Genetic Studies in Natural Populations of Forest Trees

I. Genetic Variability on the Enzymatic Level in Natural Forests of Thujopsis dolabrata[1])

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Introduction

Natural forests are not only of immense interest from scientific viewpoints, but are also of primary importance to foresters, since they are the main source of genetic and planting materials. However, only a few studies on genetic variability in natural forests seem to have been undertaken so far. This is probably because of the difficulties involved in extensive breeding experiments with long-lived and attaining large size tree species.

In recent years, the remarkable development of electrophoretic techniques for the analysis of enzymes furnishes a convenient tool for approaching that difficult object. It seems that by introducing this new method we may attack the problem of measuring genetic variability in natural forests without spending years and space. Our present study deals with the electrophoretic analysis of peroxidase in natural forests of Thujopsis dolabrata. The forests investigated in this study are composed mainly of trees of this species, though they are mixed with a few broad-leaved species such as Aesculus turbinata, Fagus crenata and Magnolia obovata.

The Thujopsis trees growing in the forests are naturally of varying age, from as young a tree as a sapling to a Methuzaleh, seemingly mcre than several hundreds or even thousand years old. A *Thujopsis* tree is in general capable of vegetative as well as sexual propagation, though the former seems to be far less frequent than the latter.

The two forests investigated are geographically isolated from each other as shown in Figure 1. The purpose of the present study was (1) to examine individual variation in peroxidase isozymes in each forest and (2) to find to what extent the two forests are genetically differentiated.

We wish to express our sincere thanks to the Forestry Agency, Ministry of Agriculture and Forestry, Tokyo, for placing at our disposal facilities and information for this study. We are also grateful to the Aomori Regional Forest Office for the many helps extended to us in our on-the-spot work in the forests. We are especially thankful to Mr. Futoshi Tashiro of the Forestry Agency, Tokyo, and Mr. Koichiro Yamada of the Regional Forestry Office, Aomori, for their valuable advices and suggestions. Thanks are due to Drs. M. Arita of Gifu University, S. Hayashi of Kagoshima University and Y. Shimada of Ishikawa Prefectural Forest Experiment Station who were kind enough to help us in carrying out this study. We also wish to thank Dr. T. Endo of the Department of Biochemical Genetics, National Institute of Genetics who was kind enough to give us valuable criticisms during our study.

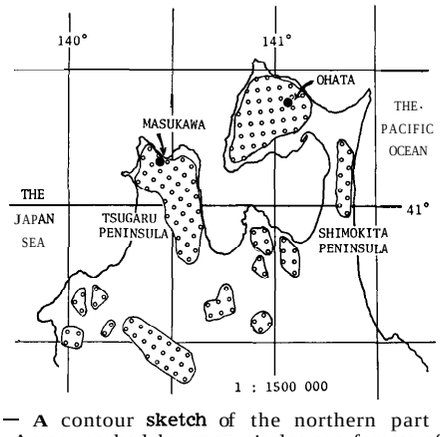

Figure 1. — A contour sketch of the northern part of Aomori prefecture. Areas marked by open circles are forests of Thujopsis dolabrata.

Materials and Methods

The two natural forests of *Thujopsis* dolabrata investigated in this study are located in Ohata in Shimokita peninsula and in Masukawa in Tsugaru peninsula in Aomori prefecture. A contour sketch of the prefecture is given in Figure 1, showing the scattered distribution of Thujopsis habitats in both peninsulas and the inland area.

From Figure 1, we find that the two facing each other promontories of the two peninsulas, where the Ohata and Masukawa forests are located, are isolated from each other by about 10 kilometer wide straits.

The field work started with mapping accurately the numbered individual trees of the forests on a section paper; at the same time measurements of tree height, stem diameter at breast height and length of the thickest bough were taken. A certain amount of needle-leaves were collected from the thickest bough of each tree for the further study of leaf characters and electrophoretic analysis in the laboratory. The characters dealt with are illustrated in *Figure* 2.

A portion of leaf samples were deep-frozen in the laboratory until needed for the electrophoretic study of the peroxidase isozymes. The technique adopted for the analysis was that developed by Smithies (1955) and Endo (1968), described in detail by Miyazaki and Sakai (1969).

