

Genetic Studies in Natural Populations of Pinus

I. Genetic Variability in Local Populations from Several Prefectures*

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INTRODUCTION

The Japanese black pine, *Pinus Thunbergii Parl.* is one of the most popular tree species growing wild in Japan. It grows mainly along the seacoast of the islands, forming a continuous belt of distribution, facing Japan Sea as well as Pacific Ocean. Little is known, however, about the extent of genetic variation among local populations. We do not know about the distance to which gene flow occurs through the dispersal of either pollen or seed. Thus we have little knowledge to what extent the pine populations are genetically differentiated.

Since 1970, the senior author and his collaborators have succeeded in ascertaining the genetic variation involved in natural populations of *Thujopsis* (Sakai, Miyazaki and Matsuura, 1971), *Cryptomeria* (Sakai and Park, 1971) and *Abies* (Matsuura and Sakai, 1972) with the aid of electrophoretic variation of isoperoxidases in leaf sap. It has been elucidated by those investigations that natural populations of forest trees involve a good deal of spatial differentiation among populations in addition to the within-population differentiation by spontaneous inbreeding.

In the present study, the writers intend to make a survey on genetic variability involved in the natural populations of *Pinus Thunbergii* with the aid of isoperoxidase variation.

The study is partly assisted by the grant for scientific research from the Ministry of Education for which the senior writer is very grateful. Collection of needle-leaves in local populations was helped by the expert foresters in Niigata, Miyagi, Tottori, Shizuoka and Hiroshima Prefectures. To all of them, the writers have to express their hearty gratitude.

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MATERIALS AND METHODS

Needle-leaves were collected on an individual tree basis in natural populations of *Pinus Thunbergii*. The places where needle-leaves were collected are shown in Figure 1.

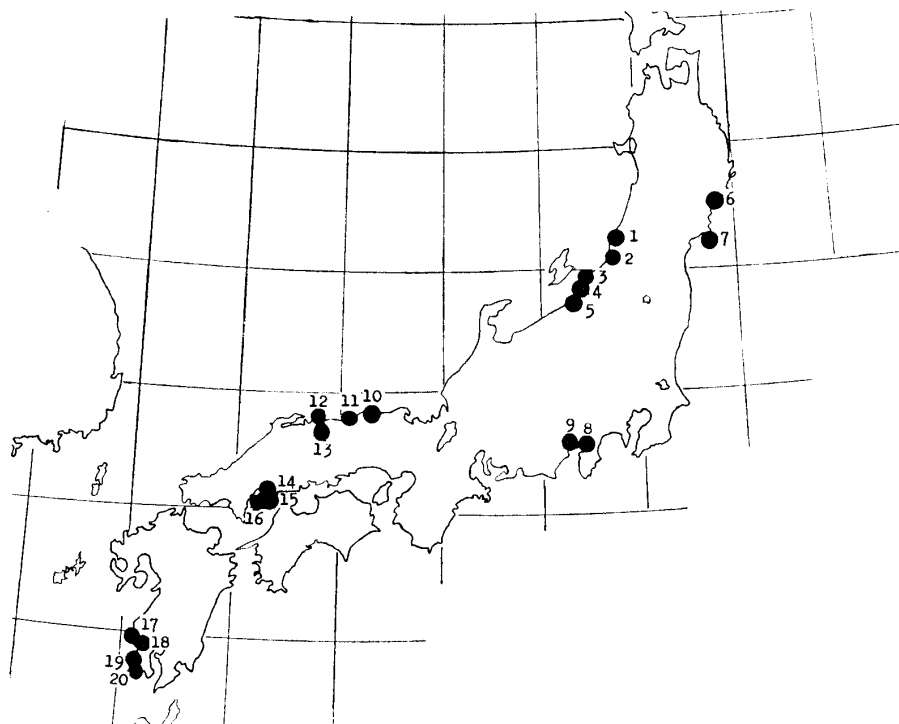


Fig. 1. A sketch map of Japanese islands showing sites of collection of needle-leaves of *Pinus Thunbergii*. 1-5 : Niigata Pref.; 6-7 : Miyagi Pref.; 8-9 : Shizuoka Pref.; 10-13 : Tottori Pref.; 14-16 : Hiroshima Pref.; 17-20 : Kagoshima Pref.

