generation segregants that maintain some characters of both pure species.

The extent and pattern of hybridisation can be determined fully only after controlled fertilization but the present study provides strong, circumstantial and statistical phenotypic evidence supporting the occurrence of hybridisation between the two taxa that are traditionally considered to be distinct species in different subsections of the genus *Pinus* (*Oocarpeae* and *Australes*).

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Inheritance of Isozyme Variations in Japanese Black Pine, Pinus thunbergii Parl.

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Summary

Inheritance of isozyme variations of 19 enzyme systems in Japanese black pine (*Pinus thunbergii* PARL.) was investigated by polyacrylamide gel electrophoresis. Analysis using haploid megagametophyte tissues was demonstrated that allozyme variants in these enzymes were encoded by a total of 120 structure genes in 37 loci. In five of these loci, *Adh-2, Me-2, Tzo-3, Tzo-4* and *Got-3*, no variation was recognized. In other 32 loci, more than two variants in each loci were found. The confirmation of 1:1 segregation ratio in seed of heterozygous trees revealed that these allozymes exhibited simple Mendelian inheritance. Three sets of isozyme were observed, that were of the same electrophoretic character and were encoded by different loci. This suggested that the homoeo-alleles caused by duplications of chromosome have been maintained well in this species.

Key words: inheritance, isozymes, allozymes, *Pinus thunbergii*, Japanese black pine, acrylamide gel electrophoresis.

Zusammenfassung

Bei *Pinus thunbergii* PARL. wurde die Vererbung von Isoenzym-Varianten bei 19 Enzym-Systemen mittels Polyacrylamid-Gel-Elektrophorese untersucht. Bei der Analyse haploider Megagametophyten-Gewebe zeigte sich, daß Allozymvarianten bei diesen Enzymen durch insgesamt 120 Strukturgene an 37 Loci kodiert werden. An fünf dieser Loci, Adh-2, Me-2, T20-3, T20-4 und Got-3 wurde keine Variation festgestellt. Bei den übrigen 32 Loci wurden mehr als 2 Varianten an jedem Locus gefunden. Die Bestätigung der 1:1 Aufspaltung in Saatgut heterozygoter Bäume zeigt, daß diese Allozyme den Mendelschen Gesetzen unterliegen. Drei Gruppen von Isoenzymen wurden beobachtet, die gleiche elektrophoretische Merkmale aufwiesen, jedoch von unterschiedlichen Genloci kodiert wurden. Dies deutet an, daß Homöo-Allele, die durch Chromosomenverdopplung entstehen, bei dieser Art vorliegen.

Introduction

Recently, isozyme techniques have been widely used in genetic studies of forest trees. They have contributed to various aspects in the practical forest tree breeding, that is (1) to identify the clone, (2) to judge the place of the seeds' origin, (3) to establish the managing skills in seed orchards, (4) to know the genetic structure in natural forest population and genetic difference between natural populations in order to manage the natural forest, and to conserve the gene resources.

The most effective use should be expected when applying the isozyme as a marker gene to grasp the genetic variation. Therefore, it is necessary to account for the inheritance of isozymes. The more markers we found, the more reliable results are expected and also, the more suitable markers to each research aim are available. Genetic analysis of isozyme has been improved rapidly using megagametophyte chiefly in conifer since BARTELS (1971) utilized it.

Genetic analysis has been carried out and a lot of loci were found in each gymnospermous species especially in *Pinus ponderosa* (O'MALLEY *et al.*, 1979; MITTON *et al.*, 1979), *P. sylvestris* (RUDIN and RASMUSON, 1973; RUDIN, 1975 and 1977; RUDIN and EKBERG, 1978), *P. contorta* (WHEELER and GURIES, 1982; DANCIK and YEH, 1983), *P. banksiana* (DANCIK and YEH, 1983), *P. taeda* (ADAMS and JOLY, 1980), *P. rigida* (GURIES and LEDIG, 1978), *P. strobus* (ECKERT *et al.*, 1981), *Pseudotsuga menziesii* (YEH and O'MALLEY, 1980), and *Picea glauca* (KING and DANCIK, 1983).