Results of Study

1. Statistical treatment of the *peroxidase* isozyme bands

Figure 3 shows a few examples of peroxidase zymograms

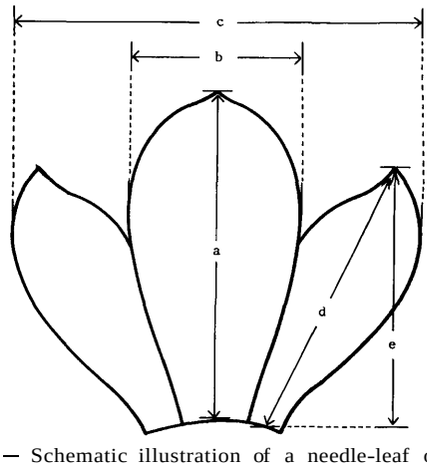

Figure 2. — Schematic illustration of a needle-leaf of Thujopsis dolabrata. — (a) Leaf length; — (b) Width of main leaf; — (c) Width of the compound leaf; — (d) Length of lateral leaf; — (e) Vertical length of lateral leaf.

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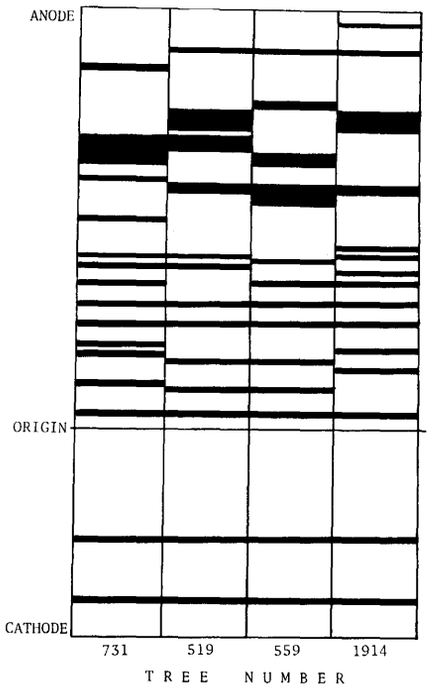

Figure 3. — A few examples of peroxidase zymograms observed in needle-leaves of *Thujopsis dolabrata.*

observed in *Thujopsis dolabrata.* There appeared many bands on the anodic side, but only two on the cathodic side. Since the two cathodic bands were uniformly present in all trees, they are left out of consideration in the present study.

A difficulty encountered in a variation study of isozymes with the unaided eye is how to count correctly the bands. We can often not decide if a band observed is really a single one or if it consists of two or more components. How can we find the correct number of isozyme bands or how to find the mistake in misreading them?

Figure 4 represents a schematic illustration of a case where two adjoining bands, *a* and *b*, show four kinds of combination: (1), both *a* and *b* bands are present, in (2) or (3) either one of them is found, while in (4), neither is seen. Let the frequency of four combinations, (1) *ab*, (2) *ao*, (3) *ob* and (4) *oo* be p, q, r, and s, respectively, where $p + q + r + s = 1$. The frequency of occurrence of *a* is $(p + q)$, and that of *b* $(p + r)$. If *a* and *b* are independent, the expected occurrence of the four combinations may be as follows: $E(ab) = (p + q)(p + r)$, $E(ao) = (p + q)(q + s)$, $E(ob) = (r + s)(p + r)$ and $E(oo) = (r + s)(q + s)$. The correlation between *a* and *b* in such a case may be zero. If *a* and *b* occur more or less in association, the frequency of (ab) will be larger than $(p + q)(p + r)$ and $r_{ab} > 0$. If, on the contrary, *a* and *b* are more prone to repel each other than at random, the frequency of (ab) is smaller than $(p + q)(p + r)$ and $r_{ab} < 0$.

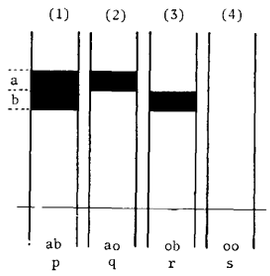

Figure 4. — Schematic illustration of four combinations for the presence and absence of two adjoining bands, *a* and *b*.

Theoretically, it may be expected that high correlation coefficients between bands might be caused by either of the following factors: (1) merely by chance, (2) by a genetic mechanism such as pleiotropy or gene linkage, and (3) by a shift in the position of a single band appearing as if there were two different bands. It is desired to find out the misread bands at this point, (3). Thus, we have calculated the correlation coefficients among 53 bands in each of the two forests, 1378 for each. The frequency distribution of those correlation coefficients is presented in *Table 1.*

The distribution of correlation coefficients in *Table 1* suggests that they are distributed normally rather than otherwise.