Collected needle-leaves were preserved in a refrigerator kept at -20°C for some time until they were squeezed to have the leaf sap taken for the study of isoperoxidase by the starch-gel electrophoresis. The method adopted for electrophoresis was the one developed by SMITHIES. The apparatus used was a horizontal gel mold. The gel was made from 60 g of hydrolyzed starch heated in 500 ml of 0.03 M borate buffer, pH 8.5, containing 0.105 sodium hydroxide and 0.9g boric acid. The electrode buffer, pH 8.5, contained 3.4 g sodium hydroxide and 18.5 g boric acid per litre.

Extract from needles of approximately 0.3 g was soaked in a small piece of filter paper which was inserted into the right half portion of a slit of the starch gel at the distance of 8 cm from cathodic edge. In the remaining left half portion, another small piece of filter paper soaked with needle-extract from a standard individual was inserted. The individual which was to serve as the standard was selected on the occasion of the examination.

The electrophoretic separation was carried out for about 115 minutes at the voltage

gradients of 350 V. The treatment-period of each gel was decided by the movement of Bromo-Phenol-Blue (B. P. B.) simultaneously set with the same gel. After the treatment, each gel was sliced horizontally and the bottom half was used for staining peroxidase isozyme by the reacting mixture. The mixture (300 ml solution) contained 1.5 ml 3 % hydrogen peroxide, 146 mg o-Dianisidin, 86 mg β -naphthol, 30 ml acetone, 30 ml 0.1 M Tris-acetic acid buffer with pH of 4.0 and 238.5 ml distilled water.

The isoperoxidase bands investigated in our material are diagrammatically represented in Figure 2. In this figure, we find two bands on the cathodic side and twelve on the anodic side.

RESULTS

The investigation started by counting the number of isoperoxidase bands in individual trees. Table 1 presents results of the study in the form of frequency distribution in twenty populations.

We find from Table 1 that there are distinct differences among prefectures in the average number of isoperoxidases per tree. For example, populations from Niigata, Miyagi or Shizuoka prefecture, or north-eastern parts of Japan manifested 4.45 to 5.54 isoperoxidase bands per tree, while populations from Kagoshima prefecture, or from south-western districts, 7.60 to 8.92 bands. Populations from Tottori and Hiroshima prefectures were intermediate, *i.e.* from 5.72 to 7.63. Thus it looks as if there is a geographical cline in the number of isoperoxidase bands manifested per individual tree.

Figure 3 and 4 are graphs showing relationship, if any, between the number of isoperoxidase bands per tree and the latitude or the longitude. Apparently, they bear so striking a resemblance to each other that it is difficult to determine whether or not the number of isoperoxidase bands is related more closely to the latitude than to the longitude. The correlation coefficients among the average number of peroxidase bands (X), the latitude (Y) and the longitude (Z) were: $r_{XY} = -0.8441$, $r_{XZ} = -0.8614$ and $r_{YZ} = +0.9117$. Hence it becomes necessary to find out to what extent X is substantially correlated with Y or Z. To answer this question, partial correlation was investigated among X, Y and Z in the twenty populations.

$$R_{XY.Z} = -0.2717$$

$$R_{XZ.Y} = -0.4167$$

It seems right to conclude from the partial correlation coefficients given above, that the number of isoperoxidase bands has something to do with the longitude rather than with the latitude.

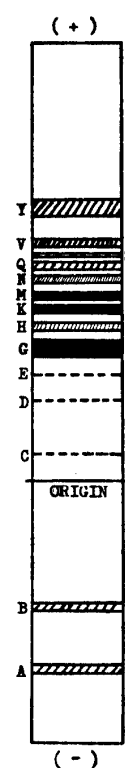


Fig. 2. A schematic representation of isoperoxidase bands in *Pinus Thunbergii* revealed in an electrophoretic starchgel stained by ortho-dianisidine solution.

Table 1. Number per tree of isoperoxidase bands in 20 native populations of *Pinus Thunbergii*.