This paper describes the inheritance of 19 enzyme systems detected from megagametophyte tissue in Japanese black pine (*Pinus thunbergii* PARL.).

Materials and Methods

Ramets of 37 plus trees were selected as phenotypically superior trees over a wide area in Japan, and one resistance clone, Sendai-sho 2 against the pine wood nematode (*Bursaphelenchus xylophilus* (STEINER and BUHRER, 1934; NICKLE, 1970) were used as materials. Wind-pollinated seeds were collected from each plus tree's ramets in several clonal orchards in the fall of 1981 and 1982 and from the ortet of Sendai-sho 2 in the fall of 1980. The seeds were air dried and stored at 4° C until the isozyme analysis. The seeds were soaked in the 4° C running water for 48 hours, and then chilled on moistened filter papers in Petri dishes at 4° C for ca. 3 weeks.

Single megagametophyte was excised from each stratified seed individually, and thoroughly homogenized in 100 μl extract buffer (50mM Tris-HCl buffer, pH 7.5 containing 5mM Dithiothreitol, 1mM NAD, 1mM NADP and 20% Glycerol) with glass micro tissue grinder. The homogenate of individual megagamentophyte was centrifuged under 5000xg, 0° C for 20 minutes. Ten microliter of the resulting supernatant per each enzyme was used for electrophoresis.

Table 1. — Composition of staining solutions and temperature for incubation used in this study.

Enzyme	Buffer	Substrate		Others		Temperature
ADH	42mM Tris-HCl,pH7.0	Ethanol	8.3%	NAD*1	1.1mM	37°C
				NBT*3	0.41mM	
				PMS*5	0.27mM	
SoDH	45mM Tris-HCl,pH8.0	D-Sorbitol	100mM	NAD	0.62mM	37°C
				MTT*4	0.22mM	
				PMS	0.059mM	
ShDH	45mM Tris-HCl,pH8.0	Shikimic acid	2.1mM	NADP*2	0.15mM	37°C
				MTT	0.22mM	
				PMS	0.059mM	
				$MgCl_2$	9.1mM	
G2D	44mM Tris-HCl,pH7.0	DL-Glyceric acid	15mM	NAD	0.60mM	37°C
				NBT	0.21mM	
				PMS	0.29mM	
MDH	46mM Tris-HCl,pH7.0	Na DL-Malate	19mM	NAD	0.63mM	R.T.*9
				NBT	0.23mM	
				PMS	0.060mM	
ME(A)	42mM Tris-HCl,pH8.0	Na DL-Malate	85mM	NADP	0.14mM	37°C
				MTT	0.21mM	
				PMS	0.055mM	
				$MnCl_2$	8.5mM	
ME(B)	43mM Tris-HCl,pH7.0	Na DL-Malate	86mM	NADP	0.14mM	37°C
				MTT	0.21mM	
				PMS	0.28mM	
6PGD	45mM Tris-HCl,pH8.0	6-Phosphogluconic acid	0.27mM	NADP	0.15mM	37°C
				MTT	0.22mM	
				PMS	0.059mM	
				$MgCl_2$	9.1mM	
G6PD	45mM Tris-HCl,pH8.0	D-Glucose-6-Phosphate	1.3mM	NADP	0.15mM	37°C
				MTT	0.22mM	
				PMS	0.059mM	
				$MgCl_2$	9.1mM	
GDH	45mM Tris-HCl,pH7.0	L-Glutamic acid	110mM	NAD	0.62mM	37°C
				NBT	0.22mM	
				PMS	0.059mM	
POD	10mM Tris-acetate, pH4.0	H_2O_2	0.03%	3-Amino 9-Ethyl-carbazole	2.0mM	R.T.
				β-Naphthol	2.0mM	
				Acetone	20%(V/V)	
TZO	47mM Tris-HCl,pH7.0	NBT	0.23mM	NAD	0.64mM	37°C
				PMS	0.31mM	
GOT	0.1M Phosphate,pH7.0	L-Aspartic acid	7.5mM	Pyridoxal-5'-phosphate	0.40mM	R.T.
		α-Ketoglutaric acid	6.8mM	Fast Blue BB salt	0.15%(W/V)	
GK	45mM Tris-HCl,pH8.0	Glucose	7.4mM	NADP	0.15mM	37°C
				MTT	0.22mM	
				PMS	0.058mM	
				ATP*6	1.5mM	
				$MgCl_2$	8.9mM	
				G-6-PD*7	15units	
PGM	45mM Tris-HCl,pH8.0	D-Glucose-1-phosphate	4.2mM	NADP	0.15mM	37°C
				MTT	0.22mM	
				PMS	0.059mM	
				$MgCl_2$	9.1mM	
				G-6-PD	15units	
Est	0.1M Phosphate,pH5.6	α-Naphtyl acetate	1.8mM	Ethanol	4.6%	37°C
		α-Naphtyl propionate	1.8mM	Fast Blue RR salt	0.10%(W/V)	
Amy	0.1M Phosphate,pH6.0	Soluble starch	0.3%	(I_2	10mM)	37°C
				(KI	14mM)	
				(Acetic acid	1.0%)	
Lap	0.2M Tris-malate,pH6.0	L-Leucine β-naphthylamide HCl	0.68mM	Fast Black K salt	0.04%(W/V)	37°C
FM	45mM Tris-HCl,pH7.0	Fumaric acid	67mM	NAD	0.61mM	37°C
				NBT	0.22mM	
				PMS	0.058mM	
				MDH*8	100units	
PGI	45mM Tris-HCl,pH8.0	D-Fructose-6-phosphate	0.60mM	NADP	0.15mM	37°C
				MTT	0.22mM	
				PMS	0.059mM	
				$MgCl_2$	9.1mM	
				G-6-PD	15units	