The question then arises: are high positive or negative coefficients of correlation really biologically significant? If a high correlation merely occurs by chance in one forest, then, the probability that the corresponding one in another forest might again be high would be very small. The contingency table for the test of coincidence of associated bands between two forests is presented in *Table 2.* It is found from *Table 2* that the χ^2 value is highly significant, indicating that pairs of bands positively or negatively associated in both forests are far more frequent than theoretically expected from random combination.

The number of coefficients which were significant and positive in both forests was 31 against the expected frequency of 14. A similar relationship was also found for coefficients which were negative in both forests, i. e. 30 against 12. Occurrence of other combinations, that is, + in one forest and 0 in the other or — vs. 0, + vs. —, and 0 vs. 0 is always less than theoretically expected. It is thus concluded that there was certainly a tendency for some isozyme bands to be positively or negatively associated more frequently than at random.

In order to find the case of (3) where a single band is mistaken for two separate ones by a shift in its position, it is necessary to examine band pairs in the + vs. + as well as — vs. — rectangles of *Table 2* in respect to their position. If two bands highly correlated are adjoining each other, one of them may possibly be a phantom of the other. Examination of correlated bands revealed that eleven "bands" among 53 were phantoms of real bands. They were

Table 1. — Frequency distribution of r among 53 isozyme bands of peroxidase in two forests of *Thujopsis dolabrata.*

	r among isozyme bands										
	—1.0∼ —0.8	—0.8∼ —0.6	—0.6∼ —0.4	—0.4∼ —0.2	—0.2∼ 0	0∼ +0.2	+0.2∼ +0.4	+0.4∼ +0.6	+0.6∼ +0.8	+0.8∼ +1.0	Total
Ohata*)	1	1	29	122	577	470	136	35	4	3	1378
Masukawa*)	1	11	34	163	477	468	174	38	11	1	1378

*) The statistically significant values for Ohata (df = 81) and Masukawa (df = 40) are roughly 0.2 and 0.3, respectively.

Table 2. — Coincidence test of correlated pairs of peroxidase isozyme bands between two forests of *Thujopsis dolabrata* growing in Ohata and Masukawa. Figures in parentheses stand for the expected frequencies based on random assortment.

		Ohata[1]			Total
		—	0	+	
	—	30	57	13	100
		(12)	(75)	(13)	
Masukawa[2]	0	122	910	140	1172
		(136)	(879)	(157)	
	+	8	67	31	106
		(12)	(80)	(14)	
Total		160	1034	184	1378

[1]) For Ohata (d. f. = 81); + : (+1.00) — (+0.20), 0 : (+0.19) — (—0.19), — : (—0.20) — (—1.00)
[2]) For Masukawa (d. f. = 43); + : (1.00) — (0.30), 0 : (0.29) — (—0.29), — : (—0.30) — (—1.00)
χ^2 = 59.77*** (d. f. =4)

thus omitted from the data, 53 bands initially counted being reduced to the final number of 42. Correlation coefficients among these 42 bands were again calculated for both forests. The number of correlation coefficients thus calculated in each population was 861; their frequency distribution is presented in *Figures 5 and 6.*

In *Figure 5,* which represents the correlation coefficients for the Masukawa forest, the fit to a normal curve is good, but the Ohata forest represented in *Figure 6* yields a significantly leptokurtic distribution with the χ^2 value 45.81 against 9 degrees of freedom. The test of coincidence of association among 861 bands between the two forests is given in *Table 3.* The test showed that the χ^2 value was 14.29 against 4 degrees of freedom which was still statistically highly significant. The largest disagreement was found in the (—/—) rectangle of *Table 3,* in which the observed

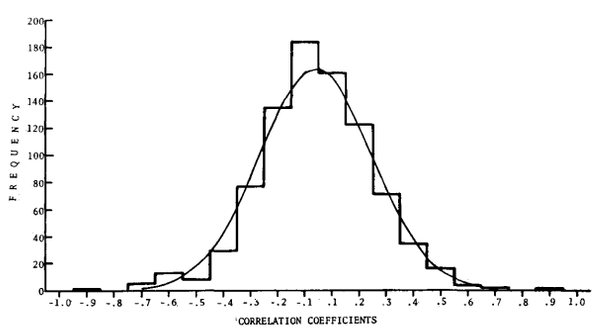

Figure 5. — Frequency distribution of coefficients of correlation among peroxidase isozymes in Masukawa forest.