Number	Prefecture	Location	Number per tree of isoperoxidase bands													Number of trees	Average	Standard deviation		
			0	1	2	3	4	5	6	7	8	9	10	11	12				13	
1	Niigata	Senami					6	27	9	2	1							45	5.22	0.823
2		Fujizuka				6	15	18	2	1								42	4.45	0.889
3		Yotsugoya		1	2	9	26	5										43	4.74	0.790
4		Mase		1	4	11	18	4	1									39	4.59	0.993
5		Osaki			1	5	20	8	3									37	5.19	0.877
		Total	2	13	46	109	28	7	1								206	4.84		
6	Miyagi	Okama	1		13	20	28	9	2								73	5.49	1.095	
7		Kurosaki		2	8	23	36	9									78	5.54	0.922	
		Total	1	2	21	43	64	18	2								151	5.52		
8	Shizuoka	Numazu	2	16	22	12	1										53	4.89	1.054	
9		Ipponmatsu		1	13	16	3										33	4.64	0.700	
		Total	3	29	38	15	1										86	4.79		
10	Tottori	Haneo			8	14	26	10	4								62	5.81	1.069	
11		Natsudomari			1	13	26	11	1								52	5.96	0.791	
12		Nawa		1	3	14	15	16	2	1							52	6.00	1.155	
13		Daisen			4	19	23	6	1		1						54	5.72	1.036	
		Total	1	16	60	90	43	8	1	1						220	5.87			
14	Hiroshima	Kure			2	4	14	6	2	1	1						30	7.30	1.291	
15		Kurahashi			1	2	12	7	3								25	7.63	0.952	
16		Ondo-Hata			6	9	10	2									27	6.30	0.912	
		Total			9	15	36	15	5	1	1					82	6.99			
17	Kagoshima	Sendai			1	4	9	10	12	1	1						38	8.92	1.260	
18		Kushikino			1	3	8	15	9	2	1						39	7.97	1.203	
19		Oura			1	1	6	8	15	7	2						40	7.60	1.293	
20		Bonotsu				3	11	23	4								41	7.68	0.756	
		Total	1	2	13	31	62	30	16	2	1					158	8.03			

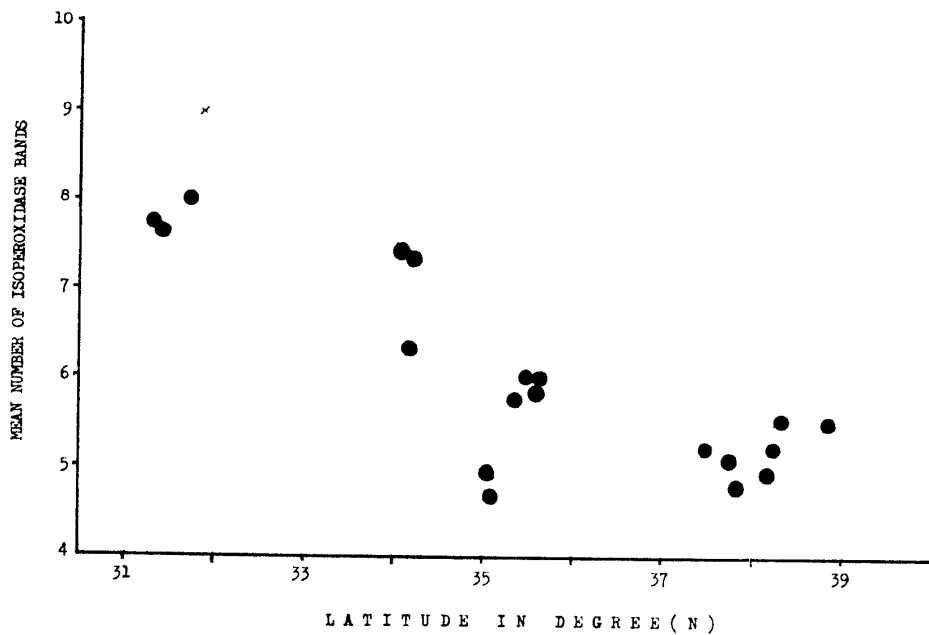


Fig. 3. Relation between the number of isoperoxidase bands in *Pinus Thunbergii* and the latitude at origin.