*1 β-Nicotinamide adenine dinucleotide; *2 β-Nicotinamide adenine dinucleotide phosphate; *3 Nitro blue tetrazolium; *4 MTT tetrazolium; *5 Phenazine methosulfate; *6 Adenosine 5'-triphosphate; *7 Glucose-6-phosphate dehydrogenase; *8 Malate dehydrogenase; *9 Room temperature

Table 2. — Locus designated and alleles detected in each locus for the 19 enzyme systems.

enzyme	Locus	Allele									
		No.	*o*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
ADH	*Adh-1*	3		26	29	30					
	Adh-2	1		33							
	Adh-3	4	null	35	38	41					
SoDH	*Sod*	4		33	34	35	36				
ShDH	*Shd-1*	3		35	38	40					
	Shd-2	8		38	40	41	42	43	44	46	49
G2D	*G2d*	3	null	25	31						
MDH	*Mdh-1*	3	null	18	19						
	Mdh-2	2		26	29						
ME	*Me-1*	6	null	13	14	15	16	17			
	Me-2	1		34							
	Me-3	2		38	40						
6PGD	*6Pg-1*	3		30	34	36					
	6Pg-2	4		32	33	34	35				
	6Pg-3	2	null	55							
G6PD	*G6p*	3		17	18	18H					
GDH	*Gdh*	2		13	15						
POD	*Px*	2	null	42							
TZO	*Tzo-1*	1		32							
	Tzo-2	1		45							
	Tzo-3	2	null	74							
	Tzo-4	8	null	79	80	82	83	84	85	86	
GOT	*Got-1*	2		13	18						
	Got-2	4	null	28	30	31					
	Got-3	1		34							
GK	*Gk*	4	null	36	38	42					
PGM	*Pgm-1*	3	null	37	40						
	Pgm-2	5		52	56	57	58	59			
Est	*Est-1*	3		28	29	30					
	Est-2	4	null	36	38	40					
	Est-3	4	null	49	51	54					
	Est-4	2	null	85							
Amy	*Amy*	4	null	43	48	51					
Lap	*Lap-1*	5		43	45	45L	46	47			
	Lap-2	5	null	58	62	62L	63				
FM	*Fm*	2		24	27						
PGI	*Pgi*	4	null	20	21	23					
19	37	120									

