TRIS	5.98 gm.		BIS	2.5 gm.
	TEMED	0.46 ml.		Water to	100 ml.
	Water to	100 ml.			
	pH 6.7				
E.	Riboflavin	4.0 mg.	F.	Sucrose	20.0 gm.
	Water to	100 ml.		Water to	100 ml.

Formulation: Mix 2 parts B, 3 parts D, 1 part E, and 4 parts F.

into the glass tubes with rubber serum caps at the bottom. A 0.5-cm. layer of distilled water was placed gently on the top of separation gel to prevent oxygen-inhibition of polymerization and to level the gel top. Polymerization of acrylamide was accomplished with the aid of fluorescent light for 30 minutes or longer. Following polymerization, the water was poured off, and the spacer-gel solution was layered on the top of the polymerized separation gel. Again, a layer of water was placed onto the spacer gel to prevent oxygen-inhibition of polymerization.

Electrophoresis was conducted in the cold room (0^{o} to 4^{o} C) using 0.005 M Tris-Glycine (pH 8.3) as reservoir buffer solution (Table 2). The protein extract from a single seedling was layered on the top of a spacer gel. 2.4 ml. of 0.001% bromophenol blue was added to the upper reservoir buffer before turning on the power supply. Each gel was applied with 5 mA. current which flows from the upper buffer (negative electrode) through gel medium down to the lower buffer (positive electrode).

When the bromophenol blue marker had migrated to the position about 0.6 cm. from bottom end of the gels, the power was turned off. The gels were relased from the glass tubes by a jet of water. The excess gel below the marker was trimmed off by a razor blade prior to staining for enzymes. This is because the bromophenol blue band often diffused or faded after staining and storage for several days

that cause difficulty in measuring the migration distance of the marker front. The acrylamide-starch mixed gels were sliced to longitudinal halves and then stained one each for leucine aminopeptidase and glutamate oxaloacetate transaminase.

Enzyme Staining, Fixation and Gel Storage

The choice of enzymes for study was largely based on the stainability and sharpness for visual observation in the gel. Therefore, a number of enzymes were screened and three were found to have detectable and repeatable banding patterns, namely, leucine aminopeptidase (EC 3. 4. 1. 1) (LAP), esterase (EC 3. 1. 1. 2) (EST), and glutamate oxaloacetate transaminase (EC 2. 6. 1. 1) (GOT).

Leucine Aminopeptidase (EC 3. 4. 1. 1)

Leucine aminopeptidase activity was located by using the method suggested by Brewbaker et al., (1968). Gels were placed in 0.5 x 4 inch test tubes containing a solution consisting of 40 mg. L-leucyl-naphthylamide hydrochloride (substrate) and 50 mg. Black K salt (diazo dye-coupler, K and K Laboratory, Plainview, N. Y.) in 100 ml. 0.2 M Tris-Maleate buffer (pH 3.6) and 0.15 M Sodium hydroxide buffer (pH 12.2). After 30 minute-incubation at 37° C, the isoenzymes are visualized as purple bands in the gel. The gels

were rinsed with distilled water and then fixed in the solution of 5 parts methanol : 1 part acetic acid : 5 parts water overnight. Finally, the gels were stored in 7% acetic acid.

Esterase (EC 3. 1. 1. 2)

Esterase was stained using the technique of Brewbaker et al., (1968). Gels were placed in 0.5 x 4 inch test tubes containing an incubation solution of 30 mg. α-naphthyl acetate (substrate) in 70% (v/v) acetone and 100 mg. Fast Blue RR salt (dye-coupler, Sigma) in 100 ml. 0.2 M Na_2HPO_4 (pH 8.8) and NaH_2PO_4 (pH 6.6) buffers. Staining is completed in 45 minutes at 37° C and is visualized as brownish-black bands. The gels were rinsed with distilled water. Semi-permanent gel was obtained by fixing the gel overnight in the solution of 6 parts methanol : 1 part acetic acid : 4 parts water, and then replacing the fixing solution with 7% acetic acid.

Glutamate Oxaloacetate Transaminase (EC 2. 6. 1. 1)

The staining method for glutamate oxaloacetate transaminase is essentially the one adopted by Gottlieb (1973a). Gels were placed in 0.5 x 4 inch test tubes. The tubes were then filled with a solution of 200 mg. α-aspartic acid and 200 mg. α-ketoglutaric acid (substrates), 100 mg. pyridoxal-5'-phosphate (cofactor), and 300 mg. Fast Blue BB salt (dye-coupler) in 100 ml. 0.1 M Tris-HCl buffer

(pH 8.0). Incubation was performed at 37° C for 10-15 minutes, and the enzyme activity was indicated by the greenish blue bands in the gels. The gels were rinsed with distilled water and then fixed in 7% acetic acid in the cold.

Zymogram Analysis and Recording

The migration distances of stained isoenzyme bands and bromophenol blue front from the spacer-separation gel interface were measured using an engineers' rule garduated in mm. Diagrammatical drawings of the banding patterns were simultaneously made on graphed paper with a scale of 10 millimeters to the centimeter.

The gels were then photographed using Kodachrome positive film.

The Rf values were calculated for isoenzyme identification. This was done by calculating the ratio of migration distance of enzyme bands to the distance of front migration. In order to test the reliability of Rf values for cataloging the enzyme bands, two replicated samples of a single protein extract were subjected to different electrophoretic runs. The Rf values for such replicated samples of single extracts were repeatable to within 1.5% Rf units. The reproducibility obtained corresponded well with the result of Hall et al., (1969). In addition, the homogenates of two seedlings with dissimilar banding patterns were mixed and subjected to an electrophoretic run.

It was found that the characteristic bands of different seedlings appeared in the gels and showed the constancy of band positions.

Statistical Treatments

Chi-square goodness-of-fit tests were employed to study the fit of the observed phenotypic frequencies to those predicted by the Hardy-Weinberg Law. Differences in allelic frequencies and proportions of estimated heterozygotes among provenances were studied by using the Chi-square test of independence and the test for homogeneity of variance for binomial distributions respectively (Snedecor and Cochran, 1967). Linear regression analyses were used to express the relationship of levels of heterozygosity and latitude or altitude of different provenances. Genetic variability patterns in terms of allelic frequencies of isoenzymes and environmental variability patterns were examined and analyzed by the methods of principal components (Kendall, 1957; Johnson et al., 1969; Press, 1972). The genetic identity and genetic distance between provenances were measured using the method developed by Nei (1972).

IV. RESULTS

Some general description of the properties of leucine aminopeptidase, esterase, and glutamate oxaloacetate transaminase are as follows:

Leucine aminopeptidase hydrolyzes peptide linkage at amino end of a peptide and, hence, results in the production of free amino acids from reserve proteins during seed germination for embryo and seedling growth. It has a wide range of substrates and overlapping reactions. In spite of vagueness in substrate specificity, LAP is a very useful enzyme in the study of electrophoretic variability. It is because the isoenzymes' size and change are rather specific, so it usually produces sharp bands in various buffers with little satellite zones. Furthermore, it is highly polymorphic (Manwell and Baker, 1970).

Esterase distributes widely in plant tissues. It hydrolyzes a variety of ester linkages. Lipids, a major reserve food in coniferous seeds and other plant cells, may be cleft by this enzyme during germination. It also is a nonspecific enzyme because it may hydrolyze, in vitro, a number of different esters due to an indiscriminate bond-breaking mechanism. The activity of such a nonspecific enzyme is exhibited by synthetic substrates such as α-naphthyl acetate, and shows overlapping reactions. Despite the multiplicity of esterase activity, the sharp resolution of isoenzyme banding on the gels and

the ease with which it can be detected have rendered it as a favored biochemical marker in the study of isoenzyme variation. For instance, Kahler and Allard (1970) concluded, from their study, that esterase isoenzymes appear to be useful research tools for studies of the extent of allelic variability within different local populations of barley, and of the geographical distribution of such variability.

Glutamate oxaloacetate transaminase catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate in germinating pine seeds (Mirov and Stanley, 1959). Thus, it may play an important role in balancing the needs of different kinds of amino acids at the expense of TCA intermediates during germination and differentiation of plant tissues. Although GOT shows a moderate degree of multiplicity in substrate specificity, its phylogenetic nature was considered to be appropriate for biosystematic study of plant species (Gottlieb, 1973a, b).

There may often be a plausible argument that enzymes possessing nonspecificities and overlapping activities, such as LAP, EST, and GOT presented here, cannot serve as valid gene markers in any genetic study because the isoenzyme bands in the gels may probably represent the alleles of unrelated or different gene loci. Nevertheless, Shaw (1965) argued that the distinction between specific and nonspecific enzymes is an operational one, and not entirely valid or meaningful because some of the esterases display activity toward a number of

synthetic substrates *in vitro*, whereas *in vivo* they may have a high degree of specificity. Brewer (1970) further postulated that if isoenzymes have overlapping activities against even a synthetic substrate, they would also have overlapping activities *in vivo* and possibly even be evolutionarily related. Therefore, the three enzyme systems chosen in this study should be appropriate for the investigation on isoenzyme variation in Douglas-fir.

For the purpose of convenience, Rf values will be expressed as percentage values rather than decimal fractions in the following discussion. That is, a fraction values of Rf = 0.21 will be expressed as Rf = 21.

A. Leucine Aminopeptidase (LAP)

Figure 1 shows the photographic zymogram of leucine aminopeptidase. A total of four isoenzyme bands was detected on the gel, namely, Rf 33, 39, 46 and 50. The migration direction of bands is from the top (cathode) down to the bottom (anode). The first band with Rf 33 is a polymorphic band (or variable band) whereas the second band of Rf 39 is monomorphic because it occurs consistently in all the individuals from all provenances examined. Shaw (1965) has noted that if electrophoretic variants show alteration in one of two bands but not in the other, then it is likely that the two bands are controlled by different genetic loci. It would appear, then, that the two different

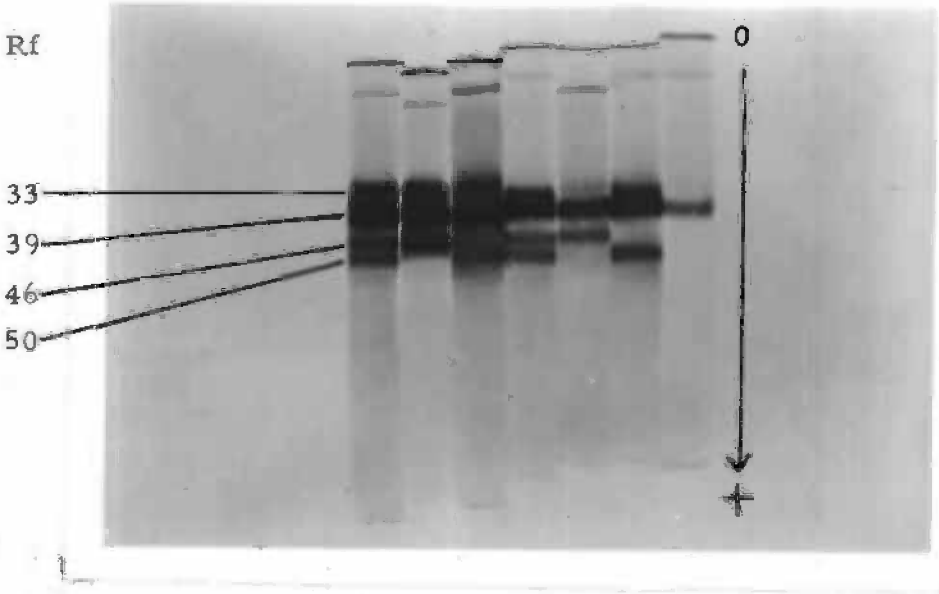


Figure 1. **Photographic zymogram of leucine amino-peptidase. The origin is indicated by 0.** The arrow shows the direction of migration toward the anode.

loci, designated as LAP-A and LAP-B (Figure 2) respectively, are responsible for the production of bands Rf 33 and 39.

The frequency of occurrence of band Rf 33 is very low in some provenances and even absent in other populations (Table 4). It is probable that a locus (LAP-A) with two alleles, one null (or silent) and one productive can account for the banding variation at Rf 33 zone. The most likely genotypes associated with observed phenotypes are as follows: those germinants exhibiting no band are homozygous for the recessive null allele; among the germinants exhibiting the single band are both heterozygotes and homozygotes for the dominant productive allele. Because it is impossible to distinguish visually between the latter two on the gels, the proposed genotypes cannot be verified by conducting a Hardy-Weinberg equilibrium analysis. Thus, the allelic variability at this locus is not sufficiently reliable for the population study, and consequently, no attempt was made to evaluate critically the isoenzyme variation at LAP-A locus.

Genetic Basis of Allelism at LAP-C Locus

The last two bands with Rf 46 and 50 were presumed to represent two codominant alleles, designated as LAP-C^{46} and LAP-C^{50} respectively, at LAP-C locus as shown in Figure 2. In addition, a null allele, designated as LAP-C^{-}, was also observed at this locus, which is responsible for the inactivation of LAP synthesis. The

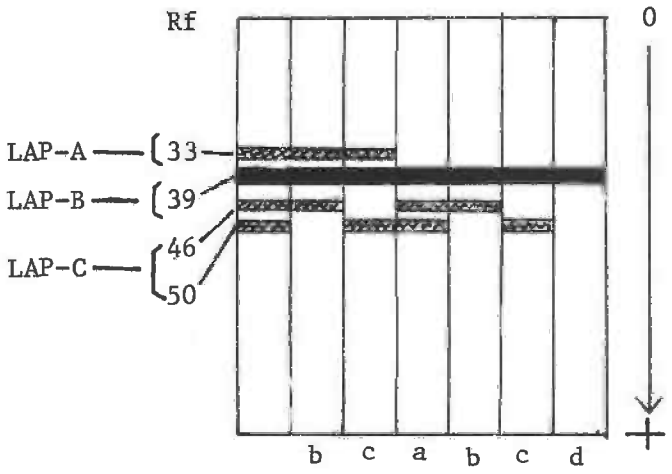


Figure 2. Diagrammatic representation of LAP zymogram. The phenotypes at LAP-C locus are indicated as: a -- (46 50), b -- (46 -), c -- (- 50), and d -- (- -).

Table 4. Frequency of Occurrence of Electrophoretic Variant Rf 33 of LAP in Douglas-fir Provenances.