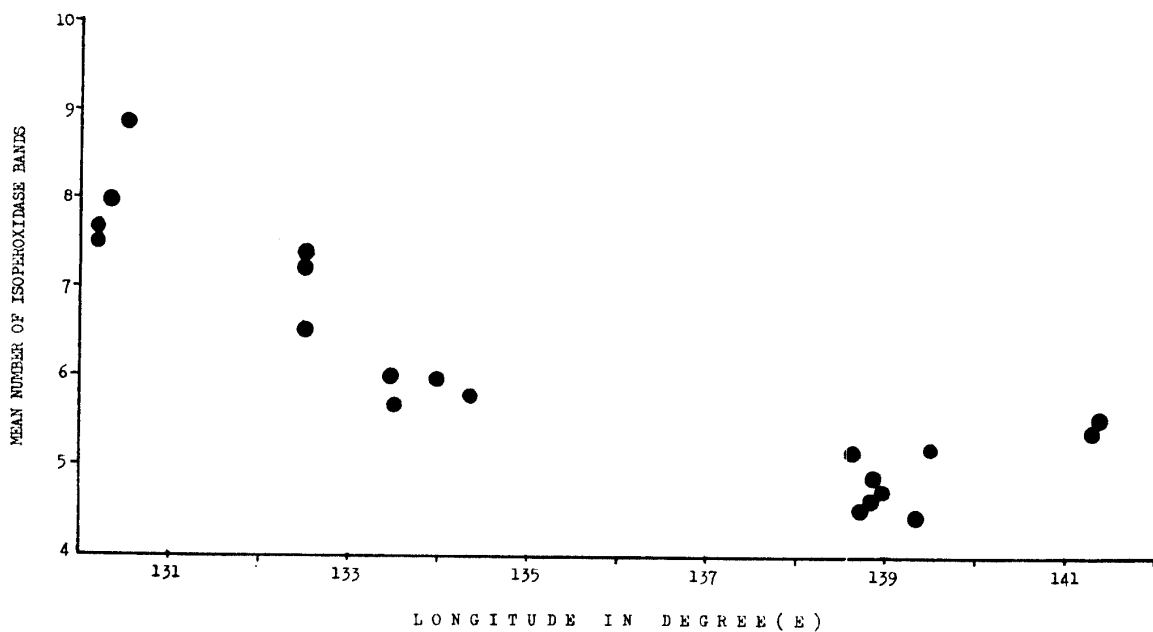


Fig. 4. Relation between the number of isoperoxidase bands in *Pinus Thunbergii* and the longitude at origin.

Incidence of each of 14 isoperoxidase bands was measured in the twenty populations, the results of which are presented in Table 2 and Figure 5. In Table 2 and Figure 5, we first notice that there are a few bands which are almost always present in every individual tree. They are those bands designated as G and M. Incidence of other bands,

Table 2 Frequency of occurrence in percent of isoperoxidase bands in 20 populations from six prefectures of *Pinus Thunbergii*.

Number	Prefecture	Location	Number of trees	Isoperoxidase band(%)													
				A	B	C	D	E	G	H	K	M	N	Q	S	V	Y
1	Niigata	Senami	45	2	2	0	0	0	100	18	96	100	89	16	0	0	98
2		Fujizuka	42	0	0	0	0	100	2	95	100	64	5	2	0	0	76
3		Yotsugoya	43	2	0	0	2	100	9	84	100	81	7	0	0	0	88
4		Mase	39	5	0	0	0	100	10	95	97	74	10	0	0	0	54
5		Osaki	37	3	3	0	0	100	14	95	100	97	27	0	0	0	81
		Total	206	2	1	0	0.5	0	100	11	93	100	81	13	0.5	0	80
6	Miyagi	Okama	73	3	4	0	3	0	100	67	67	100	96	23	1	0	85
7		Kurosaki	78	0	1	0	0	100	63	87	99	95	17	0	0	0	92
		Total	151	1	3	0	1	0	100	65	77	99	95	20	1	0	89
8	Shizuoka	Numazu	53	4	0	0	0	100	11	94	98	92	19	0	0	0	64
9		Ipponmatsu	33	12	9	0	0	6	100	12	97	97	45	6	0	0	76
		Total	86	7	3	0	0	2	100	12	95	98	74	14	0	0	69
10	Tottori	Haneo	62	6	0	0	0	100	50	90	100	95	52	3	0	0	76
11		Natsudomari	52	12	0	0	2	100	54	94	100	94	44	2	0	0	92
12		Nawa	52	13	6	0	4	2	100	60	81	98	92	44	0	0	96
13		Daisen	54	13	2	0	0	0	100	13	85	100	98	65	6	0	93
		Total	220	11	2	0	1	1	100	44	88	100	95	51	3	0	89
14	Hiroshima	Kure	30	10	0	7	7	3	100	80	97	100	100	93	33	7	97
15		Kurahashi	25	20	12	8	8	0	100	96	88	96	100	96	20	0	96
16		Ondo-Hata	27	4	0	0	0	0	100	59	100	100	93	74	4	0	96
		Total	82	11	4	5	5	1	100	78	95	99	98	88	20	2	96
17	Kagoshima	Sendai	38	97	3	0	3	3	100	74	97	82	100	100	58	0	97
18		Kushikino	39	95	0	3	0	0	100	85	100	64	100	95	31	0	100
19		Oura	40	84	0	3	3	0	100	63	100	100	100	88	13	3	90
20		Bonotsu	41	100	0	2	0	0	100	66	98	100	98	90	12	0	98
		Total	158	94	1	2	1	1	100	72	99	87	99	93	28	1	96

