Genetic Studies in Natural Populations of Forest Trees

II. Family Analysis: A New Method for Quantitative Genetic Studies1)

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In a previous paper (Sakai, Tomita and Hayashi, 1970), a method was reported for detecting clones in a natural forest of *Cryptomeria japonica* by means of a similarity index constructed on the basis of several needle characters. Thereafter, the present authors have become interested in electrophoretic peroxidase variation in trees. We found that not only detection of clones but also that of families of seed-propagated sib-trees was possible by comparing the peroxidase isozymes among trees of the same forest. Our work was conducted with natural forests of *Thujopsis* dolabrata which grew in Aomori prefecture situated in lat. 40—41° N. and long. 140° 20'—141° 20' E.

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Materials and Methods

In the two natural forests, Masukawa and Ohata, of *Thujopsis* dolabrata, our study started with accurately mapping on a section paper the numbered individual trees. Measurements were taken on individual basis of tree height, stem diameter at breast height, length of the thickness bough and number of its twigs. 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 measured were length and width of the main leaf and width of the compound leaf. A portion of leaf samples were deep-frozen in the laboratory until needed for the electrophoretic study of peroxidase isozymes. The method adopted for electrophoresis was that developed by Smithies (1955). The apparatus used was a horizontal gel mold. The gel was made from 60 g hydrolyzed starch heated in 500 ml. of 0.03 M borate buffer, pH 8.5, containing 0.105 g sodium hydroxide and 18.5 g boric acid per litre. An extract from needles weighing approximately 0.3 g was soaked in a small piece of filter paper (Toyo No 50, 5 X 18 mm^2) which was inserted into a slit of the starch gel at the distance of 8 cm from the cathodic edge. The electrophoretic separation was carried out for 95 minutes at variable voltage gradients between 100 V for the first 10 minutes followed by 400 V for the remaining 85 minutes at a temperature of 7° C. After the separation, each gel was sliced horizontally and the bottom half was used for staining the peroxidase isozymes by the reacting mixture containing 0.3% hydrogen peroxide, 0.2% benzidine acetate and 0.0625 M Tris-acetic acid buffer (0.01 M Tris and 0.0525 M acetic acid) with a pH of 4.0 (Endo, 1968). The reaction was stopped in distilled water when the bands, stained light-blue, appeared after 10 to 20 minutes.

The maximum number of peroxidase isozymes in the present species was 42; a few examples are schematically illustrated in Figure 1.

Comparison between the isozyme patterns of different trees was made by measuring number of azygous bands or the "disagreement count".

Examples of disagreement counts measured on the three zymograms of Figure *1* are as follows: Between 731 and 519 = 13; between 519 and 559 = 9; between 731 and 559 = 15.

Results of Study

During the comparative study of peroxidase isozymes among the trees, there was every indication that the disagreement counts between trees growing in the neighbourhood were often very small in comparison with those between trees growing far apart in the same forest. Such being the case, the disagreement counts, 990 in Masukawa and 3403 in Ohata, were grouped according to the distance between the trees.

Figures 2 *and* 3 represent the frequency distribution of the disagreement counts among trees growing at various

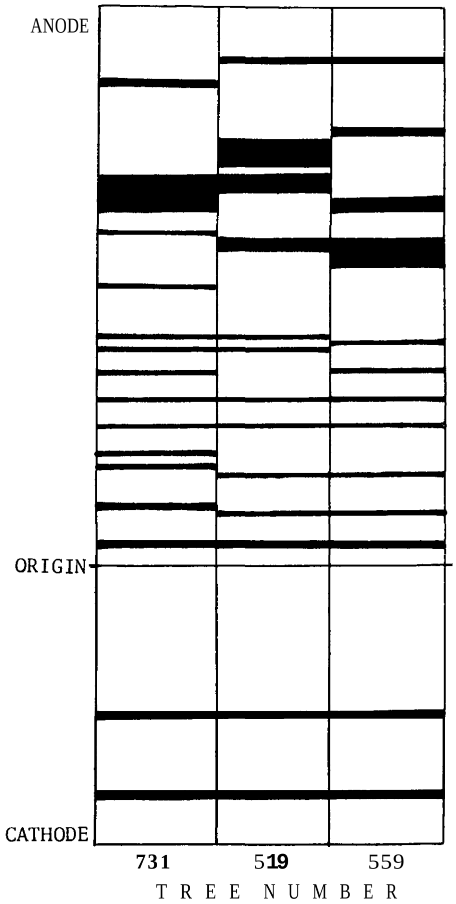

Figure 1. — A schematic illustration of three examples of peroxidase zymograms of ***Thujopsis dolabrata.*** "Disagreement counts" among them are given in the text.