	Provenance								
	A	C	D	E	F	G	H	I	J
Frequency	0.037	-	-	0.012	0.029	0.090	0.192	0,120	-

three-allele system at this locus can be rationalized on a genetic basis by postulating the genotype corresponding to each phenotype and then testing the hypothesis by comparing the observed frequencies with those predicted by a Hardy-Weinberg equilibrium in the population.

LAP-C locus gives rise to the isoenzyme banding patterns responsible for the four possible phenotypic expressions (Figure 2). When the individual germinant is heterozygous for two codominant alleles ($LAP\text{-}C^{46}/LAP\text{-}C^{50}$), both bands are present [phenotype (46 50)]. When homozygous for either codominant allele ($LAP\text{-}C^{46}/LAP\text{-}C^{46}$) or ($LAP\text{-}C^{50}/LAP\text{-}C^{50}$) and heterozygous for one productive allele as well as a null allele ($LAP\text{-}C^{46}/LAP\text{-}C^{-}$) or ($LAP\text{-}C^{50}/LAP\text{-}C^{-}$), either band 46 or 50 is present [phenotype (46 -) or (- 50)]. When homozygous recessive ($LAP\text{-}C^{-}$ $LAP\text{-}C^{-}$), the bands are absent [phenotype (- -)]. Therefore, the genotypes corresponding to the phenotypes characterized by the bands 46 and 50 are: phenotype (46 50), ($LAP\text{-}C^{46}/LAP\text{-}C^{50}$); phenotype (46 -), ($LAP\text{-}C^{46}/LAP\text{-}C^{46}$) and ($LAP\text{-}C^{46}/LAP\text{-}C^{-}$); phenotype (- 50), ($LAP\text{-}C^{50}/LAP\text{-}C^{50}$) and ($LAP\text{-}C^{50}/LAP\text{-}C^{-}$); phenotype (- -), ($LAP\text{-}C^{-}/LAP\text{-}C^{-}$). The homozygote ($LAP\text{-}C^{46}/LAP\text{-}C^{46}$) cannot be distinguished phenotypically from heterozygote ($LAP\text{-}C^{46}/LAP\text{-}C^{-}$) from known data because of the existence of the recessive null allele, $LAP\text{-}C^{-}$. This is also true between ($LAP\text{-}C^{50}/LAP\text{-}C^{50}$) and ($LAP\text{-}C^{50}/LAP\text{-}C^{-}$). In these circumstances, only phenotypes (46 50) and (- -) can be read and

interpreted as distinctive genotypes.

Using the method described by Falconer (1960), the frequencies of alleles were determined from the observed numbers of phenotypes under the Hardy-Weinberg assumption. Since it is not possible to identify the silent allele (LAP-C^-) in the heterozygous state, its frequency must be estimated from the number of homozygote (LAP-C^-/LAP-C^-) observed, i.e. frequency of LAP-C^- equals square root of the observed frequencies of LAP-C^-/LAP-C^-. The frequency of LAP-C^{46} is the difference of one and the square root of the sum of (- -) and (- 50) observed frequencies. The frequency of LAP-C^{50} equals one minus the square root of the sum of (- -) and (46 -) frequencies. Although in most cases the three estimated allele frequencies do not add up exactly to unity, they are usually very close to it for large samples. In these circumstances, it is desirable to adjust the three estimated allele frequencies so that they add up to one using a simple but ingenious device described by Li (1955):

$p' = p(1 + 1/2\ d)$, $q' = q(1 + 1/2\ d)$, $r' = (r + 1/2\ d)(1 + 1/2\ d)$.

Where p, q and r are original frequencies of LAP-C^{46}, LAP-C^{50} and LAP-C^- respectively; p', q' and r' are the adjusted ones; and $d = 1 - (p + q + r)$. Adjustment was made once in most cases, but in few cases, one more repeat was needed, using the new deviation $d' = 1 - (p' + q' + r')$.

The same methods were also employed to calculate and adjust the allele frequencies at EST and GOT loci.

Using allele frequencies given in Table 5, the expected numbers of various phenotypes or genotypes were calculated and compared to those observed in nine provenances. As shown in Table 6, agreement was fairly good except that of provenance J from Santa Cruz, California, which has highly significant Chi-square value, mainly resulting from the deficiency of observed heterozygote ($LAP\text{-}C^{46}/LAP\text{-}C^{50}$). This might have been expected since provenance J is more or less an isolated population. The general good correspondence between the expected and observed results indicates that the assumption of three-allele system is correct at LAP-C locus.

Geographic Variation of Allele Frequencies and Genic Heterozygosity

Of the three alleles that have been previously described at LAP-C locus, both $LAP\text{-}C^{46}$ and $LAP\text{-}C^{50}$ are in moderate to moderately high frequency. $LAP\text{-}C^{46}$, however, predominates in five out of the nine provenances examined (provenance A, C, D, G, I), which are located either in northern latitudes or at high elevations. $LAP\text{-}C^{-}$, the null allele, occurs as a rare allele (below 0.10) in provenances mainly from Vancouver Island of Canada, Washington, Oregon and Forest Glen of California (provenance I), and as a minor allele (above

Table 5. Allele Frequencies and Estimated Heterozygosity (H) at LAP-C, EST-B, EST-C and GOT-B Loci in Douglas-fir Provenances.

	LAP-C				EST-B				EST-C				GOT-B			
	46	50	--	H (%)	28	34	--	H (%)	52	64	--	H (%)	27	30	--	H (%)
A	.618	.322	.060	51.1	.456	.272	.272	64.4	.320	.374	.306	66.4	.367	.278	.355	66.2
C	.495	.461	.044	54.0	.581	.196	.223	57.5	.444	.412	.144	61.2	.275	.365	.360	66.1
D	.475	.466	.059	55.4	.618	.181	.201	54.5	.417	.434	.149	61.6	.445	.367	.188	63.2
E	.389	.542	.069	55.0	.535	.129	.336	58.4	.382	.394	.224	64.9	.383	.421	.196	63.8
F	.405	.515	.080	56.5	.707	.140	.153	45.7	.414	.372	.214	64.5	.329	.488	.183	62.0
G	.526	.372	.102	57.4	.519	.316	.165	60.4	.457	.416	.127	60.2	.325	.498	.177	61.5
H	.399	.497	.104	58.3	.588	.206	.206	57.0	.415	.450	.135	60.7	.421	.412	.167	62.5
I	.630	.292	.078	51.1	.821	.124	.055	30.7	.466	.449	.085	57.4	.446	.412	.142	61.1
J	.289	.499	.212	62.3	.490	.245	.265	63.0	.381	.324	.295	66.3	.227	.414	.359	64.8
X^2		56.8		8.0[a]		67.4		78.4		37.5		7.5[a]		48.6		2.4[a]
P (16 d. f.)		<.005				<.005				<.005				<.005		
P (8 d. f.)				.45				<.005				.49				.96

[a] not significant

Table 6. Observed and Expected Phenotypes at LAP-C Locus in Douglas-fir Provenances.

Provenance		Phenotype				X^2	P (2 d.f.)
		46 -	46 50	- 50	- -		
A	OBS	47	47	13	1	1.79	0.43
	EXP	42.98	49.27	15.36	0.39		
C	OBS	27	36	24	0	0.84	0.67
	EXP	25.06	39.73	22.04	0.17		
D	OBS	30	37	29	0	1.68	0.45
	EXP	27.08	42.51	26.07	0.33		
E	OBS	21	31	35	0	2.13	0.37
	EXP	17.86	36.71	32.02	0.41		
F	OBS	28	35	40	0	3.45	0.19
	EXP	23.61	42.94	35.79	0.66		
G	OBS	34	23	21	0	4.31	0.12
	EXP	29.98	30.52	16.69	0.81		
H	OBS	28	28	38	0	5.32	0.07
	EXP	22.73	37.31	32.95	1.01		
I	OBS	53	30	17	0	3.29	0.21
	EXP	49.55	36.79	13.05	0.60		
J	OBS	28	17	52	1	11.17	<0.005
	EXP	20.28	28.28	45.09	4.40		

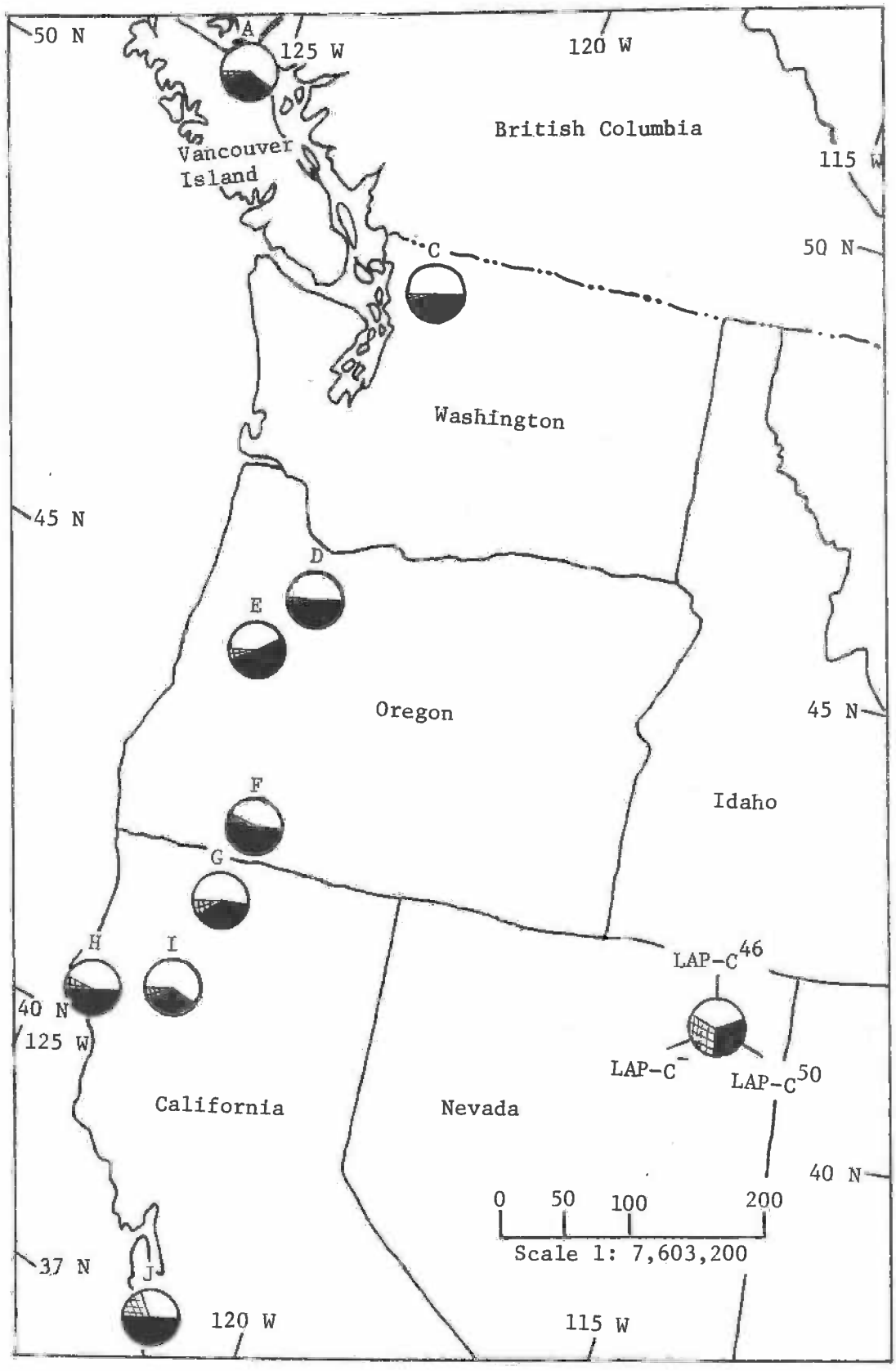
0.10) in three provenances (G, H, J) from northern California. The overall frequency of LAP-C^{46} was slightly larger than that of LAP-C^{50} (Table 5 and Figure 3).

It is interesting to mention that the drastic changes of allele frequencies at LAP-C locus were found among provenances in northern California. The frequency of LAP-C^{46} varies from 0.63 in provenance I to 0.29 in provenance J, while LAP-C^{50} and LAP-C^{-} vary in opposite direction, ranging from 0.29 to 0.5 and 0.08 to 0.21 respectively. The changes were particularly obvious between the two provenances (H and I) of the same latitude. The frequency of LAP-C^{46} in provenance I is much higher than in provenance H whereas those of LAP-C^{50} and LAP-C^{-} are in opposite trends.

A chi-square test of independence (Snedecor and Cochran, 1967) comparing allele frequencies among Douglas-fir provenances revealed highly significant differences for the LAP-C locus (Table 5). There are some obvious differences in the frequencies of different alleles from population to population. For instance, LAP-C^{46} shows a wider range of allele frequency changes, from 0.63 in provenance I to 0.29 in provenance J, than does LAP-C^{50}, from 0.54 in provenance E to 0.29 in provenance I.

The highly significant variation of alleles provided the incentive for investigating the existence of differences in heterozygosity among Douglas-fir populations at enzymatic level. Heterozygosity is defined

Figure 3. Geographic distribution of allele frequencies at LAP-C locus.


50 N
125 W
120 W
115 W
A
British Columbia
Vancouver Island
C
50 N
Washington
45 N
D
E
Oregon
45 N
F
Idaho
G
H
I
LAP-C46
40 N
125 W
LAP-C
LAP-C50
California
Nevada
40 N
0 50 100 200
Scale 1: 7,603,200
37 N
J
120 W
115 W

here as the percentage of heterozygotes in a population. The presence of null allele (LAP-C^-) makes it impossible to score the proportion of heterozygotes directly from the LAP zymogram on which the heterozygotes are represented only by the two-band phenotypes (46 50) in the case of the absence of null allele. Therefore, the heterozygosity can be alternatively estimated from the present data by taking the frequencies of all the alleles at a locus in a population, and calculating the expected frequencies of heterozygotes from the Hardy-Weinberg proportions for each provenance separately, i.e., $H(\%) = 2 (p'q' + p'r' + q'r')$, (for p', q' and r', see p. 45).

The heterogeneity with regard to the proportions of heterozygotes found in different Douglas-fir provenances was also tested by using the Chi-square test. As shown in Table 5, the change of heterozygosity from provenance to provenance is not statistically significant, but a more or less clinal increase from the north to the south was observed. Such clinal variation was also found from isoenzyme studies of certain rodents, fishes, and Drosophila as reveiwed by Avise and Smith (1974), and Wagner and Selander (1974). The percent of heterozygotes (H) was regressed on latitude (L), $H = 0.8082 - 0.5795\ L$ with a correlation of -0.686[1].