Remarks; Figures under alleles are Rf values of bands encoded by them.

The following 19 enzyme systems were analyzed in this study: Alcohol dehydrogenase (ADH; E.C. 1.1.1.1), Sorbitol dehydrogenase (SoDH; E.C. 1.1.1.14), Shikimate dehydrogenase (ShDH; E.C. 1.1.1.25), Glycerate dehydrogenase (G2DH; E.C. 1.1.1.29), Malate dehydrogenase (MDH; E.C. 1.1.1.37), Malic enzyme (ME; E.C. 1.1.1.40), 6-Phosphogluconete dehydrogenase (6PGD; E.C. 1.1.1.44), Glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49), Glutamate dehydrogenase (GDH; E.C. 1.4.1.2), Peroxidase (POD; E. C. 1.11.1.7), Tetrazolium oxidase (TZO; E.C. 1 . . . .), Glutamate oxaloacetate transaminase (GOT; E.C. 2.6.1.1), Glucokinase (GK; E.C. 2.7.1.2), Phosphoglucomutase (PGM; E.C. 2.7.5.1), Esterase (Est; E.C. 3.1.1), Amylase (Amy; E.C. 3.2.1), Leucine aminopeptidase (Lap; E.C. 3.4.1.1), Fumarase (FM; E.C. 4.2.1.2), Phosphoglucose isomerase (PGI; E.C. 5.3.1.9). Polyacrylamide vertical slab gel electrophoresis was prepared approximately according to DAVIS (1964) and ORNSTEIN (1964) method. Seven percent running gel and 3% spacer gel were used. Electrophoresis was carried out at 4° C, 12.3 mA/cm² for 140 minutes. The gels were stained and incubated in the dark using stain formulas in *Table 1*.

In heterozygous trees, allozymes are to segregate in a 1:1 ratio in the gamete level. Chi-square test was utilized to determine the goodness-of-fit to the expected 1:1 Mendelian ratio for each locus segregation.

Results and Discussion

Isozyme variations in 19 enzyme systems were surveyed in the megagametophytes from 38 clones. They were encoded by a total of 37 loci. The allelic variants found are summarized in *Table 2*. The numbers used in the table refer to the Rf (Relative to the front) values of the band controlled by each allele. When one allele controlled more