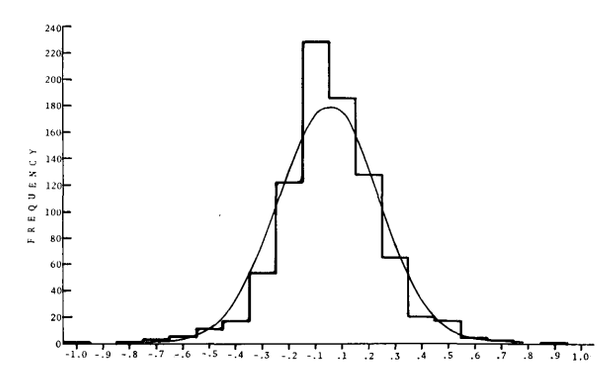

Figure 6. — Frequency distribution of coefficients of correlation among peroxidase isozymes in Ohata forest.

Table 3. — Coincidence test of association of 861 correlated pairs among 42 corrected bands in two forests of Ohata and Masukawa. Figures in parentheses stand for theoretical frequencies expected on the basis of random assortment.

		Ohata			Total
		—	0	+	
	—	14	33	9	56
		(6)	(43)	(7)	
Masukawa	0	67	587	93	747
		(78)	(575)	(98)	
	+	6	42	10	58
		(6)	(45)	(8)	
Total		87	662	112	861

χ^2 = 14.29** (d. f. = 4)

frequency was 14 against the expectation of 6. The cause of this high association is not known, but it is not our present intention to go further into this problem. The number of peroxidase isozymes thus finally identified was 42; on this basis the following study was performed.

2. Genetic variation in peroxidase isozymes in two natural forests

Of 42 peroxidase isozyme bands detected in *Thujopsis dolabrata,* 15 or 16 on the average were counted in individual trees. The frequency of trees with different numbers of peroxidase bands is shown graphically in the histogram of *Figure 7.*

In the Masukawa forest, the range of variation was between 11 and 19 with the mean number of 16.29 ± 2.17, while in Ohata, it was from 9 to 21, the mean number being 15.51 ± 2.76. The difference between the two forests in the range of variation may most probably be due to the difference in population size, i. e. 83 and 45 trees in Ohata and Masukawa, respectively. The frequency of individual bands, however, is not always the same in the two forests as *Figure 8* shows.

In *Figure 8,* the frequency in percent of bands in the Ohata forest is represented by an open bar and that of the Masukawa forest by a solid bar. We find from the figure that one band, No. 12, is present in all trees of both forests. Some bands, on the contrary, had a very low frequency in both forests, for example, Nos. 4, 16, 17, 32 and 40. It happened that band No. 31, which appeared in the Ohata forest, was not detected in the Masukawa forest.

A statistical test was conducted to find if the two forests were significantly different in respect of the occurrence of peroxidase isozymes. This was performed by calculating the measure of divergence between both populations (Berry, 1963). The measure of divergence was obtained using the formula

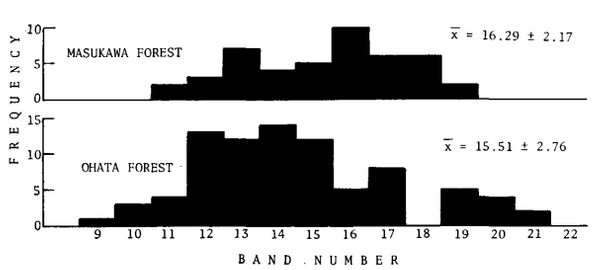

Figure 7. — Frequency distribution of trees with 10 to 22 bands of peroxidase isozymes collected from two forests of Ohata and Masukawa.