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[1] $P < .05$

B. Esterase (EST)

The isoenzyme bands of esterase of Douglas-fir germinants can be classified into two groups according to their migration rate (Figures 4 & 5). A "S" or slowly migrating region is present near the top of the gel and in this region three bands with Rf 21, 28, and 34 were recorded respectively. The band of Rf 21 is monomorphic and contributes no variation for the subsequent discussion. A monomorphic locus, designated as EST-A, with a single allele was suggested to be responsible for the sysnthesis of this band. The next two bands (Rf 28 and 34) represent two codominant alleles at a polymorphic locus, designated as EST-B, which will be discussed later in detail.

In the lower portion of the gel is a "F" or fast migrating region in which two isoenzyme bands with Rf 52 and 64 were demonstrated. These two bands, like bands 28 and 34, were presumed to represent two codominant alleles at another polymorphic locus, designated as EST-C.

Different staining intensities that exist among different isoenzyme bands of esterase within a single gel which represents a single individual germinant are presumed to be of genetic origin. Different alleles within a locus or between loci may cause differential levels of gene dosage in time or space, resulting in the unequal relative activities of isoenzymes in a gel. An exact mechanism accounting for

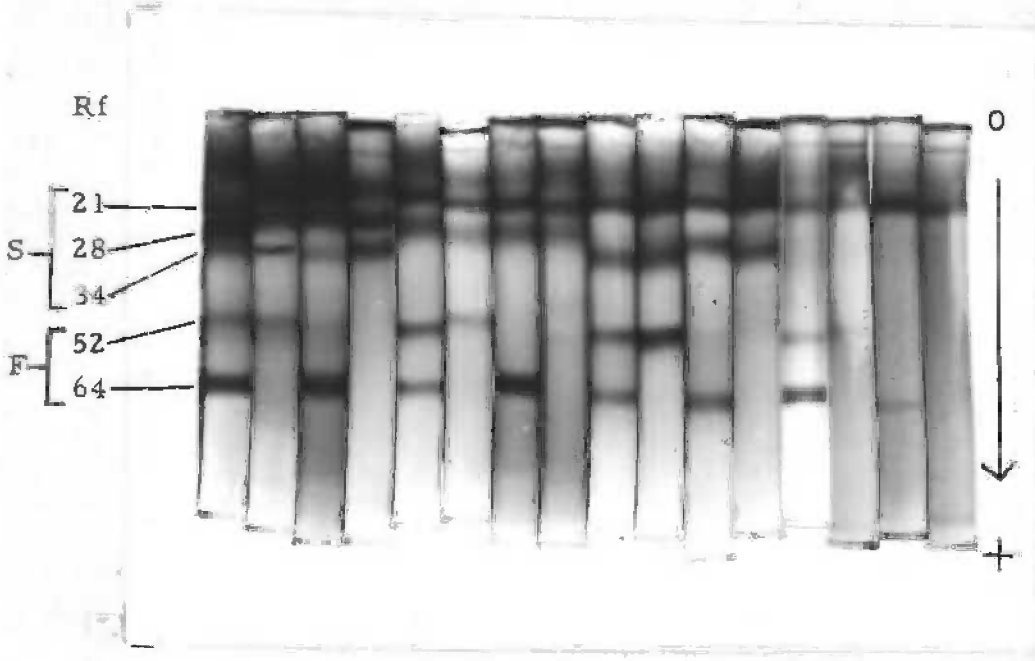


Figure 4. Photographic zymogram of esterase. The origin is indicated by 0. The arrow shows the direction of migration toward the anode.

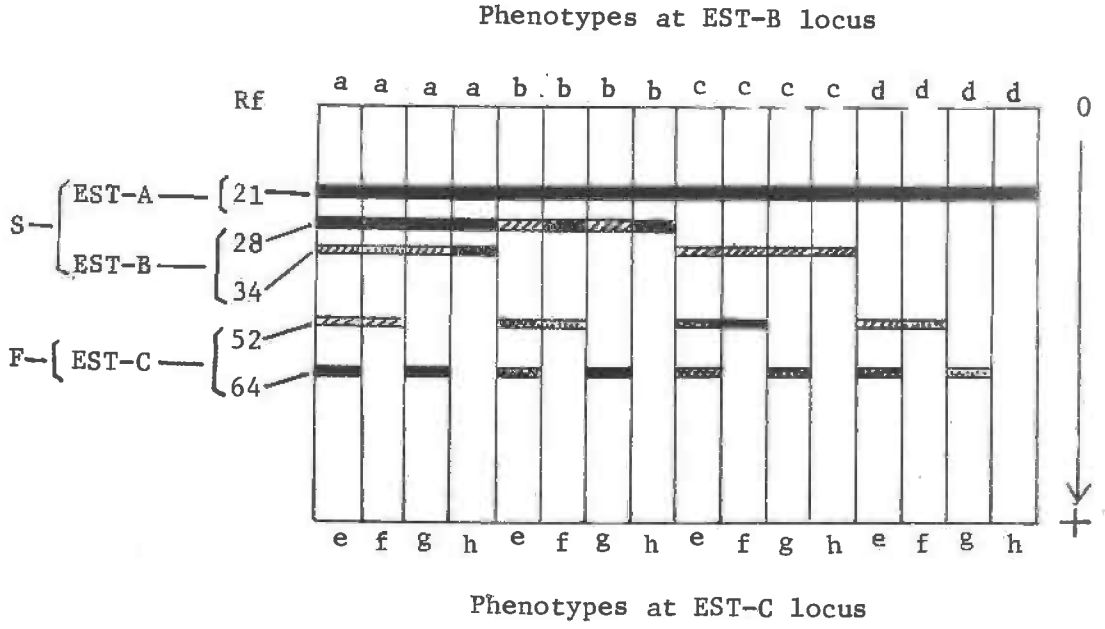


Figure 5. Diagrammatic representation of EST zymogram. The phenotypes at EST-B locus are indicated as: a -- (28 34), b -- (28 -), c -- (- 34), and d -- (- -). The phenotypes at EST-C locus are indicated as: e -- (52 64), f -- (52 -), g -- (- 64), and h -- (- -).

this hypothesis could not be formulated from the present data. The difference in staining intensity of the same band (same Rf value) was also found between gels. This is because individual young seedlings usually do not contain exactly the same amount of soluble proteins even though they are at the same developmental stage, or some unavoidable loss of soluble protein might occur during the extracting process and dialysis.

Genetic Basis of Allelism at EST-B and EST-C Loci

In addition to the two codominant alleles, designated as EST-B^{28} and EST-B^{34} respectively, a null recessive allele, designated as EST-B^{-}, was known for the polymorphic locus (EST-B) in "S" region (Figure 5). Hardy-Weinberg calculations with regard to EST-B locus were based on the assumption of a three-allele system in which double banded phenotype at Rf 28 and 34 represents the heterozygote (EST-B^{28}/EST-B^{34}); the single banded phenotype at Rf 28 represents the homozygote (EST-B^{28}/EST-B^{28}) and heterozygote (EST-B^{28}/EST-B^{-}); the single banded phenotype at Rf 34 represents the homozygote (EST-B^{34}/EST-B^{34}) and heterozygote (EST-B^{34}/EST-B^{-}); and the no band phenotype represents the homozygous condition (EST-B^{-}/EST-B^{-}).

Based on this genotypic assumption, the expected phenotypic proportions can be computed, using the allele frequencies in Table 5, and compared against the observed ones. These values, accompanied

by their Chi-square testing values, are shown in Table 7.

A wide range of Chi-square values were obtained, as in the case of leucine aminopeptidase. Also as before, the three significant Chi-square values from provenances A, C and J resulted primarily from the deficiency of observed heterozygotes (EST-B^{28}/EST-B^{34}). The second contributor to the significant Chi-square values was the excess of the observed phenotypic class of (34 -) composed of the two genotypic classes: a homozygote (EST-B^{34}/EST-B^{34}) and a heterozygote (EST-B^{34}/EST-B^{-}).

Three alleles are now known for EST-C locus in "F" region, two codominant alleles, designated as EST-C^{52} and EST-C^{64} respectively, and one null recessive, designated as EST-C^{-} (Figure 5). As before, four phenotypic expressions occurred in the "F" region of the esterase zymogram. Phenotype (52 64) represents the heterozygous genotype (EST-C^{52}/EST-C^{64}); phenotype (52 -) represents the homozygous genotype (EST-C^{52}/EST-C^{52}) and heterozygous genotype (EST-C^{52}/EST-C^{-}); phenotype (- 64) corresponds to the homozygous state (EST-C^{64}/EST-C^{64}) and heterozygous state (EST-C^{64}/EST-C^{-}); and phenotype (- -) corresponds to the homozygous state (EST-C^{-}/EST-C^{-}).

Using allele frequencies at the EST-C locus determined for nine provenances (Table 5), the expected phenotypic proportions were compared to those observed. In most of the provenances, the observed numbers showed fairly close agreement with the Hardy-Weinberg

Table 7. Observed and Expected Phenotypes at EST-B Locus in Douglas-fir Provenances.

Provenance		Phenotype				X^2	P (2 d.f.)
		28 -	28 34	- 34	- -		
A	OBS	76	12	46	2	30.89	<0.005
	EXP	61.94	33.76	30.24	10.06		
C	OBS	68	15	20	2	9.15	<0.025
	EXP	62.58	23.95	13.25	5.22		
D	OBS	63	16	14	2	3.91	0.16
	EXP	59.87	21.24	10.04	3.86		
E	OBS	86	15	16	13	1.21	0.56
	EXP	83.95	17.98	13.44	14.63		
F	OBS	85	21	9	2	0.81	0.68
	EXP	83.73	23.21	7.32	2.74		
G	OBS	41	23	21	1	2.66	0.27
	EXP	37.88	28.20	17.57	2.35		
H	OBS	64	18	18	2	5.28	0.08
	EXP	59.92	24.75	13.01	4.32		
I	OBS	81	18	5	0	2.13	0.37
	EXP	79.56	21.13	3.00	0.31		
J	OBS	80	8	42	1	39.80	<0.005
	EXP	65.45	31.43	24.89	9.23		

expectations in various phenotypic classes. These are given in Table 8 where, again, three statistically significant Chi-square values were obtained. In contrast to the results for LAP-C and EST-B loci, the statistically significant deviations from Hardy-Weinberg proportions in provenance A, D and J were primarily in the direction of heterozygote excess of the phenotypic class (52 64). The second significant contributor to the highly significant Chi-square value was the homozygote excess at phenotypic class (- -).

The genetic assumption of three-allele systems at both EST-B and EST-C loci are correct, since most provenances exhibited numerically close agreement between the observed and the expected numbers of the various phenotypic classes.

Geographic Variation of Allele Frequencies and Genic Heterozygosity

EST-B^{28} predominates over the other two alleles, EST-B^{34} and EST-B^{-} at EST-B locus in all provenances studied. It is in high frequency in high elevation provenances such as D, F and I. The frequencies of EST-B^{34} and EST-B^{-} are relatively low; ranging from 0.31 in provenance G to 0.12 in provenance I for EST-B^{34} and from 0.34 in provenance E to 0.05 in provenance I for EST-B^{-}. Both alleles show approximately equal frequencies over all the provenances except in provenance I where the silent allele, EST-B^{-}, is in

Table 8. Observed and Expected Phenotypes at EST-C Locus in Douglas-fir Provenances.

Provenance		Phenotype				X^2	P (2 d.f.)
		52 -	52 64	- 64	- -		
A	OBS	25	53	35	23	31.65	<0.005
	EXP	40.53	32.50	50.19	12.78		
C	OBS	30	45	26	4	3.76	0.17
	EXP	34.18	38.44	30.22	2.17		
D	OBS	22	44	24	5	9.34	<0.01
	EXP	28.27	34.37	30.24	2.11		
E	OBS	35	48	37	10	5.66	0.06
	EXP	41.20	39.09	43.17	6.55		
F	OBS	36	43	30	8	3.86	0.16
	EXP	40.77	35.99	34.85	5.39		
G	OBS	24	39	20	3	4.31	0.12
	EXP	27.92	32.73	23.97	1.38		
H	OBS	24	46	28	4	5.75	0.06
	EXP	29.04	38.11	32.99	1.85		
I	OBS	27	50	25	2	4.05	0.15
	EXP	30.86	43.51	28.87	0.75		
J	OBS	34	52	24	21	30.00	<0.005
		48.46	32.31	38.30	11.42		