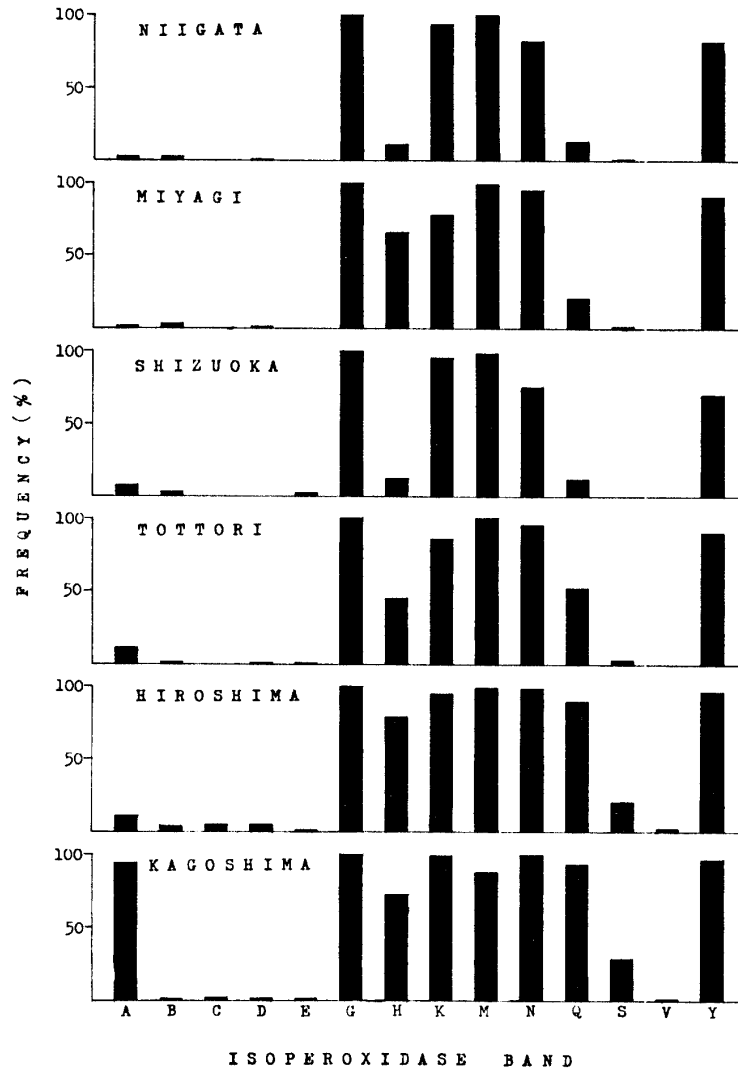


Fig. 5. Frequency of occurrence of 14 isoperoxidase bands in native stands of *Pinus Thunbergii*.

however, are variable from population to population.