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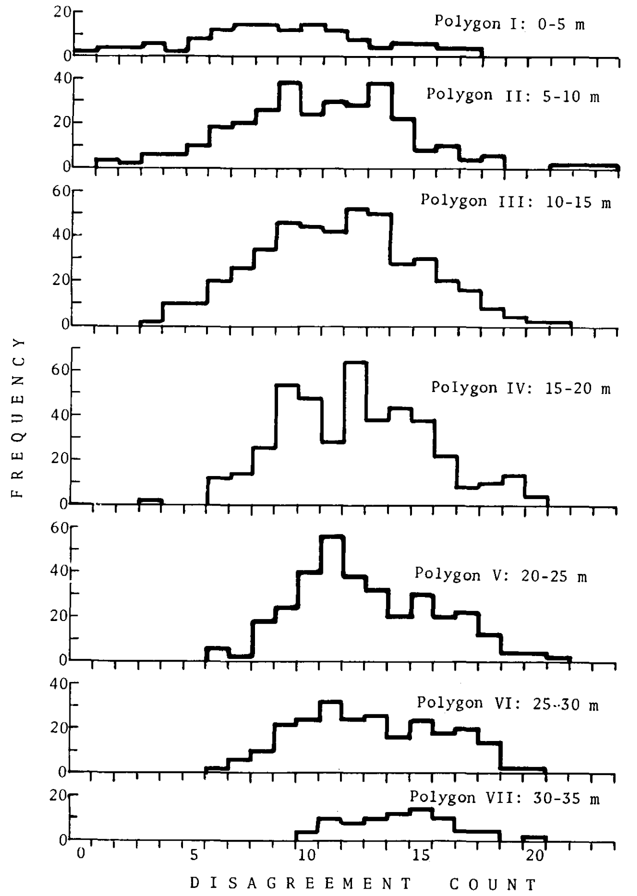

Figure 2. — Frequency distribution of disagreement counts in Masukawa Forest in relation to distance.

distances in the two natural forests. Polygon I shows disagreement counts between trees growing within a circle of 5 meter radius around each central tree. In polygon II, they occur "doughnutted" in an area between two circles of 5 meter and 10 meter radii.

Average disagreement counts in various distance groups, I—X, are shown in *Figure 4.* The horizontal broken line shows the average disagreement count among trees of unrelated forests (See *Fig. 7).* It is found from *Figures 2 and 3* that the very low counts, 0, 1, 2 and 3 are found in I, II, III and IV polygons in Masukawa, and in I, II, III, IV and V polygons in Ohata. The lower disagreement count or higher similarity in the isozyme pattern among trees growing in the neighbourhood as shown in *Figures 2, 3 and 4* might suggest either that (1) similarity in environmental conditions might induce similarity in the isozyme pattern, or that (2) genetically related individuals are standing rather in proximity than being scattered. Hypothesis (1) is not plausible considering an environmental variation which occurs within the area of the same forest.

It may be understood that genetically related individuals are growing in clusters rather than scattered in a forest. It is thus concluded that trees which show low disagreement counts, 0 to 3, may indicate that they would be in some way or other sib-trees. In order to examine if this hypothesis is correct, variance of a few vegetative characters within each group of trees classified according to the disagreement count was investigated. In *Figures 5 and 6,* we find that the within-group variances were very small when the disagreement count was so low as 0, 1, 2 and 3.