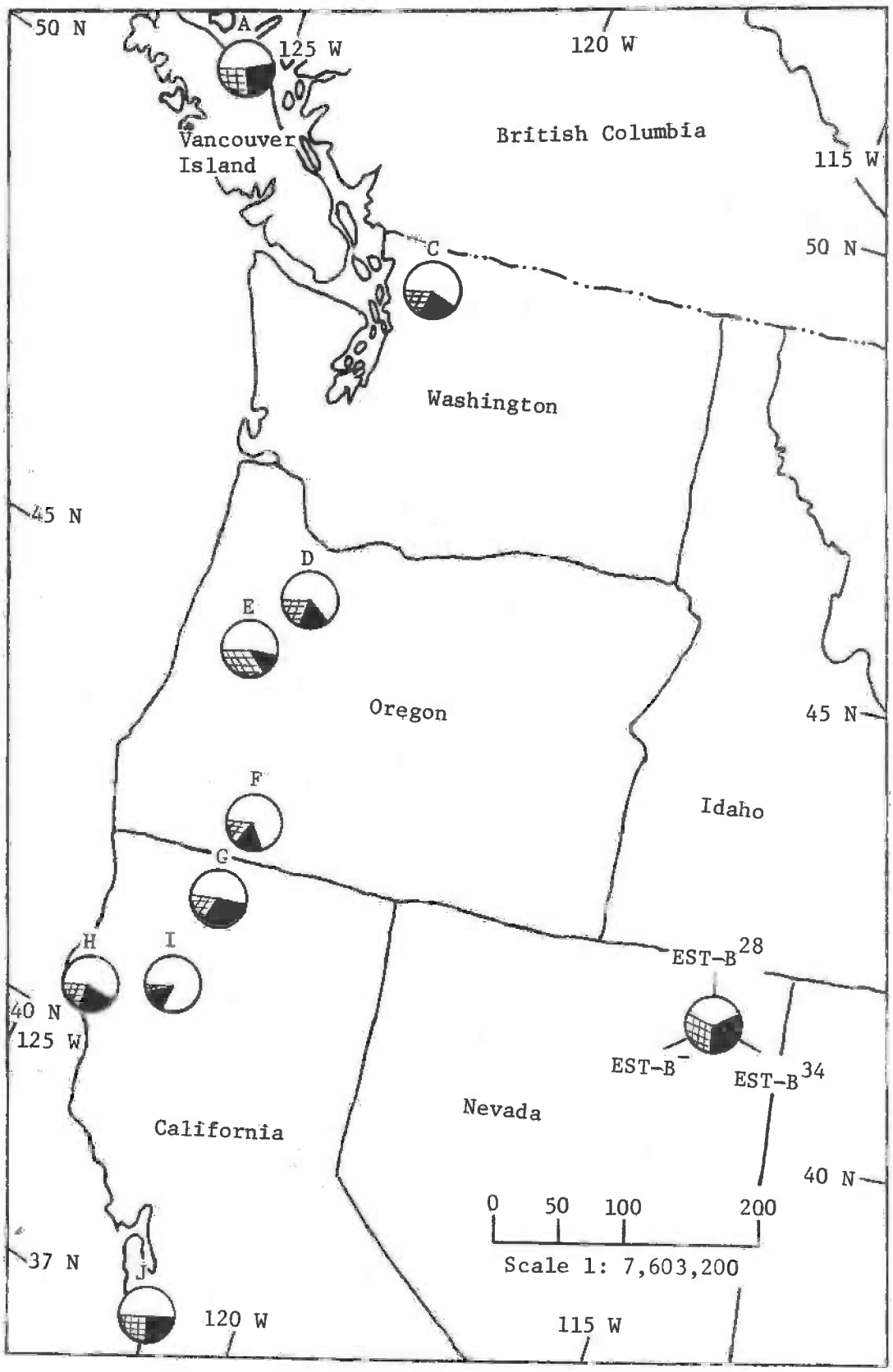
particularly low frequency. These results are shown in Table 5 and Figure 6.

As in the case of LAP-C locus, the Chi-square independence test for heterogeneity of allele frequencies at EST-B locus indicated highly significant variation among Douglas-fir provenances. Different alleles exhibited different magnitude of change from provenance to provenance.

The percentage of heterozygotes with respect to EST-B locus for every provenance was estimated using allele frequencies in Table 5. In contrast to LAP-C locus, the levels of heterozygosity at EST-B did not demonstrate a macrogeographically clinal trend, but showed a certain relationship with elevations. A linear regression analysis revealed a statistically significant ($P < .05$) negative relationship between percentage of heterozygotes and the elevation of the provenances, ($H = 0.6814 - 0.0064E$, $F = 7.11^{*}$, with 1 and 7 d.f.). The correlation between percent of heterozygotes of EST-B and elevation was -0.710. Therefore, about one-half of the inter-provenance variation in level of heterozygosity may be accounted for by linear regression on elevation. The heterogeneity Chi-square test indicated considerable differences in heterozygote proportions with regard to EST-B in natural stands of Douglas-fir.

The three alleles at EST-C locus and their frequencies are listed in Table 5. The geographic patterns of allelic variation at this

Figure 6. Geographic distribution of allele frequencies at EST-B locus.


50 N
125 W
120 W
115 W
A
Vancouver
Island
British Columbia
C
Washington
45 N
D
E
Oregon
F
Idaho
G
H
I
EST-B 28
40 N
125 W
EST-B
EST-B 34
Nevada
California
0 50 100 200
Scale 1: 7,603,200
37 N
J
120 W
115 W

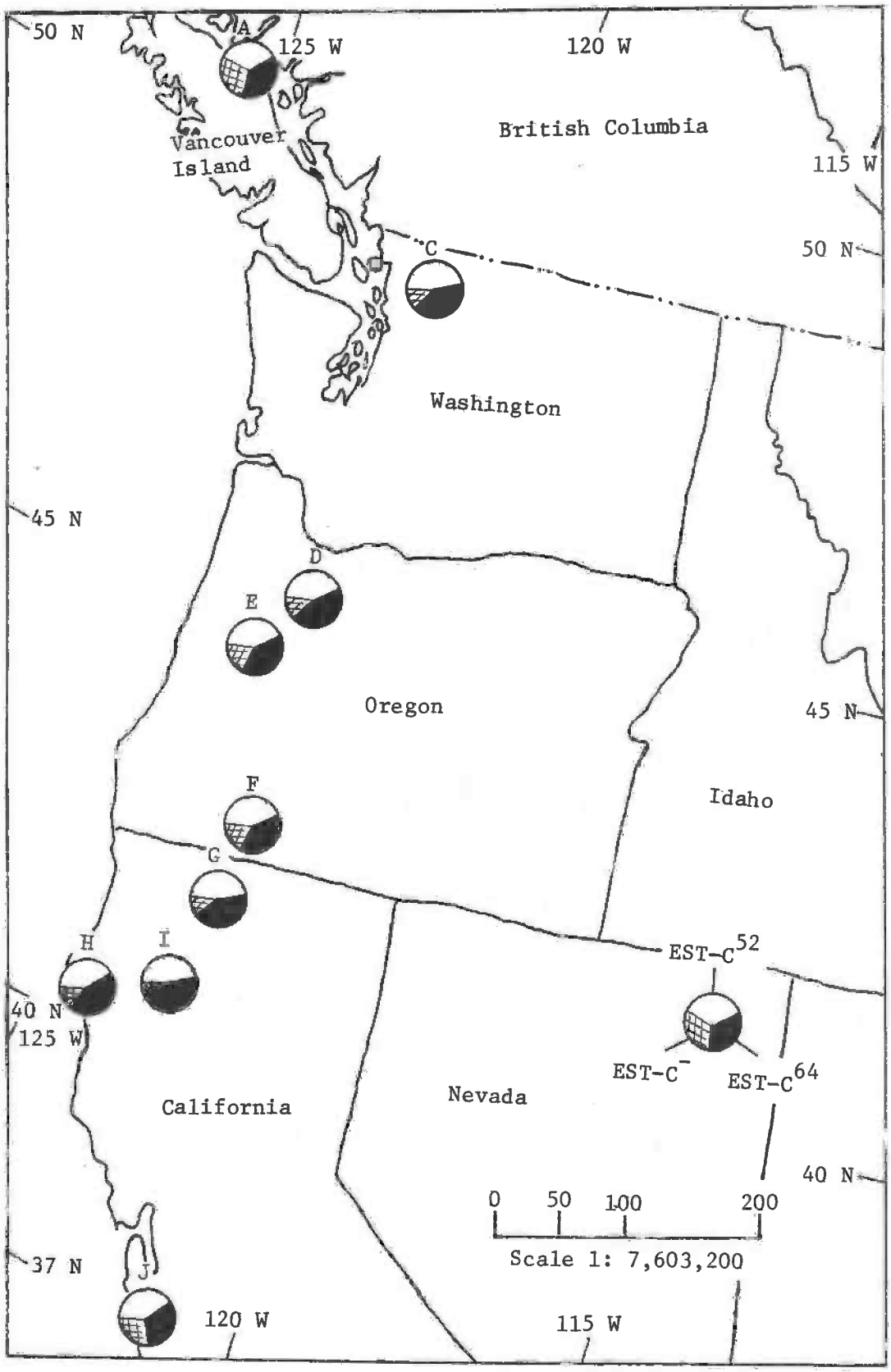
locus (Figure 7) are somewhat different from those at EST-B. In general, no particular predominant allele occurs in high frequency. Two common alleles, EST-C^{52} and EST-C^{64}, have moderate and approximately equal frequencies, and the silent allele, EST-C^{-}, is in low frequency in most provenances except the extreme northern and southern populations (A and J) in which the frequencies of three alleles are more or less equal.

It is interesting that EST-C exhibited a certain degree of geographic uniformity in genic heterozygosity, which is a common finding from the studies on a number of protein polymorphisms (largely esterases) over the distributional range of several species complex in certain rodents, marine organisms and many species of Drosophilia (Selander, Hunt and Yang, 1969; Prakash, Lewontin and Hubby, 1969; Prakash, 1969; Selander, et al., 1970; Selander, et al., 1971; Johnson and Selander, 1971; Johnson, 1971; Ayala, et al., 1972; Richmond, 1972; and Avise and Smith, 1974). However, no particular clinal changes were found as in the case of LAP-C.

C. Glutamate Oxaloacetate Transaminase (GOT)

A total of five isoenzyme bands of glutamate oxaloacetate transaminase were observed on the GOT zymogram, and their Rf values are 13, 21, 27, 30 and 36 respectively (Figures 8 & 9). The lowest band with Rf 36 is monomorphic and well defined. It represents

Figure 7. Geographic distribution of allele frequencies at EST-C locus.


50 N
125 W
120 W
115 W
A
Vancouver Island
British Columbia
C
50 N
Washington
45 N
D
E
Oregon
45 N
F
Idaho
G
H
I
EST-C52
40 N
125 W
EST-C-
EST-C64
California
Nevada
40 N
0 50 100 200
Scale 1: 7,603,200
37 N
J
120 W
115 W

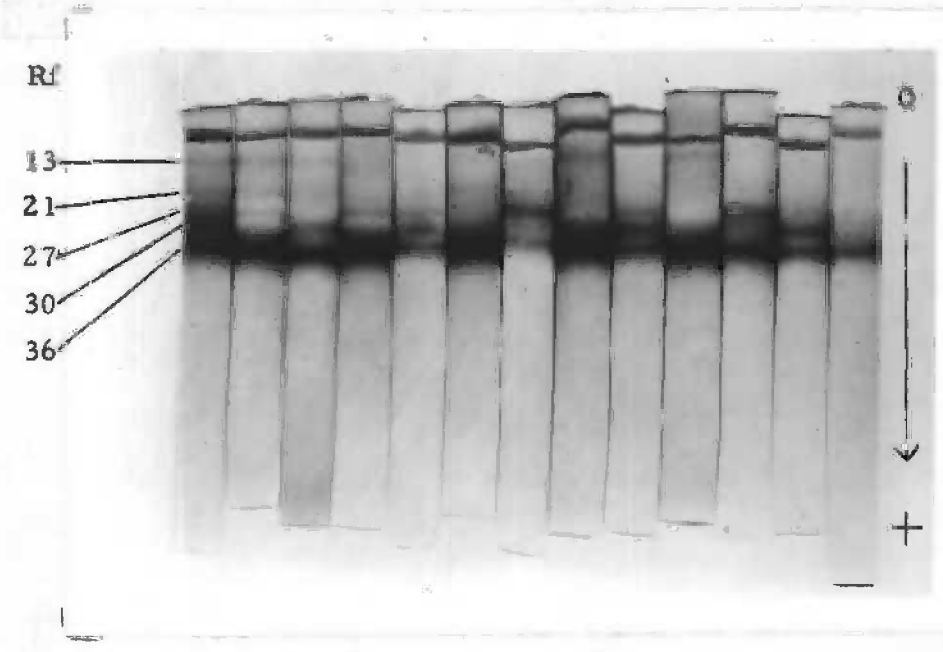


Figure 8. Photographic zymogram of glutamate oxaloacetate transaminase. The origin is indicated by 0. The arrow shows the direction of migration toward the anode.

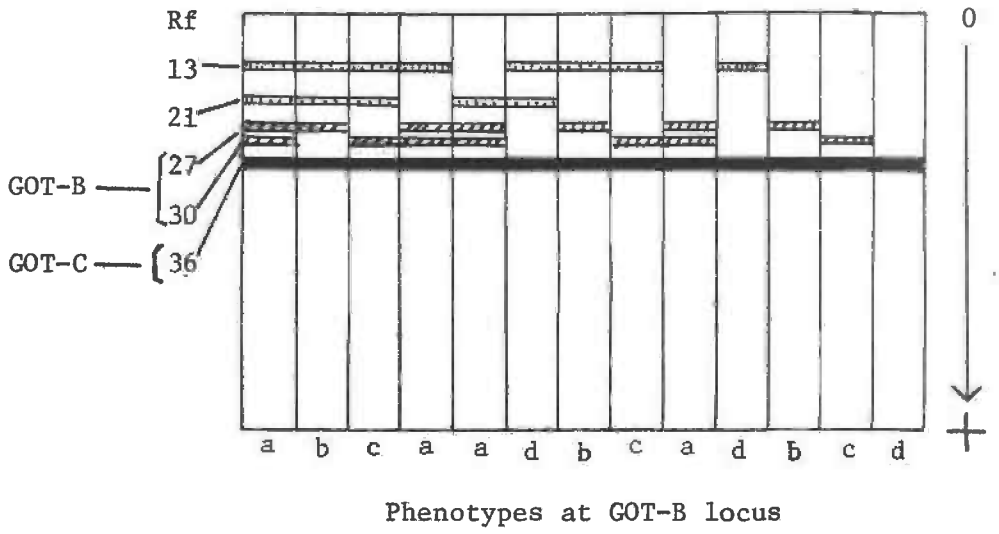


Figure 9. Diagrammatic representation of GOT zymogram. The phenotypes at GOT-B locus are indicated as: a -- (27 30), b -- (27 -), c -- (- 30), and d -- (- -).

a single allele at a monomorphic locus, designated as GOT-C. The first two bands (Rf 13 and 21) were not easily observed and scored because of reduced staining intensity and nonrepeatable occurrence. No allelic relationship could be found between Rf 13 and 21 bands, or between these two bands and others by Chi-square analysis for goodness of fit. In addition, it was realized that the efficient analysis of zymogram and the meaningful interpretation of isoenzyme variation involve the scoring of alleles at loci encoding particular enzymes rather than the mere comparison of numbers and positions of isoenzyme bands on gels (Wagner and Selander, 1974). In this connection, Rf 13 and 21 bands were excluded in the subsequent analysis of GOT variation. The two bands of Rf 27 and 30 showed clear banding patterns and were easily observed and counted. They therefore were used as one of the enzyme markers for the study of allelic variation in natural populations of Douglas-fir.

Genetic Basis of Allelism at GOT-B Locus

The bands 27 and 30 represent two codominant alleles, designated as GOT-B^{27} and GOT-B^{30} respectively at a polymorphic locus (GOT-B). In addition, a null recessive allele, designated as GOT-B^{-}, was again recorded at this locus. Four phenotypic expressions with respect to GOT-B occurred on the zymogram (Figure 9).