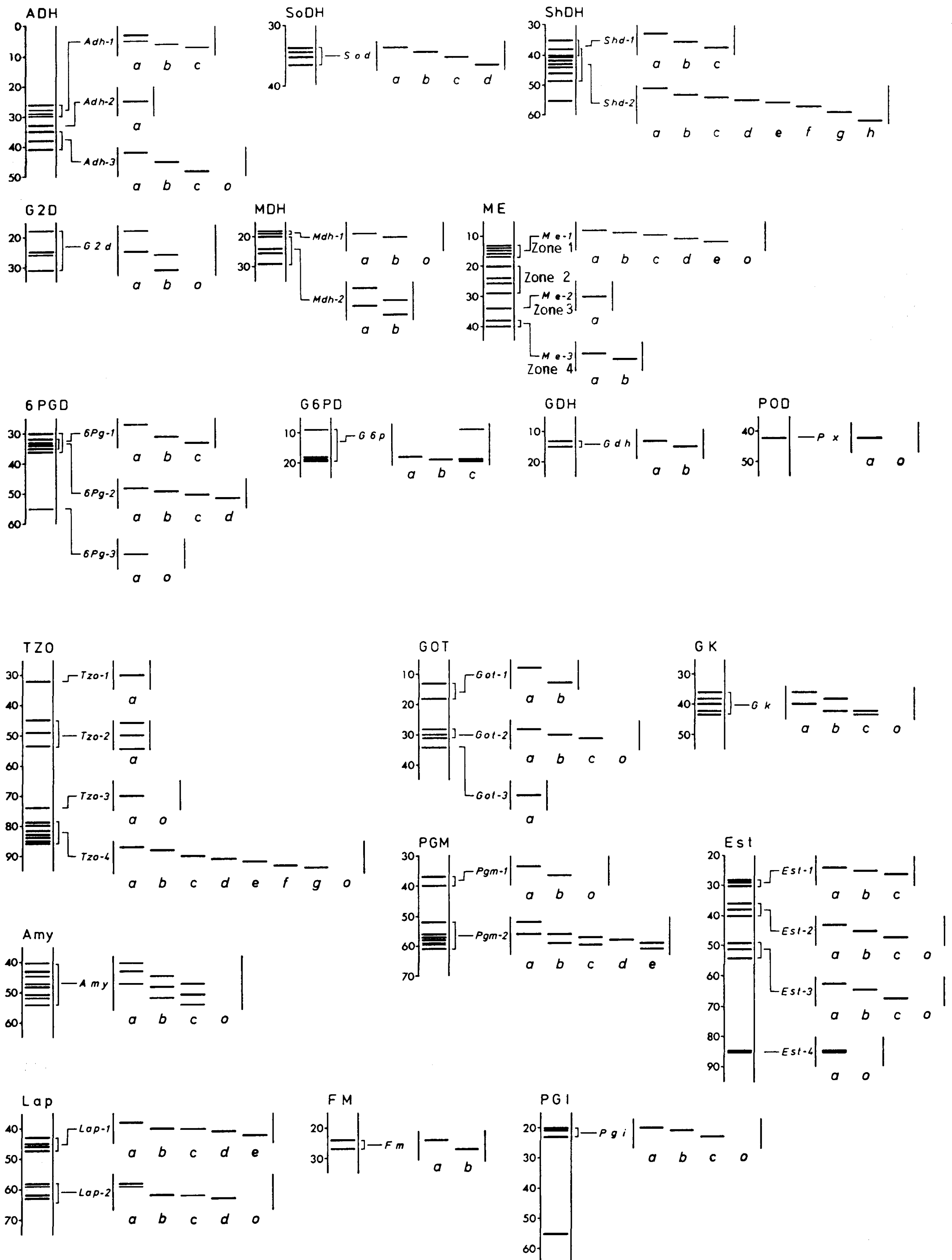

Figure 1. — Isozymes detected in 19 enzyme systems and allelic variants at 37 loci of Japanese black pine. The lower-case letters below each phenotype refer to the allele designations given in *Table 3*. The order of each zone corresponds to the order of the locus except for ME.

than two bands, the Rf value of the most active band was adopted. "Null", "H" and "L" represent non-active, high-active, and low-active band, respectively. The bands recognized in each enzyme and phenotypes of variant in each loci were presented diagrammatically in *Figure 1*.

1) *Alcohol dehydrogenase*

Eight active bands and two faint bands were identified on the gels for ADH. These active bands were divided into

Table 3. — Segregation of allelic variants in 32 polymorphic loci.