The horizontal broken lines in the Figures represent between-tree variance for the whole forest. You may find in general that there is a more or less marked difference in the magnitude of within-group variance between the count-groups 3 and 4 in Masukawa as well as in Ohata forest. These figures suggest that trees which are comparatively similar in isozyme patterns resemble each other also in vegetative characters, and they may most probably be

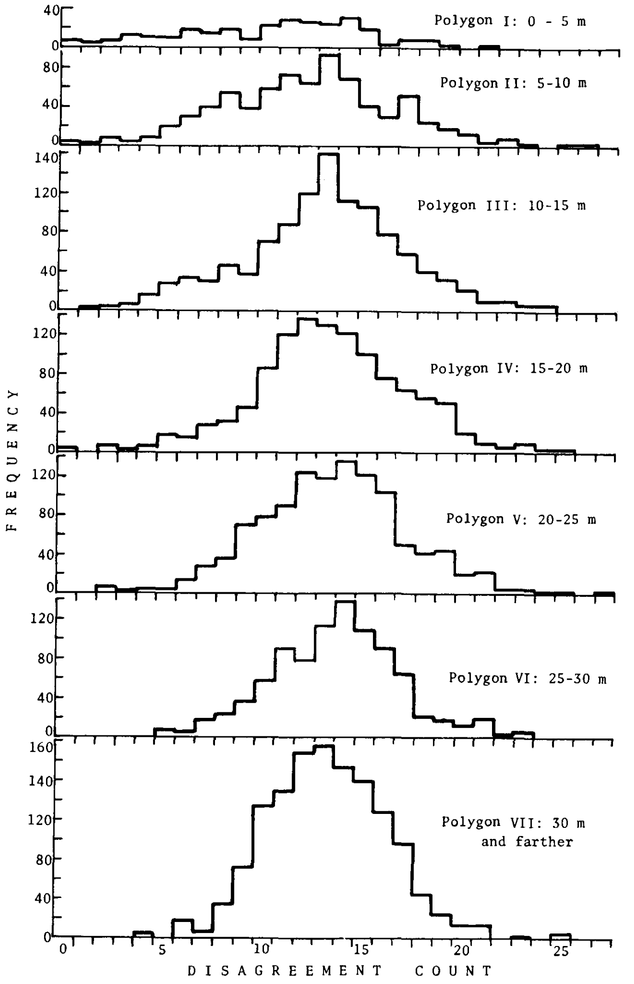

Figure 3. — Frequency distribution of disagreement counts in Ohata Forest in relation to distance.

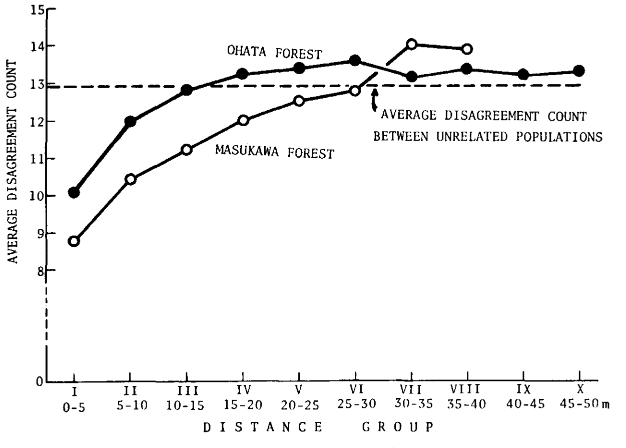

Figure 4. — Average disagreement count in various distance groups in two forests of Ohata and Masukawa.