The four phenotypes at GOT-B consist of: (27 30), two bands at Rf 27 and 30, representing heterozygous condition ($GOT\text{-}B^{27}/GOT\text{-}B^{30}$); (27 -), a single band at Rf 27, representing homozygous as well as heterozygous conditions ($GOT\text{-}B^{27}/GOT\text{-}B^{27}$) & ($GOT\text{-}B^{27}/GOT\text{-}B^{-}$); (- 30), a single band at Rf 30, representing homozygous and heterozygous conditions ($GOT\text{-}B^{30}/GOT\text{-}B^{30}$) & ($GOT\text{-}B^{30}/GOT\text{-}B^{-}$); and (- -), no band appeared at this zone, representing recessive homozygous condition ($GOT\text{-}B^{-}/GOT\text{-}B^{-}$).

Table 9 gives the results of calculations based on Hardy-Weinberg equilibrium for GOT-B phenotypes, using allele frequency data in Table 5. The observed proportions of various phenotypes show fairly good correspondence with the expected ones in most provenances except in provenances C and I, indicating that the assumption of a three-allele system at GOT-B locus is correct. The significant Chi-square value in provenance C mainly resulted from the heterozygous excess in phenotypic class (27 30) whereas in provenance I, it was largely due to homozygous excess in phenotypic class (- -).

Geographic Variation of Allele Frequencies and Genic Heterozygosity

In general, there is no particular predominant allele at GOT-B locus. The frequencies at $GOT\text{-}B^{27}$ and $GOT\text{-}B^{30}$ are moderately low to moderate in nine provenances examined. In provenances A, C

Table 9. Observed and Expected Phenotypes at GOT-B Locus in Douglas-fir Provenances.

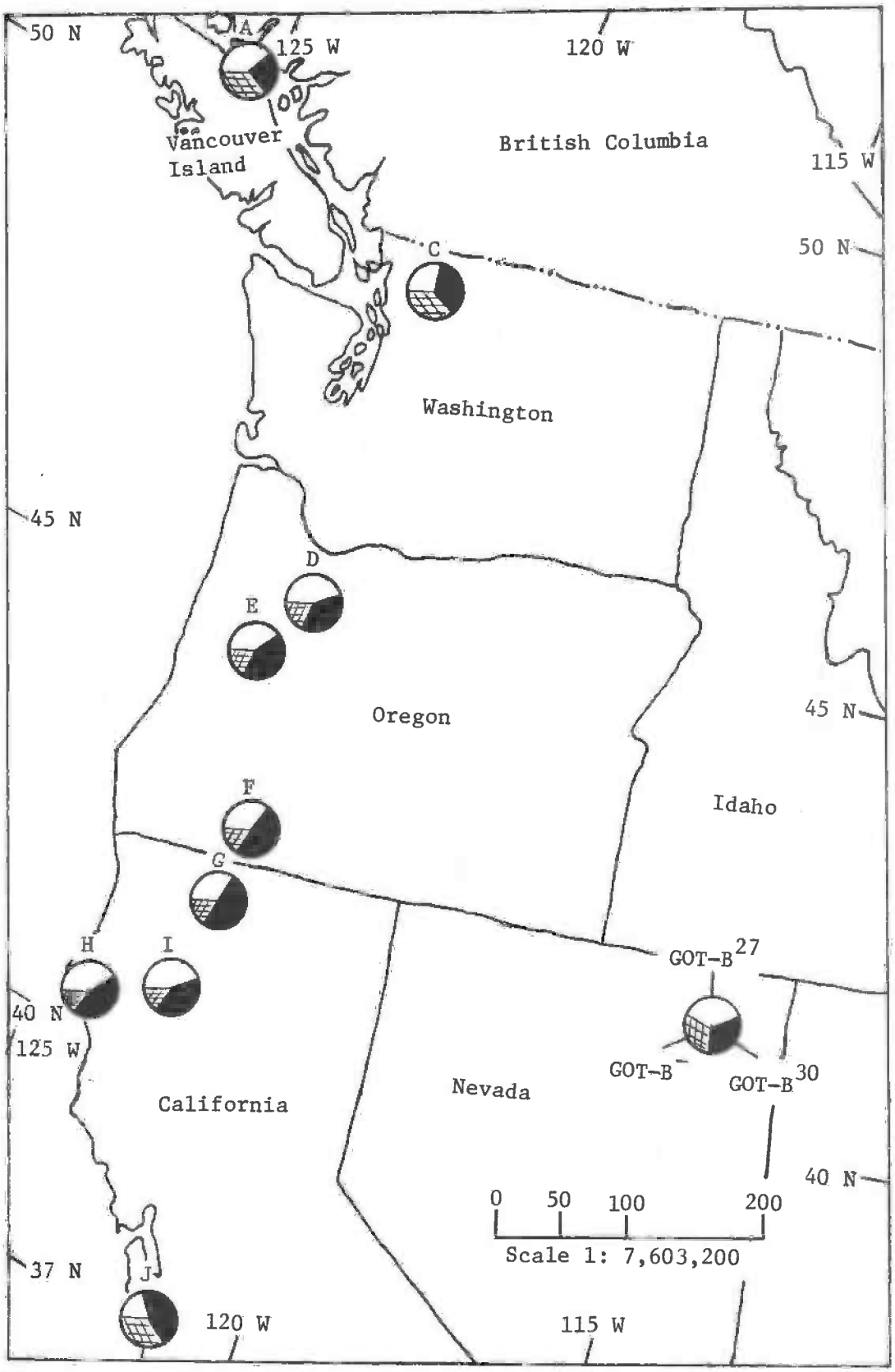
Provenance		Phenotype				X^2	P (2 d.f.)
		27 -	27 30	- 30	- -		
A	OBS	45	19	32	12	0.92	0.65
	EXP	42.37	22.05	29.63	13.59		
C	OBS	17	26	28	16	9.31	<0.01
	EXP	23.77	17.43	34.49	11.30		
D	OBS	30	39	21	6	5.62	0.06
	EXP	35.04	31.40	26.18	3.38		
E	OBS	23	33	27	5	2.22	0.35
	EXP	26.15	28.36	30.10	3.39		
F	OBS	19	41	39	6	4.91	0.09
	EXP	24.02	33.72	43.75	3.51		
G	OBS	16	27	32	3	0.37	0.84
	EXP	17.19	25.24	33.12	2.44		
H	OBS	25	40	24	5	5.44	0.08
	EXP	29.89	32.56	28.91	2.64		
I	OBS	26	47	22	5	10.13	<0.01
	EXP	32.59	36.74	28.65	2.02		
J	OBS	19	21	44	14	0.78	0.69
	EXP	21.06	18.45	45.88	12.61		

and J, the frequency of GOT-B^{-} is slightly higher than that of either GOT-B^{27} or GOT-B^{30}. Therefore, the silent GOT-B^{-} is not a rare allele as in the case of LAP-C^{-} but represents a frequent occurrence one. Figure 10 gives the illustration on the variation patterns of allele frequencies at GOT-B.

By using Chi-square test of independence, considerable differences in frequencies of three alleles at this locus were found among and within natural populations of Douglas-fir (Table 5). The variation patterns, however, does not demonstrate any obvious macrogeographic trend or relationship with elevations. A general finding with respect to each of the three alleles is that the highest frequency in one provenance is approximately twofold the amount of the lowest frequency in another provenance. This was also found for LAP-C^{46}, LAP-C^{50}, EST-B^{28} and EST-B^{34}.

As given in Table 5, the level of heterozygosity is again more or less uniform geographically. It was also found that there seemed to be a slightly lowered level of heterozygosity in high elevation populations. The correlation between the percentage of heterozygotes and elevation of provenances was $r = -0.664$ which is very close to the significant level at 5% (0.666 with 7 d.f.).

Figure 10. Geographic distribution of allele frequencies at GOT-B locus.


50 N
125 W
120 W
115 W
A
Vancouver Island
British Columbia
C
50 N
Washington
45 N
D
E
Oregon
45 N
F
Idaho
G
H
I
GOT-B 27
40 N
125 W
GOT-B
GOT-B 30
Nevada
California
40 N
0 50 100 200
Scale 1: 7,603,200
37 N
J
120 W
115 W

Genetic Identity and Genetic Distance between Douglas-fir Provenances

The geographic variation of allele frequencies at the four enzyme loci, as described previously, indicates significant differentiation among Douglas-fir provenances. It is, then, interesting to know if the degree of such a genetic differentiation parallels the geographical distance between provenances. The measures of genetic identity (I) and genetic distance (D) formulated by Nei (1972) may give the proper answer.

Genetic identity is defined as the normalized identity of genes between populations. It measures the proportion of genes that are common in the two populations under study. When I equals zero, two populations have no common alleles. When I equals one, two populations have the same allele in identical frequencies. In definition, genetic identity is equivalent to genetic similarity which is regularly used in numerical taxonomy.

The genetic distance is the natural logarithmic function of genetic identity ($D = -\log_e I$) and is used to measure the accumulated allelic substitutions per locus. It is linearly associated with geographical distance between populations in some migration models. It is also linearly related to the evolutionary time between populations under reproductive isolation in case the rate of gene substitution is constant per year. In summary, the measures of genetic identity and

genetic distance can effectively provide an index for the degree of genetic differentiation between populations in time and space.

To express the overall genetic identity and genetic distance between all pairs of nine provenances, allele frequency data from four polymorphic loci (LAP-C, EST-B, EST-C and GOT-B) and three monomorphic ones (LAP-B, EST-A and GOT-C) were used to calculate the coefficients of identity and distance. The matrices of coefficients are presented in Table 10. The values for genetic identity (or the degree of identity of alleles) between populations are quite high and, as a consequence, genetic distance is small. This is because both indices are complements each of the other to unity. Similar findings were also noted in Swedish populations of Norway spruce (Picea abies) by Bergmann (1973b).

The results presented here follow a general trend that the shorter the geographical distance between two provenances the larger the similarity value (or the smaller the distance value). It is particularly obvious for the trend between provenances from Vancouver Island down to Oregon. Three provenances (G, H, J) in California showed somewhat of a deviation from this general trend. They possessed higher similarity value to provenance A and, hence, lower distance value than did a northern provenance (F) (see first row from top and first column from left). But on an average, the genetic distance values in California provenances are higher than those of

Table 10. Coefficients of Genetic Identity and Genetic Distance between Douglas-fir Provenances Based on Four Polymorphic and Three Monomorphic Loci of Isoenzymes.

Provenance		Provenance A	C	D	E	F	G	H	I	J
		Genetic Identify								
A			0.987	0.983	0.979	0.971	0.982	0.977	0.964	0.977
C		0.013		0.993	0.990	0.990	0.990	0.992	0.977	0.986
D		0.017	0.007		0.994	0.994	0.992	0.998	0.987	0.978
E		0.021	0.010	0.006		0.992	0.985	0.995	0.967	0.987
F		0.029	0.010	0.006	0.008		0.988	0.995	0.983	0.985
G		0.018	0.010	0.008	0.015	0.012		0.992	0.981	0.979
H		0.023	0.008	0.002	0.005	0.005	0.008		0.981	0.983
I		0.036	0.023	0.013	0.033	0.017	0.019	0.019		0.947
J		0.023	0.014	0.022	0.013	0.015	0.021	0.017	0.053	

Provenance — Genetic Distance

Oregon provenances, indicating an increasing degree of genetic differentiation in parallelism to increasing geographical distance between Douglas-fir provenances.

Isoenzyme Genotype - Environment Relationship

The described findings on the allelic variation at four isoenzyme loci have demonstrated that there is a considerable amount of genetic variability at the molecular level in the natural populations of Douglas-fir. Such a genetic variability is so complex that a simple underlying pattern(s) could not be readily realized by describing individual allelic changes from provenance to provenance.

In order to better understand the overall integrated patterns of variability in each of the four polymorphic loci, principal component method was applied to analyzing the gene frequency data. A principal component is the definition of a new variable as a linear function of original variables. This new variable can be obtained by first constructing the variance-covariance matrix for allele frequencies of every locus. The first component is derived as the characteristic vector corresponding to the largest characteristic root of the matrix. The percentage out of the total variation, which could be explained by this component, can be calculated from the estimated value of the largest characteristic root. From the characteristic vector, one can see the relative amount and direction of allelic variation. Similarly,

a second component can be obtained as the characteristic vector corresponding to the second largest characteristic root of the variance-covariance matrix.

Let us consider first the variability patterns in leucine aminopeptidase. The variance-covariance matrix for the LAP alleles was computed and is given in Table 11. The numerical values of two components which correspond to the two largest characteristic roots of the variance-covariance matrix were derived from the matrix. The first component accounts for more than 95% and the second one for about 5% of the total variability in the LAP-C allele frequencies. As a result, all of the variability in the LAP-C allele frequencies is accounted for by these two components (Table 12).

Table 11. Variance-Covariance Matrix for LAP-C Locus.

Allele	46	50	--
46	0.01249	-0.00902	-0.00347
50		0.00804	0.00098
--			0.00249

Table 12. Estimates (E) of the Two Largest Characteristic Roots of the Matrix for LAP-C Locus and Their Respective Percent of Variation.

Component	E	% Variation
First	0.01955	95.3
Second	0.00097	4.7
Total		100.00

One can see the directions and amount of changes in allele frequencies at LAP-C from the characteristic vectors corresponding to the two largest characteristic roots of the matrix. As seen in Table 13, the first LAP-C component can be primarily attributed to a larger change in one direction of LAP-C^{46} accompanied by a somewhat smaller change in the opposite direction of LAP-C^{50}. For the second LAP-C component, the frequencies of LAP-C^{46} and LAP-C^{50} vary in one direction but in reversed-order quantities in contrast to those in first component. This is simply because LAP-C^{-} is a rare allele and, hence, there are no more than two alleles with appreciable frequency at this locus. In this connection, the variability patterns are essentially a reflection of the orthogonal increase and decrease in frequencies of LAP-C^{46} and LAP-C^{50} from provenance to provenance. The amount and direction of LAP-C^{-} change is too small to be effectively detected by principal component analysis.