We may compare incidences of A band among different populations as an example. In the north-eastern or eastern prefectures, *i. e.* Niigata, Miyagi and Shizuoka, the incidence is so low as 2, 1 and 7% respectively, while in Tottori or Hiroshima prefecture, it is 11% and in Kagoshima prefecture it is so high as 94% (See Table 2 and also Fig. 5).

The similar tendency is also found in other bands such as H, Q and S. It is worthy of noticing that the incidence of a given isozyme band increases in general from north or north-eastern to south or south-western prefectures just as the number of isozyme band does, although the band H seems to be a different case: its incidence is estimated to be so high as more than 60% in two populations of Miyagi prefecture.

DISCUSSION

It is a matter of common knowledge that natural populations of a forest tree species involve a good deal of genetic variation between as well as within populations. A number of papers dealing with geographic variation have been published and they seem to have aroused the interests of forest tree breeders of the world to perform the provenance-tests on an international scale.

We may mention several examples of genetic variation in natural populations of forest trees. GENYS (1968) investigated growth characters of seedlings of *Pinus strobus* collected from 119 natural stands distributed between 49° N to 35° N. Findings from this experiment are that some characters such as 1000 seed weight, growth rate, terminal bud formation, etc. were negatively correlated with the latitude of origin, while germination rate was negatively correlated with the elevation. Geographic variation in growth characters in seedlings of *Pinus strobus* was also investigated by WRIGHT, LEMMIEN and BRIGHT (1970), or in *Pinus resinosa* by YAO, PITCHER *et al.* (1971).

It was found by ALLEN (1969) that eight local populations of *Pinus echinata* were variable with regard to respiration and some other physiological characters of seedling roots. Of interest in his paper was that the root respiration was positively correlated with the annual temperature at seed origin. Growth of roots in seedlings was investigated in four geographic populations of *Pinus nigra* by RÖHIG (1969). He failed, however, to detect significant difference associated with seed provenances. BROWN (1969) made a comparative study on root development in seedlings of *Pinus sylvestris* collected from 54 provenances in Europe. He found that development of tap and lateral roots or root length was variable among provenances. MASCHNING (1971) found that the number of cotyledons in *Pinus contorta* was variable among provenances. It was positively correlated with seed weight which was negatively correlated with the latitude of provenance. Wood formation of xylem characteristic in *Pinus banksiana* was also different among the provenances (KENNEDY 1971).

A few more papers reporting genetic variation in natural populations of forest trees other than *Pinus* may be cited. MORGENSTERN (1969) investigated genetic variation in seedling characters in *Picea mariana* in order to ascertain if genetic differentiation in this species is either clinal or clinal and ecotypic. His conclusion was that variation in *Picea mariana* was essentially clinal and he could obtain little evidence for ecotypic variation. LESTER (1970) investigated geographic variation in *Abies balsamea*. He found that populations of the species were very variable with regard to seedling height, growth rate and frost resistance. He also found that intra-population variation was as great as inter-population variation. OKADA, MUKAIDE *et al.* (1973) reported result of their study on genetic variation in local populations of *Abies sachalinensis* in northern Japan. They found that growth characters of seedlings were very variable among populations. Of interest was that the number of layers of inner bud scales in relation to winter coldness was distinctly different among the provenances.

In other coniferous species, we may mention two papers published by Wright and his colleagues. One is an investigation performed by WRIGHT, KUNG and their collaborators (1971) on geographic variability in native stands of *Pseudotsuga menziesii*. According to them, the populations exhibited very large differences in growth rate and date of bud-

set. They concluded that the coastal and interior populations were very distinct genetically. Another work is study on geographic variation in *Larix leptolepis* conducted by FARNSWORTH, GATHERUM *et al.* (1972).

They found that stem form, time of growth initiation, damage by spring frost, time of leaf fall, and growth rate were different among the provenances. Of special interest was that the correlation between climate at places of origin and performance were too weak to indicate strong cause-and-effect relationships. Thus, they ascribed most of the variations to genetic drift.

Reviewing the papers dealing with geographic variation in forest trees as described above, we find that some characters are apparently related to the climatic or ecological conditions of provenance, but others are most likely not, though variation from provenance to provenance is apparent. In other words, there are doubtlessly those characters whose inter-provenance variation might be ascribed to genetic drift rather than to ecotypic variation.