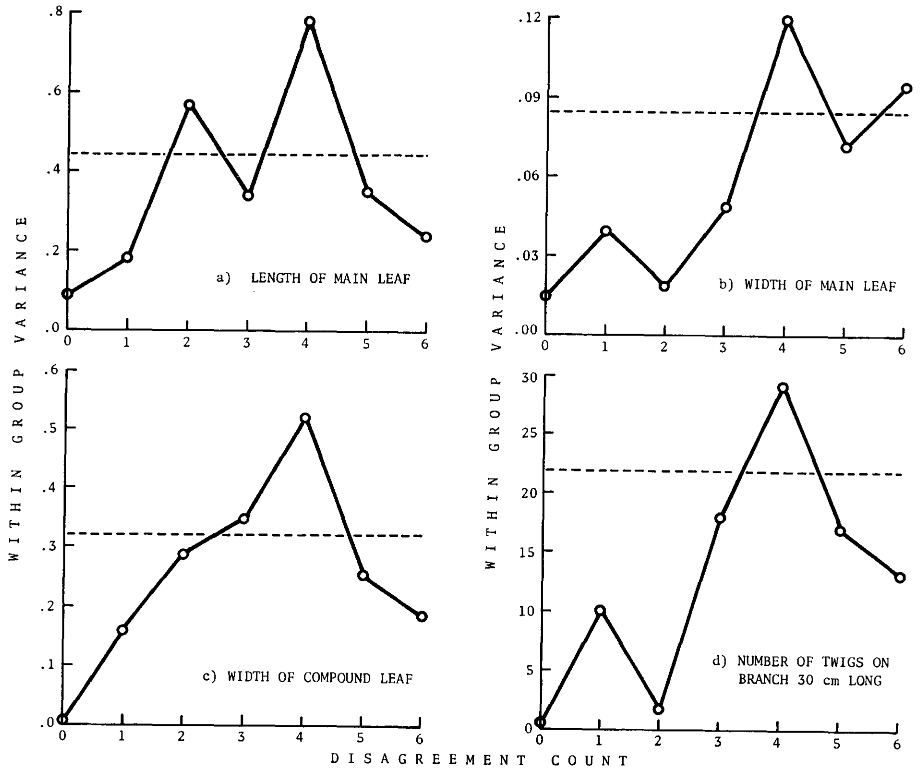

Figure 5. — Variance of four vegetative characters among trees with various disagreement counts of peroxidase isozymes in Masukawa Forest.

genetically related members of the same family. The next problem was to find whether so low a disagreement count as 3 could occur among non-related individuals between different populations. To solve this problem, the disagreement count was examined between trees of Masukawa and those of Ohata forest. These two forests were isolated from each other by about 10 kilometres wide straits. It has been demonstrated by SAKAI, MIYAZAKI and MATSUURA (1971) that they were genetically different.

In *Figure 7*, the frequency distribution of the disagreement counts between trees of Masukawa and those of Ohata forest is represented. It shows that there occurred as low a disagreement count as 3 in four cases among 3432 counts. They may most probably be the result of chance occurrence

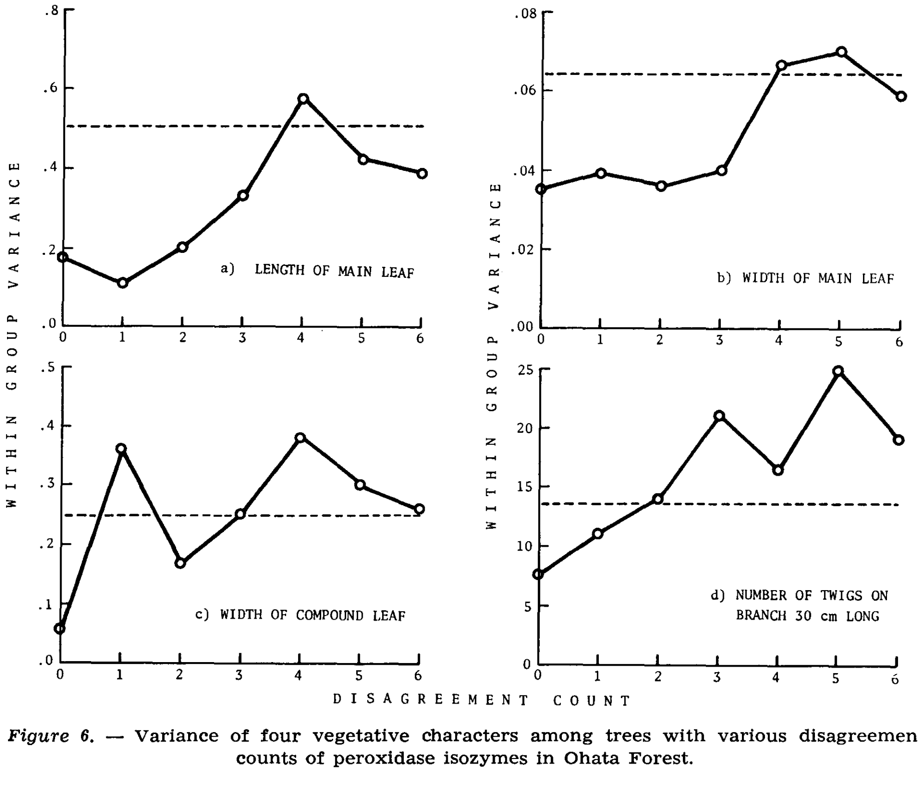

Figure 6. — Variance of four vegetative characters among trees with various disagreement counts of peroxidase isozymes in Ohata Forest.