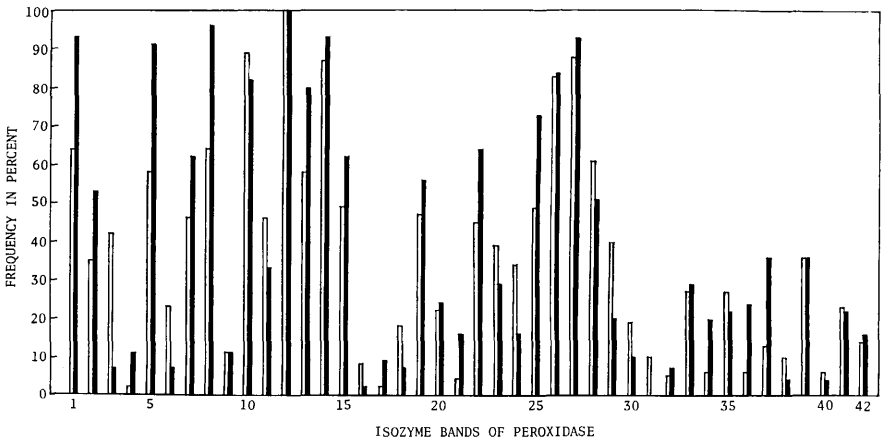

Figure 8. — Frequency in percent of 42 isozyme bands of peroxidase in two forests of Ohata (open bar) and Masukawa (black bar).

$$D^2 = \sum_j [(\Theta_{1j} - \Theta_{2j})^2 - \left(\frac{1}{n_1} + \frac{1}{n_2}\right)]$$

where $\Theta_{ij} = \sin^{-1}(1 - 2p_{ij})$, in which p is the frequency of occurrence of j-th band in i-th forest, and n_i is the number of investigated trees in the same forest. The statistical significance of the measure was tested by comparing it with its variance, i. e. $4\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\sum D^2$.

For further details, the reader may consult BERRY, 1963.

The test revealed that the measure of divergence was statistically significant at the 1% level of probability, $t = 7.27$, with the number of degrees of freedom 41. Since the isozyme pattern is considered to be the reflection of the genotype, we are led to the conclusion that the two populations are genetically differentiated from each other.

3. *Variation in leaf characters*

Several characters of needle-leaves as illustrated in *Figure 2* were measured in the two forests. The intra-forest variation is given in *Table 4*.

It is found from *Table 4* that among six quantitative characters of needle-leaves, two, i. e. vertical length of the lateral leaf and leaf thickness were significantly different between the two forests. The vertical length of the lateral leaf was larger in Masukawa than in Ohata, while as to the thickness of the leaf, the relation was reversed. Looking at other two characters measuring length, it is found that leaf length and vertical length of lateral leaf were more or less larger in Masukawa than in Ohata, though the difference was too small to be regarded as statistically significant

The measurement of the leaf characters allows us to estimate intra-tree and inter-tree variances on the basis of the analysis of variance *(Table 5)*.

It is found from *Table 5* that both forests were not very different from each other with regard to population parameters.

Discussion

Population-genetic studies in forest tree species are rather scanty due to many difficulties encountered in the experimental work with large-sized and long-lived trees. WRIGHT (1962), as well as LIBBY, STETTLER and SEITZ (1969) described in general the problems of geographic variation in forest trees, and discussed the importance of migration, isolation, population size and maintenance of genetic variability.

The object of the present study was to measure the genetic variability in two natural forests of *Thujopsis dolabrata*. They are located in two facing each other promontries of two peninsulas separated from each other by

Table 4. — Variation in six leaf characters in two forests of *Thujopsis dolabrata*. The number of trees investigated is 45 and 83 in Masukawa and Ohata forests, respectively.

Character		Class interval	Class value									Mean	σ	Difference
			-4	-3	-2	-1	0	1	2	3	4			
(a) Leaf length	m	0.4mm	1	3	7	7	9	12	4	2		5.92	0.67	0.15
	o		3	11	15	16	11	20	4	1	2	5.77	0.72	
(b) Width of main leaf	m	0.2mm		6	3	12	12	10	1	1		3.21	0.29	0.07
	o		2	5	18	26	20	9	3			3.14	0.26	
(c) Width of compound leaf	m	0.4mm		2	5	9	16	7	5	1		6.97	0.55	0.20
	o				6	10	30	23	9	5		7.17	0.49	
(d) Length of lateral leaf	m	0.4mm		1	1	4	12	17	5	5		5.32	0.54	0.16
	o			2	2	12	34	18	10	5		5.16	0.54	
(e) Vertical length of lateral leaf	m	0.4mm	1		2	10	17	8	5	2		4.51	0.52	0.39**
	o			6	22	24	14	13	3	1		4.12	0.50	
(f) Leaf thickness	m	0.2mm	2	4	12	15	10	2				1.25	0.24	0.17**
	o			6	13	26	19	10	5	3	1	1.42	0.30	

Table 5. — Within-tree ($\sigma_w 2$) and between-tree ($\sigma_B 2$) components of variance and the estimates of genetic variability in six leaf characters in two forests of Masukawa and Ohata. The unit of measurement is milimeter.