The study on the electrophoretic variation of enzymes in organisms has made a great progress in recent years, especially for surveying genetic variation in natural populations of wild animals including insects, fishes and birds. We assume for an isoenzyme that: (1) its appearance in the zymogram is governed by the relevant gene, being little affected by usual environmental conditions, (2) each of the isoperoxidase bands is free from any selective pressure, that is, selectively neutral, and (3) the appearance of the isoenzyme patterns is repeatable except for slight variation due to an experimental error.

In connection with the second assumption, *i. e.* selective neutrality of individual isoenzyme band, there are a few papers stating opposite opinions.

In *Drosophila*, WILLS and NICHOLS (1971) investigated the effect of octanol dehydrogenase isozyme on heterosis in the fly. They found that male flies heterozygous for ODH gene were heterotic on a media including octanol, and they concluded that genes for isoenzymes might have a bearing on fitness of the fly. POWELL (1971) found in *Drosophila willistoni* that varied environments increased the frequency of heterozygosity in genes for 22 enzymes. They concluded that some, at least, of polymorphic proteins should not be neutral in natural selection. GAINES, MYERS, and KREBS (1971) made a comparative study for fitness among genotypes for transferrin polymorphism in vole populations. They concluded that an allele for the polymorphic transferrin was related to fitness of the animal. TSAKAS and KRIMBAS (1970) reported that the esterase phenotype of the olive fruit fly, *Dacus oleae*, was related to the resistance of the fly against the insecticide dimethoate, though in their later paper, they appeared to have rejected this hypothesis. Notwithstanding those reports, we are at present inclined to accept the view that each isozyme would be selectively neutral.

In forest tree genetics, there are several papers dealing with isozymes. RASMUSON and RUDIN (1971) examined esterase isozymes in needles of *Pinus sylvestris*. They found that the isoesterase patterns were clone-specific and that little influences of grafting or location were observed. FERET (1971) made an investigation on esterase and peroxidase zymograms in F_1 , S_1 and open-pollinated progeny of several trees of *Picea glauca*. Although he failed to find out any genetic basis for isozymes, he could realize that isoenzyme patterns in progeny were correlated with those of parental trees.

In 1969, MIYAZAKI and SAKAI reported that vegetatively propagated *Cryptomeria* varieties could be identified by the aid of isoperoxidases and the usefulness of electrophoretic variation in forest tree breeding has been further exploited.

In natural stand of forest tree species, investigation on the occurrence of vegetative propagation, on the one hand, and detection of the members of seed propagated families, on the other, have been made on the basis of electrophoretic variation of peroxidase in *Thujaopsis dolabrata* and *Cryptomeria japonica* (SAKAI, IWAGAMI and MIKAMI 1971; SAKAI, IYAMA *et al.* 1972; SAKAI and MIYAZAKI 1972).

Taking an extensive view in natural populations of animals and plants, isozymes are of great use for the study of genetic diversity. For an example, we may mention the work of JOHNSON (1971) on *Drosophila*, who was able to find a large amount of genetic variability involved in fly populations inhabiting dispersed islands in Pacific Ocean. The same use can also be made for forest trees. In fact, a few works have been published in recent years.

SAKAI, MIYAZAKI and MATSUURA (1971) investigated genetic variation in two populations of *Thujaopsis dolabrata* located in two peninsulas separated by about 10 km wide straits. It was found from this study that two populations were different from each other on the basis of either isoperoxidases or some leaf characters.

SAKAI and PARK (1971) made a study on genetic differentiation among three subpopulations of *Cryptomeria japonica* which were separated from each other at about 1 km each. From the statistical analysis of incidences of isoperoxidase bands, it was concluded that the three subpopulations were genetically different from each other.

MATSUURA and SAKAI (1972) investigated six populations of *Abies sachalinensis* in Hokkaido. Comparison among populations was made on the average number of isoperoxidase bands, its intra-forest variability, number of lost bands, incidence of each band and the extent of disagreement between isoenzyme patterns among trees within and between populations. On the basis of these comparisons, a hypothesis has been presented concerning the propagation routes of *Abies sachalinensis* in Hokkaido.