Two components, derived from the variance-covariance matrix for EST-B locus (Table 14), accounted for all of the variability in the EST-B frequencies as shown in Table 15. The pattern of variation described by EST-B first component is basically that as EST-B^{28} varies with high quantity in one direction, EST-B^{34} changes with moderately low quantity in the opposite direction. The second EST-B component indicated a pattern in which the frequencies of EST-B^{28} and EST-B^{34} vary similarly in one direction (Table 16).

Table 13. Characteristic Vector for First and Second Components of LAP-C Locus.

Component	Allele		
	46	50	--
First	0.787	-0.617	--
Second	0.617	0.787	--

Table 14. Variance-Covariance Matrix for EST-B Locus.

Allele	28	34	--
28	0.01304	-0.00548	-0.00756
34		0.00444	0.00103
--			0.00653

Table 15. Estimates (E) of the Two Largest Characteristic Roots of the Matrix for EST-B Locus and Their Respective Percent of Variation.

Component	E	% Variation
First	0.01570	89.8
Second	0.00178	10.2
Total		100.00

Table 16. Characteristic Vector for First and Second Components of EST-B Locus.

Component	Allele		
	28	34	--
First	0.899	-0.437	--
Second	0.437	0.899	--

Component analysis was also run for the EST-C alleles. The variance-covariance matrix for EST-C locus is given in Table 17. As in the case of EST-B, two components were derived as the characteristic vectors corresponding to the two largest characteristic root of the variance-covariance matrix. The first EST-C component, which accounts for 80.4% of the variability, is mainly attributable to more or less equal changes in the same direction of the frequencies of EST-C^{52} and EST-C^{64}. A subtle decrease in the frequency of EST-C^{52} in one direction contrasted with a little increase in EST-C^{64} frequency in the opposite direction formed the pattern by the second EST-C component which entails 19.6% of the variation. These results were illustrated in Tables 18 and 19.

Table 17. Variance-Covariance Matrix for EST-C Locus.

Allele	52	64	--
52	0.00206	0.00113	-0.00319
64		0.00171	-0.00284
--			0.00603

Table 18. Estimates (E) of the Two Largest Characteristic Roots of the Matrix for EST-C Locus and Their Respective Percent of Variation.

Component	E	% Variation
First	0.00303	80.4
Second	0.00074	19.6
Total		100.00

Table 19. Characteristic Vector for First and Second Components of EST-C Locus.

Component	Allele		
	52	64	--
First	0.760	0.650	--
Second	-0.650	0.760	--

By employing the same technique, two components for the GOT-B locus were obtained in corresponding to the two maximum characteristic roots of the matrix of Table 20. The first component accounts for 60.3% of the variation while 39.7% is taken into account by the second component. The two GOT-B components together account for 100% of the variation in GOT-B frequencies (Table 21). The basic pattern revealed by first component is that GOT-B^{27} tends to have a large change in one direction while GOT-B^{30} has a moderately small change in the opposite direction. But the two allels exhibit the same direction of change of frequencies with reversed magnitude in quantity expressed by the second GOT-B component when compared with the variability pattern for the first component (Table 22).

Table 20. Variance-Covariance Matrix for GOT-B Locus.

Allele	27	30	--
27	0.00575	-0.00080	-0.00495
30		0.00439	-0.00360
--			0.00855

Table 21. Estimates (E) of the Two Largest Characteristic Roots of the Matrix for GOT-B Locus and Their Respective Percent of Variation.

Component	E	% Variation
First	0.00612	60.3
Second	0.00402	39.7
Total		100.00

Table 22. Characteristic Vector for First and Second Components of GOT-B Locus.

Component	Allele		
	27	30	--
First	0.907	-0.420	--
Second	0.420	0.907	--

The geographically clinal variation of LAP-C heterozygosity and the significant association of EST-B heterozygosity with elevation seemed to suggest a differential fitness of these two gene loci to some latitude- or altitude-associated environmental variables. In order to study the associations more completely, principal component and correlation analyses were also used to determine certain climatographic data for patterns of variability and their relationship with isoenzyme variability.

The climatographic data in connection with nine provenances were obtained from U. S. Weather Bureau Records published in "Climatography of the U. S." and Climate of British Columbia -- Tables of

temperature, precipitation and sunshine (Appendix Table 1). The data were Studentized to remove the effects of measurement on different scales such as degree of latitude, feet of elevation, inches of rainfall, degrees Fahrenheit, and days of growing seasons. Such a linear transformation is desirable in order to put the data on to the same scale and be able to make direct comparisons. The component analysis is also very sensitive to scale changes (Johnson, 1969). The transformation consists simply of substracting the mean and dividing by the standard deviation of each variable. The transformed variables have the same variances. Thus, the variance-covariance matrix for the Studentized variables (Table 23) is the correlation matrix for the original variables.

Since seven climatographic variables were involved in the examination of variability pattern of environments, more components were synthesized for environmental variation pattern than for that of isoenzymes (Table 24). Each component is a function of all the individual environmental variables to simultaneously formulate integrated expressions of the variability among the seven variables. The two largest components (1st. and 2nd.) together account for a large part (83.7%) of environmental variability, so that they might be used for a meaningful correlation analysis with isoenzyme components.

Table 25 shows the directions of changes of various environmental variables. The main pattern of variability described by the

Table 23. Variance-Covariance Matrix for Studentized Environmental Data.

	LAT	ELEV	ATAIN	ATEMP	JATEMP	JUTEMP	FFGS
LAT		-0.017	0.494	-0.626	-0.575	-0.309	0.205
ELEV			-0.215	-0.704	-0.784	0.318	-0.947
ARAIN				-0.187	-0.011	-0.508	0.135
ATEMP					0.915	0.206	0.822
JATEMP						-0.182	0.908
JUTEMP							-0.260
FFGS							

LAT: Latitude

ELEV: Elevation

ARAIN: Annual rainfall

ATEMP: Average annual temperature

JATEMP: Average January temperature

JUTEMP: Average July temperature

FFGS: Frost-free growing season

Table 24. Estimates (E) of the Seven Characteristic Roots of the Matrix for Environmental Data and Their Respective Percent of Variation.

Component	1st.	2nd.	3rd.	4th.	5th.	6th.	7th.
E	3.738	2.121	0.632	0.471	0.028	0.010	0.001
% Variation	53.40	30.30	9.03	6.73	0.39	0.14	0.01

Table 25. Characteristic Vectors for First and Second Components of Environments.

Component	LAT	ELEV	ARAIN	ATEMP	JATEMP	JUTEMP	FFGS
First	0.246	0.446	0.008	-0.484	-0.509	0.067	-0.493
Second	0.497	-0.265	0.568	-0.202	-0.021	-0.541	0.162

first component which accounts for 53.40% of the variation shows that increases in elevation and, to a lesser extent, latitude are associated with decreases in mean annual temperature, mean January temperature and growing season. This pattern largely comprises the altitudinal and, to a lesser degree, geographically north-south clinal variations in temperature. The second component which accounts for 30.30% of the variation indicates that increases in annual rainfall, latitude and, to a less extent, growing season are accompanied by the decreases in mean July temperature and, to a lesser degree, elevation as well as mean annual temperature. This pattern is less clear in a simple geographic interpretation but seems to be related to the local topography-oriented weather variation.

Results of the simple correlation analysis between the genetic component functions of four loci and two environmental component functions are shown in Table 26. There are three statistically significant correlations, namely, those between second component of GOT-B and second component of environment; between second component of EST-C and second component of environment; and between second component of LAP-C and first component of environment.

The correlations between the genetic component functions at four loci are presented in Table 27. Only three significant correlations were found, namely, between second EST-C and first GOT-B components; between first EST-C and second GOT-B components;

Table 26. Correlations of Principal Component Functions between Isoenzyme Loci and Environments.

		LAP-C		EST-B		EST-C		GOT-B	
		1st.	2nd.	1st.	2nd.	1st.	2nd.	1st.	2nd.
Environment	1st.	0.437	0.628^{+}	0.499	0.024	0.409	0.099	0.379	0.239
	2nd.	0.226	0.349	-0.333	-0.387	-0.406	0.755*	0.560	-0.774*

Critical Correlation Coefficients: $r\binom{7}{0.10} = 0.582^{+}$

$r\binom{7}{0.05} = 0.666^{*}$

$r\binom{7}{0.01} = 0.798^{**}$

Table 27. Correlations of Genetic Component Functions at the Four Loci.

		LAP-C		EST-B		EST-C		GOT-B	
		1st.	2nd.	1st.	2nd.	1st.	2nd.	1st.	2nd.
LAP-C	1st.	-	-						
	2nd.	-	-						
EST-B	1st.	0.164	0.248	-	-				
	2nd.	0.553	-0.457	-	-				
EST-C	1st.	0.293	0.242	0.589^{+}	0.407	-	-		
	2nd.	0.326	0.429	-0.079	-0.213	-	-		
GOT-B	1st.	0.478	0.389	0.326	-0.105	0.264	0.874^{**}	-	-
	2nd.	-0.140	0.054	0.514	0.204	0.594^{+}	-0.204	-	-

Critical Correlation Coefficients: $r\binom{7}{0.10} = 0.582^{+}$

$r\binom{7}{0.05} = 0.666^{*}$

$r\binom{7}{0.01} = 0.798^{**}$

and between first EST-B and first EST-C. The lack of more significant correlations between genetic-genetic or genetic-environmental patterns seems to be due to the small number of samples (provenances) included in the study. This will be discussed in greater detail in the following chapter.

V. DISCUSSION

The modern concept of evolution was described as "changes in the frequency of genes in a population" (Solbrig, 1966; Mettler and Gregg, 1969). The rate of evolution is dependent upon the amount of genetic variability, i.e., the extent of differences in gene frequencies, in evolving populations (Hubby and Lewontin, 1966). The most obvious reflection of this intraspecific genetic variability is polymorphism in certain forms within the species, and the most fundamental reflection of polymorphism at the molecular level is variation in proteins, especially enzymes.

The presence of enzyme polymorphism implies the existence of a corresponding polymorphism at a locus (or some loci) responsible for the synthesis of such enzymes. Thus, isoenzymes are considered as primary products of gene action. The extent of genic polymorphism at single loci is regularly expressed as the frequencies of occurrence of multiple alleles which are represented by isoenzyme bands.

If, as modern evolutionary theory postulates, the extent of genic polymorphism determines the rate of evolution, then the extent of genetic variation is an essentially experimental parameter for every species. Direct determination of the extent of genetic variability in populations may be experimentally ascertained with the use of electrophoretic technique in resolving the multiple molecular forms of enzymes in the gel media.

Patterns of Geographic Variation in Alleles and Genic Heterozygosity

A great volume of data regarding the geographic variability of Douglas-fir with respect to many characters other than enzymes has been reported (Ching and Hermann, 1973; Griffin, 1974). Quite a few of these variations have been shown to be of regional clinal patterns and of adaptive importance.

It is of importance to investigate the geographic patterns of allelic variability and genic heterozygosity, and to examine if such geographic variations at enzymatic level parallel those in morphological, phenological, and other biochemical characters in Douglas-fir. If this were the case, the integrated patterns of genetic variability and evolutionary process, which may be synthesized from molecular, organismic, and populational levels, may provide a better basis for a selection improvement program.

A. Allele Frequencies

With no exception, a monomorphic locus with a single allele was detected in each of the three enzyme systems examined in this study. When isoenzymes are used to study geographic variations, only polymorphic loci are, in many cases, included in the discussion relevant to geographic variability of organisms. The monomorphic locus is regularly ignored because it contributes almost no differences

in allele frequencies from population to population. Nonetheless, it does not mean that monomorphic loci may be genetically "idle."

On the inspection of each of zymograms (Figure 2, 5 and 9), an assumption concerning the origin of polymorphic loci may, first of all, be made that variable (polymorphic) loci may have arisen from invariable (monomorphic) ones through mutation within a single gene (intragenic variation) or through duplication of a single gene, which then underwent mutation (intergenic variation) since the monomorphic locus was represented by a well-defined and intensely stained band which consistently occurred in all germinants from all provenances studied.

The proposed allelic relationships at four polymorphic loci (LAP-C, EST-B, EST-C, and GOT-B) are primarily based upon the nature of phenotypes and statistical analyses of the observed phenotypic frequencies. The generally good correspondence of the observed phenotypic proportions with the expected ones (Table 6, 7, 8 and 9) in most of the nine provenances has justified the allelic assumptions in each of four loci. Ideally, further confirmation of this relationship can be enhanced with the progeny data involving crossings and selfings between the individual trees by observing the Mendelian segregations of progenies with respect to isoenzyme phenotypes or genotypes. In this study, the failure to attain sufficient selfing stocks made such a confirmation difficult.

Within provenances, the predominance of some alleles over the others, in terms of frequency of occurrence, was observed at four polymorphic loci although to a different extent. Such allelic variations within populations may result from balancing selection with strong intensities and with the direction of selection different for various alleles.

Chi-square tests of independence of allele frequencies of four variable loci give mostly highly significant X^2 values ($P < 0.005$). The remarkable changes of allele frequencies among Douglas-fir populations are so complex that it is difficult to associate the allelic variations with latitude, altitude or other environmental variables. However, one can speculate that the marked differentiation in allelic frequencies between Douglas-fir populations from distinct ecogeographical areas is expected if selection favors particular alleles at some places and other alleles at other sites.

In the studies of a number of plant species, including both inbreeders and outbreeders, Allard and Kahler (1971) also concluded that extensive allelic variability of esterase and anodal peroxidase is the rule both within local populations and also among different populations on both micro- and macrogeographical scales.

Since allelic frequency variation may reflect not only different adaptive responses to environmental variables, but also differences in initial genetic composition and the effects of varying restrictions

in gene flow (Wheeler and Selander, 1972), it is then not surprising that simple and clearly defined associations between allele frequencies and climatographic variables such as latitude, altitude, precipitation, temperature, soil type, etc. cannot be easily explored in Douglas-fir populations.

The lack of a pronounced pattern of relationship of allelic variations with environments may be open to an argument about the existence of selective action on allelic variation. However, at least one piece of evidence led us to be less inclined to invoke genetic drift as an appropriate explanation for the geographic distribution of these alleles as a whole in the present study. This is the widespread occurrence of alleles, though in different frequencies and with no local fixation of alleles in any provenance. Even though LAP-C^- is rare, it is apparently widely distributed throughout the provenances examined. Data in Table 5 are obviously incompatible with the drift operation which usually results in the fixation or exceptionally high frequency of certain alleles and complete loss of others in natural populations.