	$\sigma_w 2$		$\sigma_B 2$		H*)	
	Ohata	Masukawa	Ohata	Masukawa	Ohata	Masukawa
(a) Leaf length	0.35	0.42	0.44	0.34	0.55	0.45
(b) Width of main leaf	0.04	0.07	0.06	0.09	0.58	0.56
(c) Width of compound leaf	0.45	0.45	0.16	0.22	0.26	0.33
(d) Length of lateral leaf	0.33	0.43	0.18	0.17	0.36	0.29
(e) Vertical length of lateral leaf	0.32	0.44	0.21	0.21	0.40	0.32
(f) Leaf thickness	0.10	0.06	0.07	0.05	0.42	0.43

*) H is estimated by $\frac{\sigma_B^2}{\sigma_w^2 + \sigma_B^2}$, which is a rough estimate of genetic variability.

a 10 kilometer wide channel in the north and fields, villages and towns in the south *(Figure 1)*. They may probably be little affected by migration from one to the other by seed or pollen. To what maximum distance a pollen grain of *Thujopsis dolabrata* can travel can not be said, but the distance which could seriously affect the genetic constitution of another conifer population seems to be less than a few hundred feet (WRIGHT 1962). It is reported by BANNISTER (1965) that unopened cones of *Pinus* species could travel to distant shores by sea currents, but it may be needless to take this into account in the present situation.

If a single population had been divided into two subpopulations and they had long been spatially separated from each other, they might show genetic differentiation, even if their habitats were not different as to environmental conditions. In the present study, an approach to the problem was made by the aid of peroxidase isozymes observed in the starch-gel electrophoresis of squeezed sap of leaves. In addition, a few quantitative leaf characters were also investigated.

The electrophoretic analysis of enzymes has recently proved to be a useful tool in genetic investigations of various organisms. SHAW (1965), WHITT (1967) and SHANNON (1968) gave general reviews on isoenzymes in animals and plants. According to them, variation in a single enzyme is the rule rather than an exception, and the fact that enzyme activity remains normal in spite of minor structural variations allows a number of interesting applications in genetical research. As a matter of fact, many studies on variation and genetics of isoenzymes in various animal and plant species have appeared of late. Among animals examples are found in *Drosophila* species (LEWONTIN and HUBBY 1966, JOHNSON, RICHARDSON and KAMBYSELLIS 1968), butterflies (JOHNSON and BURNS 1966), fishes (KOEHN and RASMUSSEN 1967, FUJINO and KANG 1968 and FUJINO 1969), frogs (SALTHE 1969), birds (GRUNDER 1968) and small mammals (NIELSEN 1969, SEMEONOFF and ROBERTSON 1968). Studies in agricultural crop plants are described by SHANNON, 1968 and WHITT, 1967. A few papers reporting genetic studies on isoenzymes in rice appeared recently (CHU, 1967 and SHAHI *et al.* 1969, a and b).

In forest trees, however, few studies in biochemical genetics have been reported except the investigations on terpenes (HANOVER 1966, see also LIBBY, STETTLER and SEITZ 1969) and polyphenols (THIELGES 1969) in *Pinus* species. In 1969, probably the first paper on the use of zymography in forest tree breeding appeared (MIYAZAKI and SAKAI 1969). The authors arrived at the conclusion that peroxidase isozyme pattern in *Cryptomeria japonica* was useful in identifying clonal varieties.

In the first part of the present paper, identification of individual isozyme bands by the aid of within-tree correlations among them is described. If any two bands are positively or negatively significantly correlated, then they may indicate either of the following three possibilities:

(1) Occurrence merely by chance of a high correlation between two bands which are primarily by no means correlated.

(2) Genetic causes such as pleiotropy or gene linkage.

(3) Shift in the position of a band, sometimes in combination with variation in its activity.

In the case of (1), bands may merely by chance show themselves as highly correlated. Since the possibility that the same pair of bands in another population would be again by chance highly correlated should be of a rare occurrence, it will be detected by a comparative investigation with other populations.