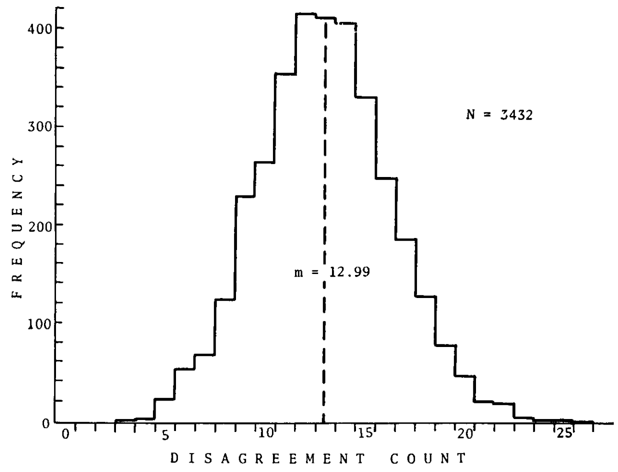

Figure 7. — Frequency distribution of disagreement counts between two geographically isolated populations.

rather than a phenomenon of biological significance. The frequency of occurrence was only 0.12% or one in one thousand. On the basis of the results of study described above, especially from those in *Figure 5 and 6,* we conservatively conclude that disagreement count 0 would indicate vegetative propagules, while the disagreement counts 1, 2, and 3 seed-propagated sib-trees. Some of the trees having 4 and higher disagreement counts might happen to be sibs, but they were disregarded in this study for safety's sake.

We have been thus able to carry out a family analysis in a natural forest and to construct on this basis the family map as shown in *Figures 8 and 9.*

Figures 8 and 9 show the family maps of the two forests, Masukawa and Ohata. A triple line in the figure indicates that trees connected by it are individuals propagated vegetatively, while trees connected by single solid lines are seed-propagated progeny. The encircled letters are the designations of different families.

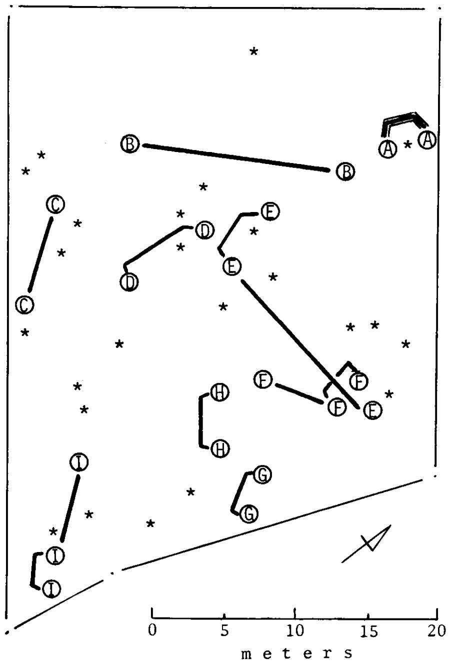

Figure 8. — Family map of 45 trees of different ages in a natural forest in Masukawa. Trees connected with triple bars: clonal propagules. Trees connected with single solid bars: seed-propagated sib-trees. Stars: orphaned trees with no relatives in the forest.