Locus	Family	genotype S/F	sample size	Segregation S	:	Segregation F	Chi-square (P)
Adh-1	Kawanabe 15	*a/b*	83	37	:	46	0.98 (0.34)
	Kimotsuke 13	*a/b*	15	7	:	8	0.07 (0.80)
	Hamamatsu 102	*b/c*	12	8	:	4	1.33 (0.26)
Adh-3	Oki 102	*a/b*	110	53	:	57	0.15 (0.71)
	Sendai-sho 2	*b/c*	89	45	:	44	0.01 (0.92)
	Hamamatsu 102	*b/o*	12	8	:	4	1.33 (0.26)
Sod	Minamata-sho 101	*a/b*	78	36	:	42	0.46 (0.51)
	Motoyoshi 104	*b/c*	12	7	:	5	0.33 (0.57)
	Yamamoto 104	*b/d*	12	6	:	6	0
Shd-1	Gohtsu 102	*a/b*	12	7	:	5	0.33 (0.57)
	Oki 102	*b/c*	110	53	:	57	0.15 (0.71)
Shd-2	Sendai-sho 2	*a/c*	89	52	:	37	2.53 (0.12)
	Motoyoshi 101	*a/d*	114	43	:	71	6.88 (0.02)
	Yatsuka 102	*a/g*	89	49	:	40	0.91 (0.35)
	Minamitakaki 102	*a/h*	78	36	:	42	0.46 (0.51)
	Kawanabe 41	*b/c*	52	28	:	24	0.31 (0.59)
	Oki 102	*b/d*	110	57	:	53	0.15 (0.71)
	Yamamoto 104	*c/f*	12	6	:	6	0
	Minamimatsuura 117	*c/g*	78	49	:	29	5.13 (0.03)
	Oki 103	*c/h*	114	56	:	58	0.04 (0.86)
	Minamata-sho 101	*c/h*	78	35	:	43	0.82 (0.38)
	Gohtsu 102	*e/f*	12	6	:	6	0
G2d	Motoyoshi 101	*a/b*	114	67	:	47	3.51 (0.07)
	Asakuchi 101	*a/b*	78	33	:	45	1.85 (0.18)
	Oki 101	*a/o*	12	8	:	4	1.33 (0.26)
Mdh-1	Minamitakaki 102	*a/b*	78	46	:	32	2.51 (0.12)
	Nishiuwa 102	*a/o*	12	8	:	4	1.33 (0.26)
Mdh-2	Minamitakaki 102	*a/b*	78	40	:	38	0.05 (0.83)
	Minamata-sho 101	*a/b*	78	40	:	38	0.05 (0.83)
Me-1	Motoyoshi 101	*a/e*	114	57	:	57	0
	Minamimatsuura 117	*b/d*	96	48	:	48	0
	Yatsuka 102	*b/d*	89	47	:	42	0.28 (0.60)
	Hamamatsu 101	*c/d*	12	3	:	9	3.00 (0.09)
	Oki 102	*d/e*	6	2	:	4	-*
	Ohkawa 102	*d/o*	12	7	:	5	0.33 (0.57)
Me-3	Oki 103	*a/b*	114	50	:	64	1.72 (0.20)
	Asakuchi 101	*a/b*	78	48	:	30	4.15 (0.05)
6Pg-1	Oki 102	*a/b*	110	56	:	54	0.04 (0.85)
	Kimotsuke 8	*a/b*	124	51	:	73	3.90 (0.06)
	Takaoka-sho 101	*a/c*	12	7	:	5	0.33 (0.57)
6Pg-2	Minamimatsuura 108	*a/b*	108	54	:	54	0
	Minamimatsuura 101	*a/c*	78	39	:	39	0
	Kawanabe 41	*a/c*	67	33	:	34	0.01 (0.91)
	Kawanabe 57	*a/d*	86	46	:	40	0.42 (0.53)
	Kimotsuke 8	*a/d*	124	64	:	60	0.13 (0.73)
	Minamimatsuura 111	*b/d*	78	34	:	44	1.28 (0.27)