Genetic mechanisms described in (2) may cause correlation among isozyme bands: Pleiotropy of genes, on the one hand, and linkage, coupling or repulsion, on the other. Correlation between bands due to pleiotropy may possibly be found in every population, while that due to linkage would be variable among populations in dependence upon the amount of linkage disequilibrium involved.

Shift in the position of a band described in (3) may be detected by examining if the seemingly two bands, highly correlated, are associated with each other.

At the start, we provisionally counted 53 bands in the zymogram of *Thujopsis dolabrata*, anticipating that some of them might have to be discarded after adequate examination. The total number of correlation coefficients obtained with 53 bands, was 1378 for each forest. They were found to distribute approximately normally with the mean values of about zero *(Table 1)*. The number of r's exceeding a statistically significant point was 20—25% in either forest. Let us now examine the meaning of those significant correlations, positive or negative.

The χ^2 test presented in *Table 2* revealed that there was certainly an excess in (+, +) as well as (—, —) rectangles over the theoretical expectation. Pairs of bands held in those two rectangles were then examined to find if the members of each pair occurred in conjunction in the zymogram. After this examination, eleven bands were discarded, or 42 bands were finally established.

On the basis of these 42 bands, 861 correlations were recalculated for each population, and they were tested again for coincidence. We find in *Table 3* that negative correlations among bands were still more frequent than randomly expected; the reason for it can not be told at present.

Comparison between the two populations as to the number of isozyme bands possessed per individual tree revealed no apparent difference between them. Frequency of occurrence of each band, however, was not always similar in both populations and the statistical test showed that the two forests could be hardly considered as similar. Dissimilarity was also found in two of the six leaf characters.

Thus, it is concluded that the two forests of *Thujopsis dolabrata*, which are geographically not very remote from each other, but more or less completely isolated by 10 kilometer wide straits, are genetically differentiated on the enzyme level as well as in leaf characters.

It is true that geographic variability in forest trees has been one of the key subjects of study in forest genetics. A few examples of late years are the reports of J. W. WRIGHT and W. I. BULL (1963) and GENYS (1968) for pines, IRGENS-MOLLER (1968) for douglas-fir, and EINSPAHR and BENSON (1967) for quaking aspen. Genetic variation between sub-populations within a limited area, however, has been little investigated. As a matter of fact, CALLAHAM (1967) in his discussion on geographic variation in forest trees, confessed that considerably less is known about genetic differences between adjacent populations than about differences between distant populations of forest trees. It is considered that the zymographic technique as described in the present paper may effectively help us in carrying forward such research work as pointed out by CALLAHAM.

Conclusion

Electrophoretic peroxidase analysis was performed in two natural populations of *Thujopsis dolabrata*, which are separated by 10 kilometer wide straits. At the start of the experiment, 53 bands were provisionally counted. Intra-tree correlations were measured among those 53 bands in order to find out the false ones which are the results of a shift of a single band. Thus 42 bands were finally identified. Some of them were found to be still negatively correlated, though no enquiry into the underlying causes had been undertaken.

The isozymes possessed by an individual tree were much fewer than 42, the average number being 15 to 16. Comparison between two populations with regard to the occurrence of the peroxidase isozymes showed that they were significantly different. The same also holds for some leaf characters.

Thus, it is concluded that the two natural stands of *Thujopsis dolabrata* which are separated by 10 kilometer wide straits are genetically differentiated. Natural populations of a forest tree species usually occupy a larger area of land, running over mountains, valleys, rivers or streams, towns and villages, etc. To what extent differentiation occurs among sub-populations within a land area is an important and interesting problem in forest genetics.

Summary

The present paper mainly reports our finding of genetic variation in leaf peroxidase components of *Thujopsis dolabrata* as demonstrated by starch-gel electrophoresis. At first, as many bands as were apparently different were counted. The number attained was 53. Then, by measuring intra-tree correlation among the bands, the possibly non-existent bands were discarded from the count. The final bands identified were 42.

Two natural forests of *Thujopsis dolabrata* which are separated by straits 10 kilometers wide were examined to find out if they were statistically different with regard to the occurrence of those 42 bands. Several leaf characters were also compared between the two forests. It has been concluded from this study that the two natural forests were genetically differentiated from each other.

Literature Cited

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