In the present study, 20 populations of *Pinus Thunbergii* collected from six prefectures were investigated for the number of isoperoxidase per tree and the frequency of occurrence of each of 14 bands. Of great interest was the fact that number per tree of isoperoxidase bands was variable among populations, and there was an indication that trees from north-eastern districts had smaller number of isoperoxidase bands than south-western trees (Table 1 referring also Figure 1). Running parallel with this, incidence of each isoperoxidase band tended to increase toward the south-western prefectures.

Such a trend for an increase toward south-west in total number as well as individual incidence of isoperoxidase bands may be either the result of genetic drift occurring during the propagation of pines or the result of natural selection by climatic or other conditions requesting, for example, higher activity of the enzyme in the south-western districts. We are not in a situation to answer this question at present, but the computation of partial correlation between total number of isoperoxidases per tree and latitude or longitude at provenances is likely to suggest that it is related to the longitude rather than to the latitude. It may hint that local increase in number of isoperoxidases may be due to the genetic drift rather than the natural selection.

Genetic variability detected in natural populations of organisms may be divided into: (1) Phylogenetic variation; (2) Geographic variation; (3) Ecological variation; (4) Distributional variation; (5) Family variation; and (6) Individual variation.

The phylogenetic variation is one which occurs due to the difference in phylogenetic relationship. CHYLARECKI and GIERTYCH (1969) investigated genetic variation in populations of *Picea abies* in Poland. On the basis of measurements on 17 characters of the cone, they were able to divide populations of the species in Poland into four groups which were different from each other with regard to their phylogenetic origin. This may serve as an example.

Geographic variation is one which is caused in relation to geographic condition, *i. e.* latitude, altitude, or in some cases, longitude. It is generally accepted that growth rhythms may be related to latitude of seed origin.

Ecological variation is genetic variation caused as the response to ecological conditions of the habitats, while the distributional variation is caused by mechanical spreading of propagules of the plant, or brought forth as the result of the so-called random genetic drift. Family variation is brought about by breeding behavior of the plant. Vegetative propagation, self-fertilization or crossing among genetically related individuals accompanied by localized distribution of the family may bring about genetic differentiation of subpopulations or families within a population. Occurrence of such a within-forest differentiation has been reported by SAKAI and MIYAZAKI (1972) in *Thujopsis dolabrata*.

Individual variation is genetic variation among individuals brought about by genetic segregation or gene mutations.

Taking above-mentioned classification for granted, the clinal variation detected in total number of isoperoxidase bands per tree or the frequency of occurrence of several bands in six prefectures in *Pinus Thunbergii* may be considered to be one of the distributional variations, probably attributable to the random genetic drift in the course of propagation and spread of the species.

CONCLUSION

It was found from this study that natural populations of *Pinus Thunbergii* growing in seaside districts of Japanese islands were variable among themselves with regard to electrophoretic variation of peroxidase in the squeezed sap of needle-leaves. The inter-population variation included the total number of isoperoxidase bands manifested per individual tree and the frequency of occurrence of several isoperoxidase bands. Both variations were quite distinct from prefecture to prefecture with an apparent tendency to increase from north-eastern prefectures to south-western. Investigation of correlations between the total number of isoperoxidase bands per tree and the latitude or the longitude indicated that this clinal variation was correlated with the longitude rather than with the latitude. It was concluded that genetic variation detected in the isoperoxidase pattern in natural populations of *Pinus Thunbergii* formed a geographical cline most probably as the result of random genetic drift taking place in propagation and spread of population.

SUMMARY

Twenty populations of *Pinus Thunbergii* collected from six prefectures in Japan were investigated for isoperoxidase variation in the squeezed sap of needle-leaves. Fourteen bands in all were identified in the starch-gel. Total number of isoperoxidases manifested by individual trees, however, was two to twelve, and the average was variable among populations with an increasing tendency toward south-western prefectures. This increase seemed to be more related to the longitude than to the latitude. Another finding from this study was that several isoperoxidase bands were variable with regard to their frequency of occurrence in populations. There appeared again a tendency that this frequency also increased from north-eastern to south-western prefectures. We were able to forward a hypothesis in this study that *Pinus Thunbergii* populations which were widely scattered in the seaside districts of Japanese islands were genetically differentiated in respect of the isoperoxidase contents, which were considered to have arisen through the random genetic drift occurring during the spreading-period of the species.

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