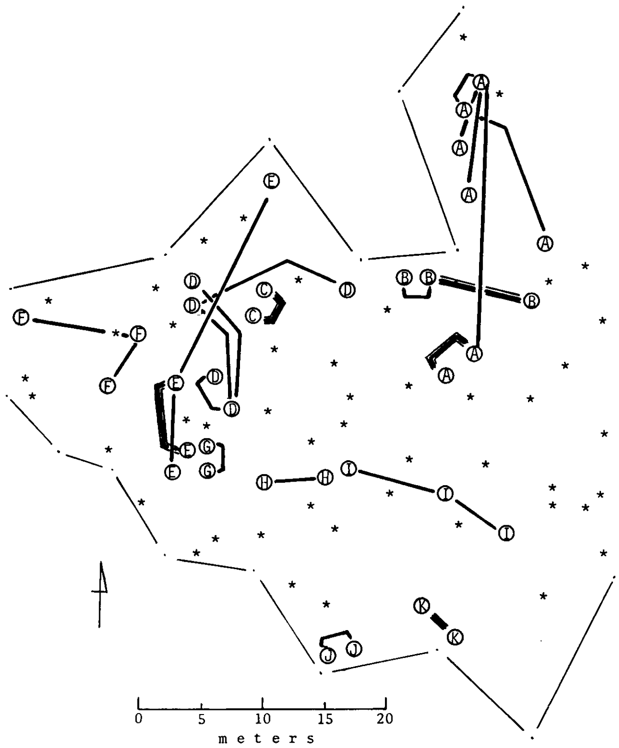

Figure 9. — Family map of 83 trees of different ages in a natural forest in Ohata. Trees connected with triple bars: clonal propagules. Trees connected with single solid bars: seed-propagated sib-trees. Stars: orphaned trees with no relatives in the forest.

Variability in vegetative characters in clones and families is shown in *Figures 10 and 11* for two characters, i. e. the number of twigs on a 30 cm branch and the width of compound leaf. In order to estimate the genetic parameters of some quantitative characters, the analysis of variance of some leaf characters and the number of twigs on 30 cm branches was conducted for between-family and within-family items.

In this analysis of variance, members of the same clone except one and all orphaned trees, that is, trees which grew alone without any relatives, were discarded. Addition of orphaned trees, however, did not seriously affect the estimation of parameters.

In the ordinary genetic study, the between-family variance component or $\hat{\sigma}^2_B$ is genetically interpreted as $1/4\ \sigma^2_G$ for the half-sib family on the assumption that the members of the population have been and are random-mated. In the present case, however, this assumption seems not to be applicable, because $4 \times \hat{\sigma}^2_B$ was in every case larger than $\hat{\sigma}^2_W$ which is assumed to be $\sigma^2_E + 3/4\ \sigma^2_G$. As will be described later, there is reason to assume that inbreeding either by self-fertilization or by hybridisation between sib-trees has occurred in the natural forest of *Thujopsis dolabrata,* bringing forth a more or less high degree of homozygosity.

If this is actually the case, then the seed-progeny from a single mother tree or members of a maternal family may exhibit great similarities, and we may not be right to adopt an ordinary genetic interpretation of $\hat{\sigma}^2_B$ and $\hat{\sigma}^2_W$. It may be possible in some cases that clones might be unconsciously involved in seed-propagated families though, so far as we could detect them, they were beforehand discarded.

Taking into consideration this partial uncertainty, we are to use a new genetic parameter which will be use in selection work, namely the degree of family likeness or the intrafamily correlation, M, expressed as

$$M = \frac{\hat{\sigma}^2_B}{\hat{\sigma}^2_B + \hat{\sigma}^2_w}$$

This is the same as intraclass correlation in the statistical sense, but a new designation here recommended may help the tree breeder in the practical use of this parameter.

Discussion

Natural forests are widely distributed over the earth. They have probably lasted for an infinite number of generations naturally reproducing by seed or clones Accordingly, natural forests are without exception a very complex mixture of trees of different ages, of different species and of different densities.

Natural populations of some animals, e. g. small mammals, as rats and mice, or insects, for instance, *Drosophila* and butterflies, have been quite often the subject of genetic study, greatly contributing to the development of the sciences of genetics and evolution. Those animals are suited for experimental study, because when captured in nature they are liable to propagate in a relatively short time in the laboratory and observations on adults can be easily made. Trees are, on the contrary, not at all suitable for such a breeding experiment. It is needless to say that trees require several decades to reach maturity, they require a large area of land for growing so many individuals usually necessary in genetic study. Thus, we should probably abandon making any genetic approach to natural forests by conventional methods.