B. Genic Heterozygosity

The geographic variations of genic heterozygosity at four loci are more definite than allelic frequency patterns, and hence provide a relatively appropriate index to the degree of genic variation in

Douglas-fir provenances. In contrast to variation patterns of individual alleles, three out of four loci demonstrated a certain degree of geographic uniformity of heterozygote proportions among nine provenances (Table 5). Only EST-B locus showed conspicuous geographic variation in the degree of heterozygosity.

Geographic uniformity in genic heterozygosity at enzymatic level has been reported in natural populations of a great variety of animal species as cited previously. The causal basis for this uniform pattern has been mostly described as the effects of migration or gene flow through a series of subpopulations in many generations which are then maintained by some form of directional or balancing selection. This statement seems also to be applicable to explain the heterozygosity variation in Douglas-fir.

Although Silen (1962) concluded that Douglas-fir pollen from isolated tree did not travel very far, the same study also indicated that large amounts of pollen can be dispersed over considerable distances from the source points in a large continuous stand, particularly during a year of heavy production. Apparently, there is potential for gene flow through a series of subpopulations and hence the neighborhood size or panmictic unit of Douglas-fir is relatively large. Under this circumstance, geographic uniformity in genic heterozygosity could be expected. However, one point of view should be clear: "uniform pattern" does not necessarily mean "identical

pattern" because directional selection relevant to geographic gradients of environments would be responsible for the subtle changes of degree of heterozygosity.

The overall pattern of geographic variation in degree of heterozygosity at LAP-C locus is a north-south cline of increase (Table 5), showing significant relationship with latitude ($r = -0.686^*$). This suggests that natural selection with some latitude-associated environmental gradients, perhaps temperature or photoperiod, is responsible for the maintenance of genic variability at LAP-C locus.

Elevation is the better single variable responsible for the remarkable changes in level of heterozygosity at EST-B locus and, to a lesser extent, at GOT-B locus (Table 5). An increase in elevation is associated with significant decrease in heterozygotes at EST-B locus and slight decrease of GOT-B heterozygotes, indicating a negative effect of some selective pressures imposed by edaphic or climatic factors on the maintenance of high level of heterozygosity in Douglas-fir. The drastic changes of edaphic and climatic factors, particularly soil type and summer moisture stress, in mountainous areas would result in lessening the panmictic unit and barring gene influx from the proximity to some degree. As a consequence, the degree of heterozygosity in high-elevation provenances would be decreased. Neither latitude nor elevation seems to be effective in describing the variation pattern of heterozygosity at EST-C locus.

The finding that variation of genic heterozygosity at some loci is more associated with latitude than elevation and some the reverse does not seem to be compatible with random drift process, but coupled with smoothing of gene flow and natural selection.

C. Parallelism between Degree of Genic Heterozygosity and Physiological Characters

It is noteworthy that clinal changes of enzymes with latitude or elevation in this study parallel clinal variations in a number of other traits of Douglas-fir. However, some reservations must be kept in mind, that variation patterns of isoenzymes are not directly comparable with other characters unless the relationship between functional differences of isoenzymes and physiological process of plants can be convincingly established. The most interesting thing is that a lowered level of genic heterozygosity with increasing elevation in the present study coincides with the diminishing early growth with increasing altitude in the reports by Sweet (1965), and Hermann and Lavender (1968). It seems reasonable that growth potential of Douglas-fir is connected with the degree of heterozygosity in the gene pool.

The Santa Cruz population is more or less geographically isolated, so that the heterozygosity level should theoretically be low owing to probable genetic drift effects. But the situation is just the

reverse. Without exception, the degree of heterozygosity at four polymorphic loci investigated were all relatively high (>60%). Such an unexpected high level also paralleled the uncommon phenomena of phenology, i. e., early budburst, and high growth rate together with fast germination rate observed by Griffin (1974) and Sweet (1965) respectively. The former author ascribed this unusually early budburst to a short and indefinite dormancy period, and the latter speculated that Douglas-fir in this area probably has developed in response to selective pressures distinct from those in other provenances.

The distributional range of Douglas-fir in California has been delimitated by Zavarin and Snajberk (1972) as the area of intergradation between var. menziesii and var. glauca, based on these authors' terpene study. If the proposition of the positive correlation between growth potential and heterozygosity level is true, it would be reasonable to speculate that extensive hybridization between the two varieties since the Wisconsin glaciation, accompanied by geographic semiisolation has remodelled the genetic structure of the Santa Cruz population. The gene pool of this population has been enriched by gene influx from var. glauca, and then has an increased degree of heterozygosity. As a result, a certain degree of heterosis would occur in terms of very active development and growth.

In summation, there is a trend of reducing heterozygosity with increasing altitude and to a lesser extent latitude. This trend is paralleled by a general physiological function of Douglas-fir observed by Griffin (1974) who concluded, after reviewing a number of literature references, that there is trend of reduced growth and increasing tolerance to drought and cold as one proceeds from coast to inland, and to a lesser extent from south to north -- a trend paralleled by increasing severity of climate. Therefore, it would be probable that an increasing level of heterozygosity in the gene pool of Douglas-fir might strengthen the capacity of trees to stimulate active development and growth, and an increasing degree of homozygosity could help attain more specific genetic adaptability for survival under severe climatic selection pressures.

Feret (1970) also found a significant correlation between family isoenzyme variability and growth variation, and between isoenzyme variability and mean height growth of young white spruce (Picea glauca), and he concluded that enzyme variation could be used as a predictor of family growth responses and that heterozygosity is an important determinant of height growth.

D. A Consideration of the Adequacy of Heterozygosity Level Relevant to a Study on Population Structure of Douglas-fir

The level of heterozygosity at individual loci presented here

may be underestimated. Douglas-fir, an obligate outcrosser, is believed to possess as high as 80% or more of heterozygotes in its natural populations (Ching, personal communication). Such a low estimate may be attributable to the limited effectiveness of electrophoretic techniques and histochemical staining, because certain vague diffused zones often unavoidably occur on the zymogram, like a zone above Rf 21 on the esterase zymogram (Figure 4).

A combination of electrophoretic technique and isoelectric focusing has been reported to increase the resolution of proteins and thus can enable researchers to resolve additional protein variants from bands that appeared to represent single protein species when analyzed by gel electrophoresis alone (Kolata, 1974). When more alleles at a locus are detected, the degree of heterozygosity will be increased.

Another factor which may be responsible for the underestimate of heterozygosity is the small sample size. In the present study only seven loci (four polymorphic and three monomorphic ones) were drawn out of the whole genome which comprises many thousands of gene loci in Douglas-fir. Thus one could not study accurately the genetic architecture of the whole genome by extrapolation from data on a small number of loci. For a given provenance, when values of percent of heterozygosity at various loci were summed up and then averaged by the number of loci involved, including monomorphic

ones which contribute no heterozygosity, oftentimes only about 34% of heterozygosity was obtained. Obviously more enzyme loci are needed to assess accurately the genetic structure of Douglas-fir populations.

Genetic Differentiation in Douglas-fir at the Enzymatic Level

A. Disadvantage of Conventional Methods of Evaluating Genetic Differentiation between Tree Populations

The genetic differentiation in populations of forest trees largely depends on the extent of pollen and seed transport. As was pointed out by Wright (1962):

> If the pollen and seed dispersion distance are great and the stands are dense, the gene flow is rapid; the entire population remains essentially uniform. If the pollen and seed dispersion distance are short and the population is sparse, gene flow is limited; the genetic differentiation within populations can occur.

Pollen, a haploid gamete, regularly travels much farther than does seed, a diploid zygote. Thus, pollen transport distance is more essential than seed dispersion distance to the study of genetic differentiation between tree populations. Under these circumstances, Wright (1962) presented several formulae of neighborhood size to estimate the extent of genetic differentiation in populations of continuously distributed tree species of different geographical locations.

The estimation of number of breeding trees in a neighborhood is based on the pollen dispersion distance and the assumed population density in terms of the number of flowering trees per square foot of areally continuous range or per linear foot of linearly continuous population.

Study of pollen transport has been undertaken for not only theoretical estimation of amount of genetic diversity in forest tree populations, but also practical management of seed orchards. Distance of pollen dispersion is affected by many factors such as fall rate of pollen grains, air temperature, atmospheric humidity, velocity of winds and their turbulence, and topographic conditions. Field investigations of pollen dispersal, therefore, are laborious and time consuming, and provide only a rough approximation of dispersal distance. Furthermore, pollen dispersal study has resulted in two opposing views on the migration distance -- long distance versus short distance transport. The estimation on amount of genetic differentiation in tree populations consequently may often be biased. All these arguments have arisen solely based on pollen dispersion without considering a direct analysis of the genetic background in a forest tree population.

B. New Approach to the Estimation of Genetic Differentiation between Douglas-fir Provenances

With the aid of electrophoresis of proteins and enzymes, the

coefficients of genetic identity and genetic distance can be calculated from allele frequencies of isoenzymes, and these coefficients may provide a new and quick approach to the quantitative measurement of comparative genetic diversity in forest trees of wide ranging distribution such as Douglas-fir. In addition, it would be interesting to know if divergence measured in this way can reflect the influence of such factors as ecological variation, population size or amount of isolation.

The foregoing discussion is based on individual loci. By contrast, the coefficient data of genetic identity and genetic distance listed in Table 10 demonstrate a synthesized feature of genetic diversity among Douglas-fir provenances.

A perusal of the table reveals that the pairs of provenances from Vancouver Island and Oregon are obviously differentiated in parallelism to latitude, indicating that genes did not transfer over a great distance in a short time, but moved gradually through a series of subpopulations. The association with latitude may reflect the important role of macrogeographic environmental gradients on modulating the genetic differences of Douglas-fir populations.

Irregular changes of values of genetic identity and genetic distance among California provenances imply that the effect of migration has been obscured by other limiting factors including external environmental variables and to a lesser extent internal genetic

constitution. Also, data based on a small number of loci might give a biased and nontypical picture.

The most striking distinctions were evidenced between two provenances of almost the same latitude (H and I). Provenance H is at a coastal location separated from inland provenance I by only 26 miles. But drastic changes in elevation and climates (particularly, frost-free growing season and the mean January temperature) have created quite different sites. Differentiation between these two provenances is extensive and reflects the adaptation of various genotypes to different ecological situations. Even though migration may exert a certain degree of influence, strong differential climatic selection could reduce its effect. Griffin (1974) has revealed that there were significant differences in seed characteristics, early growth and phenology between the coastal and inland seedlings, and concluded that to some degree complex microgeographic differentiation among Douglas-fir populations in northern California might have occurred due largely to vigorous differential selection pressures imposed by variable topography and climates. In general, local differentiation is more pronounced in California populations than in those at other geographic locations as evidenced by the present isoenzyme data and Griffin's study.

On the average, California provenances demonstrated more divergence from the Vancouver Island population than did Oregon

ones. It would then appear likely that with time, genetic differences would gradually accumulate in such a way that the greatest differences could be found between the northmost and southmost populations of Douglas-fir.

Adaptive Significance of Isoenzyme Polymorphism

A. Controversy between the Neutrality Theory and Natural Selection

The adaptive nature of isoenzyme variability in natural or experimental populations of animal and plant species has been a key-point of contention between neutrality and selection hypotheses ever since surprisingly high degrees of genic variation were revealed by electrophoresis of proteins and enzymes.

Proponents of neutralism argued, based on theoretical considerations, that small differences in tertiary structure of variant proteins and enzymes are sufficient to influence electrophoretic mobility but not to affect significantly the functioning of the protein and enzyme. Since isoenzymes which represent electrophoretically different alleles are assumed to be functionally identical, they are thought to affect the adaptedness of organisms identically, the distinctions among them thus being neutral to natural selection pressures.

On the contrary, the hypothesis of natural selection postulates, based on experimental evidence, that a great deal of genetic variation at the enzymic level may be maintained by natural selection because increasing degree of environmental heterogeneity mostly resulted in more genetic variation at enzyme loci (Kolata, 1974). Furthermore, different degrees of enzyme polymorphism may reflect different level of physiological function, some functional classes of enzymes being far more variable than others (Johnson, 1974).

B. Adaptive Nature of Isoenzymes in Douglas-fir

The overall results in this study are not compatible with the former theory, but are consistent with the latter one that some combined form of balancing or directional selection is the main factor controlling genic variation in natural populations of Douglas-fir.

In an attempt to stress the importance of migration, Kimura and Ohta (1971) noted that similar gene frequencies between widely separated populations are compatible with functional neutrality of isoenzymes. There may be no doubt that migration often takes place between populations of Douglas-fir. It is also possible that even a low rate of migration is sufficient to nearly equalize allele frequencies among populations unless selection strongly favors alternative alleles in different populations. Furthermore, migration rate high enough to retain the high degree of polymorphism observed

within provenances would almost certainly reduce differences between populations to low levels even in the face of vigorous selection in different directions in distinct populations. However, the striking differences in frequencies of most alleles among nine provenances are accordingly inconsistent with the neutrality theory, but do show evidence of selective significance.

Another piece of evidence of the selective importance of polymorphism is the significant association of genic heterozygosity at the LAP-C and EST-B loci with latitude and elevation respectively, exhibited by regression analyses. Such a significant relationship as well as the parallelism between the level of genic heterozygosity and the growth potential or adaptability for survival may lead us to the conclusion that natural selection plays a much more important role than migration or genetic drift in constructing the genetic makeup of Douglas-fir.

Regression analysis could not reveal all of the associations between isoenzyme variability and environments, due simply to the limited effectiveness of this statistical technique. In order to attempt to find stronger and more specific relationships between environment and isoenzyme genotypes, more detailed climatographic data were examined and correlated with genetic factors by the principal component method which is considered to complement regression analysis.

Data of Table 26 indicate that except EST-B, at least one component of each locus is significantly correlated with some environmental factors, suggesting that some loci must be selectively important. However, it does not mean that climate directly causes changes of allele frequencies but climate imposes a great influence over such a large portion of the total environment that the gene-environment correlation must be biologically meaningful.

It is interesting to note that when using linear regression analysis, significant relation was found between elevation and genic heterozygosity at EST-B locus, but no prominent association between EST-B gentypes and environment was detected by component method. It would appear probable that the effect of elevation on the allelic variation at EST-B was confounded by other environmental factors, or the nonsignificant relationship could be due to the limited effectiveness of the analyzing method.