The present development of industry in Japan has induced too much emigration from the country side or mountain villages, resulting in a heavy shortage of laborers for artificial planting. Under such circumstances, the importance of natural forests or extensive forestry will become greater than before, replacing laborious artificial planting or intensive forestry. A genetic study of natural forest will have to be exploited without delay. The electrophoretic variation of peroxidase in tree species has allowed us to identify families in natural forests, and we are able to find the genetic nature of quantitative or physiological characters in those forests.

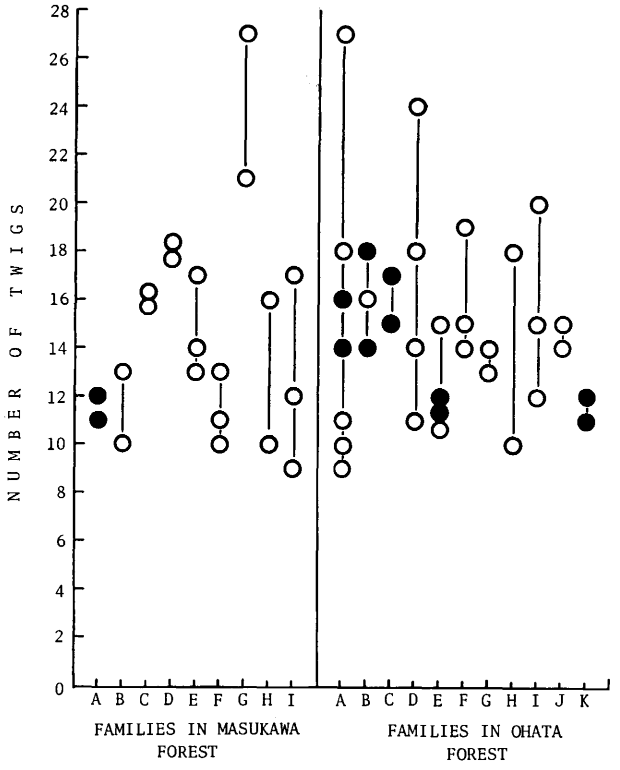

Figure 10. — Variability in number of twigs on 30 cm. branch in clones (solid circles) and families (open circles).

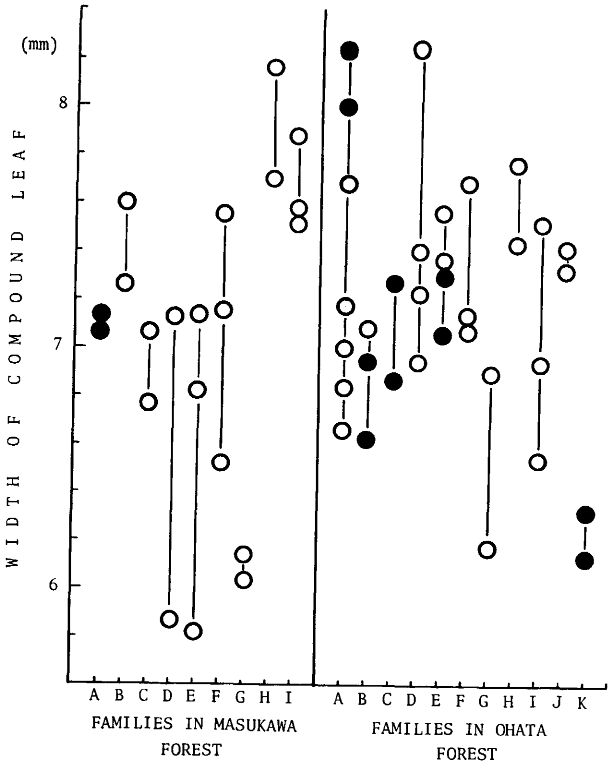

Figure 11. — Variability in width of compound leaf in hypothetical clones (solid circles) and families (open circles).

A character is considered to be heritable if its conformation is similar in the sib-trees within a family. In other words, an efficient selection of trees will be made by taking into account members of the family and comparing them with other non-related trees. Or, selection of a mother tree can be made with certainty when a given character is found in its sib-trees.

In this study, it was of interest that trees growing in proximity were found to show a high similarity in the isozyme patterns of peroxidase. Granting that environmental variations as found within an actual forest should not influence the phenotypic appearance of the isozymes, it can be concluded that genetically related trees may grow in proximity possibly forming clusters in the natural forest. It is expected that the genetic contributions from the maternal and paternal sides would be of the same importance to the isozyme pattern of the offspring.

The higher similarity in the isozyme pattern among trees growing within a circle of 20 or 25 meters may indicate that the pollen parent must have been standing in the same area. Were it not so, or if pollen had come freely from any tree in the forest, then trees showing similar isozyme patterns, should have been found not only within the distance of 20 or 25 meters but also on other remote places. If inbreeding is usual in forests of *Thujopsis dolabrata*, it may be concluded that its trees in natural forests may be more or less homozygous. We have had to define a new parameter M for the degree of family likeness, which would mediate between true heritability as considered mostly in animal breeding and its estimation in the broadest sense by comparing a seed-propagated forest with a forest consisting of single clone.

Heritability values in forest trees can be measured in various ways (see Hattemer, 1963; Namkoong, Snyder and Stonecypher, 1966; Burley, Burrows, Armitage and Barnes, 1966).

However, most of those methods can hardly be useful in natural forests, because trees in natural forests are of varying age, from as young a tree as 30 years old to so old a tree as 3000 years or older. The method of family analysis developed in this study will be useful for genetic study of quantitative as well as physiological characters in natural forests. The estimation was quite successful for Masukawa forest, but in Ohata, three or two out of four characters failed to yield successful estimate of M. We do not know at present why the estimation failed, the answer must wait until forther study.

Sakai, Hayashi and Tomita (1970) have published a paper dealing with detecting different clones by means of the identity index measuring the extent of similarity in several vegetative characters among trees. It seems, however, that the use of peroxidase isozymes would be far better than that of vegetative characters, since comparative measurements of vegetative characters may allow us to identify clone members, but not family members.

Conclusion

On the basis of an electrophoretic variation in the peroxidase, genetically related trees of different ages growing in a natural forest could be identified. They are grouped into several families according to their relationships. The reasons why we did use the isozyme patterns as a decisive trait, are that (1) they are affected by environmental conditions, (2) trees growing in proximity show similar patterns which can not be found in trees growing in remote places, (3) variation in vegetative characters among trees which are similar in the isozyme pattern is small in comparison with that among trees selected at random.

There was an indication that trees in natural forests of *Thujopsis dolabrata* were more or less homozygous, which presumably stood in the way of adopting the methods of estimation of heritability generally used by zoologists who assume random or some other types of mating. Thus, we had to define a new parameter, M, for the degree of family likeness or the intrafamily correlation computed by the formula

$$M = \frac{\hat{\sigma}^2_B}{\hat{\sigma}^2_W + \hat{\sigma}^2_B}$$

The present technique of separation of families in a natural forest will be called "the family analysis".

Summary

1. Two natural forests of *Thujopsis dolabrata* were investigated for the electrophoretic peroxidase variation.
2. It was found that trees alike in the isozyme pattern were growing within a circular area of about 20 or 25 meter radius. It is considered that genetically related trees are growing in proximity and are not scattered.
3. Variation in a few characters among trees of similar isozyme patterns showed that the variance of the characters among those trees was so small that they had to be considered to be members of the same family.
4. There is an indication that trees in a natural forest of *Thujopsis dolabrata* are more homozygous than expected from random mating.
5. The technique of "family analysis" described in this paper may enable us to carry out a genetic analysis of physiological and quantitative characters in a natural forest.

Zusammenfassung

Die Peroxidase-Variation von 2 natürlichen Beständen von *Thujopsis dolabrata* wurde elektrophoretisch untersucht. Bäume mit ähnlichem Isoenzym-Muster standen auf einer Fläche von etwa 20 bis 25 m Radius, d. h. genetisch verwandte Bäume wuchsen in unmittelbarer Nachbarschaft. Die Variation bei einigen Merkmalen dieser Bäume mit ähnlichen Isoenzym-Mustern war so klein, daß sie derselben Familie angehören konnten. Dies besagt, daß die Bäume eines natürlichen Bestandes von *Thujopsis dolabrata* homozygoter sind, als bei einer Zufallspaarung erwartet werden sollte. Die beschriebene Technik der "Familien-Analyse" macht es möglich, physiologische und quantitative Merkmale in einem natürlichen Bestand genetisch zu analysieren.

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