On the other hand, genetic variations at GOT-B and EST-C loci did not reveal a prominent association with environment by regression analysis, but were shown to have a significant level of correlation with environment by component method. For LAP-C locus, genetic variability exhibited a significant degree of relation with latitude-associated environment by regression approach, but such an association was reduced to 10% significance or even to the nonsignificant level by component analysis.

Principal component approach, simple regression analysis and linear multiple regression analysis all have been employed to assess the relationship between environmental factors and genic variation of isoenzymes in a number of animals and plants. But none of them has fully explored all of the remarkable gene-environment associations. Thus the statistical techniques for detecting, measuring and testing these associations have not reached full effectiveness. Which technique is suitable for which particular enzymic locus is still unknown. Nevertheless the general descriptions of the adaptive significance of the three enzymes in this study, revealed by either component method or regression analysis, should be satisfactory in spite of the weakness of statistical techniques.

Tomaszewski, Schaffer and Johnson (1973) noted that simple correlation analysis cannot exploit all of the relationships between the environmental and genetic components because, unlike canonical correlation analysis, it just characterizes the genetic and environmental data rather than maximizing the correlations. Based on this consideration, an attempt was made to use the method of canonical analysis to see if correlation between genetic and environmental patterns in the data can be maximized. Unfortunately, data from only nine provenances was found to be inadequate for analysis by this method (Rowe, personal communication). However, it is very likely that more extensive sampling (i. e. more provenances) over

the distributional range of Douglas-fir would reveal to a larger extent the existence of selectively adaptive importance of isoenzyme polymorphism in this tree species.

In view of the fact that latitude, elevation and climatic parameters are the gross indicators of a host of correlated climatic and biotic variables, it is not at all surprising that the results obtained by the correlative approach used in the present study were often not fully explainable. Similar difficulty in explaining the geographic pattern of allelic variation in an adaptive sense has been encountered in many isoenzyme studies of Drosophila and various vertebrates, for the same reason as described above (Wagner and Selander, 1974). In a study of geographic variation in growth as well as phenological characters of Douglas-fir seedlings from California, Griffin (1974) also presented some reservations about the interpretation of results derived from the approach of correlation analysis, owing to the fact that patterns of genetic variability have evolved in response to past environmental situations and hence can only be assessed indirectly. Explanation of isoenzyme polymorphism with an adaptive significance can be convincingly established only when functional differences of enzymes and proteins can be related to the distribution of relevant environmental factors.

Another plausible interpretation for the difficulty of detecting association between isoenzyme variability and climatographic

variables is that genetic environment may be as important as external environment in controlling the genetic structure of populations, and hence the effect of climatic selection may be sometimes obscured by genetic environment. Genetic environment represents a genetic background in which the extent and sorts of gene interactions, or the degree of linkage and inversion in chromosomes have been developed and maintained during the evolutionary process. Cytogenetic study of Douglas-fir revealed that inversions, nondisjunction or reciprocal translocations may have been involved in karyotype variation and the production of extra chromosome (Christiansen, 1963; Thomas and Ching, 1967; Owens, 1967; Ching and Doerksen, 1971; Doerksen and Ching, 1972). Whether these enzyme loci discussed here happened to reside on an inverted segment, extra chromosome or other linked group of genes remains to be investigated.

If all allelic variations were selectively important and affected by the same selective factors, then gene-gene correlations should be found theoretically. As shown in Table 27, correlations exist between EST-C and GOT-B, EST-B and EST-C, but no associations were found between EST-B and GOT-B, LAP-C and others. This indicates that four enzyme loci are not always influenced by the same selective factors and hence do not behave uniformly in response to environmental regulation, or selection may act on each locus independently. Gottlieb (1971) also noted that natural selection

would be expected to act in different ways at different loci and in different populations.

Relevance to Genetic Improvement of Douglas-fir

Breeding for high potential of height growth has been taken as an important goal in genetic improvement programs of Douglas-fir at Oregon State University (Ching, 1959; Ching and Bever, 1960; Ching, 1965; Rowe and Ching, 1973). Additionally, the importance of survival, particularly at high elevation sites, has been stressed by Silen (1966) for stand volume productivity at harvest stage. The present results have exhibited parallel patterns of the degree of genic heterozygosity with growth potential and adaptability for survival. This parallelism poses a vital problem concerning the physiological role of enzyme polymorphisms on growth rate and survival of Douglas-fir from various seed sources. The solution of such a problem requires the collaboration of physiologists and geneticists.

Johnson (1974) postulated that the level of heterozygosity at an enzyme locus may reflect the regulatory role of the enzyme, and enzyme polymorphisms may increase fitness by providing a means of metabolically compensating for a varying environment.

McNaughton (1972) gave experimental evidence of natural selection for enzymic thermal insensitivity in ecotypic populations of Typha latifolia by examining malate dehydrogenase and glycolate

oxidase. Ecotypic differentiation of malate dehydrogenase, although classified as a nonregulatory enzyme by Johnson (1974), was evidenced by interaction among activation energies, assay temperatures, and current growing conditions. Glycolate oxidase between ecotypes differs in substrate affinity, with a significant tendency toward lower affinity in the cool climate population at low assay temperatures. This decline in affinity leads to low-substrate-level extension of intrinsic thermal insensitivity of the protein at low temperature, as suggested by the author. Therefore, enzyme properties may change substantially based on genetic adaptation of the populations, as regulated by current growing conditions and the resulting internal metabolic state.

Just as in the case of the complementation of mitochondria and cytochrome c oxidase, which is relevant to practical breeding of maize, there may be some possibilities of complementation (intraallelic and/or interallelic complementation) resulting in a certain degree of heterosis by producing super efficient enzymes. This would be attrative in view of the many agricultural examples of positive heterosis of increasing growth rate, particularly at the younger stage (Manwell and Baker, 1970). However, complementation may not always result in the improvement of all characters; some properties may be improved whereas others worsened within the same hybrid protein.

The above discussion may enlighten the feasibility of investigating the functional role of isoenzymes on survival and growth rate of Douglas-fir seedlings. Enzymes involved in mitochondrial activity, photosynthesis, cellulose synthesis, etc. would be a good choice for study. A multidimensional method involving the investigation on a locus-by-locus basis with regard to genetic, environmental, biochemical and physiological considerations in some form of combination would also be of value.

Linkage study between various isoenzyme loci is one component of this combination, and can be incorporated in inbreeding and outbreeding programs of Douglas-fir. Linkage relationships between enzyme loci will provide information which can help to locate the gene loci on particular chromosomes and hence to further the understanding of the genetic environment as mentioned above. For practical purposes, such research may help to explore the question of correlation between isoenzyme complementation and hybrid vigor in heterozygotes. Breeding for hybrid vigor in offspring to attain superior growth rate and adaptability is the ultimate aim of most tree breeders.

Kinetic studies on various enzyme variants (genotypes) are another component of the combination. Kinetic properties of isoenzymes include substrate specificity and affinity, initial velocity, activation energy, Michaelis constant, isoelectric point, thermal stability, etc. The biological basis for maintenance of enzyme

polymorphism and subsequent mechanisms of plant responses to environmental regulation may be clarified by studying the differences in kinetic properties of isoenzyme genotypes from different sites. This can provide better understanding for natural selection acting on enzyme loci and a better estimate of the differential fitness values of different genotypes.

In order to carry out the multidimensional approach, such techniques as gel electrophoresis, paper chromatography, immunochemical test, isoelectric point determination, and spectrophotometric (or polarographic) assay of enzyme activity can be used sequentially in the study. In addition, experiments should be conducted to examine tissue specificity of isoenzymes and to determine isoenzyme alterations in developing tissues. Comparative developmental studies of various enzymes in the same tissues or between different tissues will allow a better understanding of differential gene action and a better choice of experimental material with appropriate developmental stages for the genetic analysis of isoenzymes.

The working program should start first with continuous inbreeding to produce inbred lines which are homozygous with respect to electrophoretically distinct genotypes or phenotypes. The feasibility of producing homozygous inbreds in Douglas-fir has been confirmed by Orr-Ewing (1969). At the same time, hybridization between inbred lines can be conducted in order to detect if isoenzyme

complementation occurs in the hybrids. The archives of inbred families should be established on different planting sites for the investigation of various kinetic properties of different enzyme genotypes, and for the comparison between functional differences of isoenzymes and physiological phenomena of trees.

Chemical identification of different enzyme loci which appear in the same gel, and detection of physiochemical properties of various alleles at a locus (Chao and Scandalios, 1969 and 1971), seem also to be attractive to the future study of isoenzymes in Douglas-fir. However, the usefulness of these techniques is dependent on the chemical nature of enzymes.

When many more enzymes systems are investigated and sufficient background information is accumulated, the knowledge gained could ultimately complement a selection program for Douglas-fir in such a way that adaptability of offspring to different planting sites may be accurately evaluated in a relatively short time and without large scale of continuous field experiments.

VI. SUMMARY AND CONCLUSION

Individual seedlings with cotyledons fully elongated, grown from open pollinated seeds of nine provenances of Douglas-fir were examined by discontinuous gel electrophoresis for evidence of polymorphism in three enzyme systems, namely, leucine aminopeptidase (LAP), esterase (EST), and glutamate oxaloacetate transaminase (GOT). The types and quantity of gel media vary as to the reproducibility and clarity of zymograms of the different enzymes. For esterase, 7.5% of polyacrylamide was used, and a mixed gel medium of 7.5% polyacrylamide and 0.5% starch was used for leucine aminopeptidase and glutamate oxaloacetate transaminase.

A total of four isoenzyme bands was detected on the zymogram of LAP, namely, Rf 33, 39, 46, and 50. Based on frequency distribution and goodness-of-fit test, three loci were suggested to account for the occurrence of these four bands. Rf 33 represents a productive allele at LAP-A locus which was not critically evaluated for allelic variability because of the scarcity and low reproducibility of this allele. A monomorphic locus (LAP-B) was represented by Rf 39 allele, which had a consistent occurrence in all individuals from nine provenances examined. Rf 46 and 50 are the codominant alleles at the LAP-C polymorphic locus, which also contains a recessive null allele.

The esterase phenotypic expression appears to be determined by a three-locus genetic background, and is represented by five bands: Rf 21, 28, 34, 52 and 64. Rf 21 is a single allele at monomorphic EST-A locus and contributes no variation for the population study. Polymorphic EST-B has two codominant alleles represented by Rf 28 and 34, while Rf 52 and 64 are the two alleles at the EST-C locus. In addition, both of these polymorphic loci possess a corresponding null allele.

Polymorphism in the GOT system appears to be dependent upon two-locus expression. GOT-B is a polymorphic locus with Rf 27 and 30 plus one recessive allele. GOT-C locus is monomorphic and is represented by a single Rf 36 allele.

Phenotypic proportions at four polymorphic loci were calculated and their postulated genotypes were tested for agreement with expected Hardy-Weinberg equilibrium values. The correspondence between observed and expected frequencies is general good except that some deviations occur in high elevations and northerly as well as southerly populations, due to heterozygote- and/or homozygote-excess.

Variation of frequencies of most alleles are significantly different among the nine provenances. The geographic patterns of overall allelic variability at LAP-C, EST-B, EST-C and GOT-B loci are to some degree too diversified to be easily interpreted within an adaptive

framework, indicating that allelic variation may reflect not only distinct adaptive responses to various environmental variables but also differences in original genetic composition among populations and the effects of varying degrees of restriction in gene flow.

By contrast, the geographic variability of genic heterozygosity at four polymorphic loci is more simple and definite than allelic frequency patterns, and shows a certain degree of geographic uniformity or clinal change except that at the EST-B locus. The effects of gene flow through a series of subpopulations plus some forms of balancing as well as directional selection relevant to macrogeographic gradients of environments would be responsible for the uniform pattern or clinal variation in genic heterozygosity. Variation in degree of genic heterozygosity at EST-B and to a lesser extent at GOT-B is associated with elevation, suggesting a negative effect of selective pressures imposed by local drastic edaphic and climatic change on the maintenance of heterozygosity level in mountainous populations.

The incidental parallelism between variation patterns of genic heterozygosity and general physiological phenomena would be suggestive that a high level of heterozygosity in the gene pool of Douglas-fir might increase the capacity of trees to promote active growth and development, and a high level of homozygosity could help attain more specific genetic adaptability for survival under severe climatic conditions. However, the relationship between genic variability

and plant growth or adaptedness should not be overemphasized until the functional differences of isoenzymes can be related to the changes of environmental variables in the natural habitats of Douglas-fir.

Using data of allele frequencies, genetic identity and genetic distance among provenances were calculated. The results suggest that degree of genetic differentiation among populations of Douglas-fir is generally parallel to increasing geographical distance.

Association of isoenzyme variation with climatographic factors, revealed by principal component method and correlation analysis, suggests that some loci are selectively important. That not all of the gene-environment relationships can be explored is probably attributable to the fact that climatographic variables are crude parameters and hence any association between isoenzymes and environments may be sometimes biased. However, correlation data are obviously suggestive that more intensive sampling over the distributional range of Douglas-fir would exhibit more gene-environment associations. Genetic environment would be another factor which could confound the effects of climatic selection, and hence the adaptive significance of isoenzyme variability may sometimes be obscured.

The physiological functions of multimolecular forms of enzymes in connection with height growth rate and adaptability for survival of Douglas-fir were stressed. Thus, a multidimensional approach involving the study on a locus-by-locus basis with regard to genetic,

environmental, biochemical and physiological considerations in some combination was proposed for the practical application in genetic improvement programs of Douglas-fir.

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Appendix Table 1. Climatographic Data Representative of Nine Provenances.

PROV	LAT (^{o}N)	ELEV (ft.)	ARAIN (in.)	ATEMP (^{o}F)	JATEMP (^{o}F)	JUTEMP (^{o}F)	FFGS (day)
A	50.25	900	85.52	48.4	35.7	61.3	217
C	48.48	1750	33.50	46.7	32.4	63.3	173
D	45.17	3600	45.90	41.3	28.7	57.5	86
E	44.50	1900	37.67	48.1	34.7	61.7	146
F	42.47	3000	19.78	47.0	31.2	69.0	119
G	41.93	2693	17.76	51.6	33.8	71.6	146
H	40.23	996	47.01	54.9	47.7	60.9	277
J	37.13	568	28.40	57.1	49.7	63.0	271

LAT: Latitude

ELEV: Elevation

ARAIN: Annual rainfall

ATEMP: Average annual temperature

JATEMP: Average January temperature

JUTEMP: Average July temperature

FFGS: Frost-free growing season