6Pg-3	Oki 102	*a/o*	110	55	:	55	0
G6p	Minamimatsuura 111	*a/b*	42	27	:	15	3.43 (0.07)
	Oki 103	*a/c*	114	57	:	57	0
	Kimotsuke 8	*a/c*	124	65	:	59	0.29 (0.60)
Gdh	Oki 102	*a/b*	110	54	:	56	0.04 (0.85)
Px	Minamimatsuura 111	*a/o*	78	45	:	33	1.85 (0.18)
	Minamitakaki 102	*a/o*	78	38	:	40	0.05 (0.83)
	Minamata-sho 101	*a/o*	78	35	:	43	0.82 (0.38)
	Kawanabe 76	*a/o*	36	21	:	15	1.00 (0.33)
Tzo-3	Sendai-sho 2	*a/o*	89	47	:	42	0.28 (0.60)
Tzo-4	Kimotsuke 8	*a/c*	36	17	:	19	0.11 (0.75)
	Saidaiji 101	*a/g*	12	5	:	7	0.33 (0.57)
	Kawanabe 15	*b/c*	83	42	:	41	0.01 (0.92)
	Kawanabe 41	*b/c*	67	34	:	33	0.01 (0.91)
	Oki 103	*b/d*	114	51	:	63	1.26 (0.27)
	Ohkawa 105	*b/f*	12	2	:	10	5.33 (0.03)
	Minamimatsuura 117	*c/d*	96	52	:	44	0.67 (0.42)
	Yatsuka 102	*c/d*	89	45	:	44	0.01 (0.92)
	Kimotsuke 13	*c/d*	15	8	:	7	0.07 (0.80)
	Kanazawa-sho 102	*d/e*	12	7	:	5	0.33 (0.57)
	Ojika 103	*d/g*	12	5	:	7	0.33 (0.57)
	Hamamatsu 102	*g/o*	12	7	:	5	0.33 (0.57)
Got-1	Kimotsuke 8	*a/b*	178	85	:	93	0.36 (0.56)
Got-2	Oki 103	*a/b*	114	55	:	59	0.14 (0.72)
	Ojika 103	*b/c*	12	5	:	7	0.33 (0.57)
	Kunisaki 105	*b/o*	12	6	:	6	0
Gk	Minamimatsuura 117	*a/b*	96	47	:	49	0.04 (0.84)
	Yatsuka 102	*a/b*	89	52	:	37	2.53 (0.12)
	Kawanabe 15	*a/b*	65	34	:	31	0.14 (0.72)
	Asakuchi 101	*a/c*	78	35	:	43	0.82 (0.38)
	Motoyoshi 104	*b/o*	12	7	:	5	0.33 (0.57)
Pgm-1	Gohtsu 102	*a/o*	12	6	:	6	0
	Motoyoshi 101	*b/o*	114	68	:	46	4.25 (0.05)
	Asakuchi 101	*b/o*	78	46	:	32	2.51 (0.12)
Pgm-2	Kawanabe 15	*a/b*	83	43	:	40	0.11 (0.75)
	Minamimatsuura 108	*a/c*	108	52	:	56	0.15 (0.71)
	Minamimatsuura 101	*a/c*	78	40	:	38	0.05 (0.83)
	Motoyoshi 104	*a/d*	12	6	:	6	0
	Minamimatsuura 117	*b/c*	96	46	:	50	0.17 (0.69)
	Asakuchi 101	*b/e*	78	41	:	37	0.21 (0.66)
Est-1	Kawanabe 41	*a/c*	67	37	:	30	0.73 (0.40)
	Minamimatsuura 111	*b/c*	72	35	:	37	0.06 (0.82)
	Minamitakaki 102	*b/c*	54	29	:	25	0.30 (0.59)
	Minamata-sho 101	*b/c*	72	31	:	41	1.39 (0.25)
	Kimotsuke 8	*b/c*	178	86	:	92	0.20 (0.66)
	Kimotsuke 13	*b/c*	15	9	:	6	0.60 (0.45)
Est-2	Hakui 107	*a/c*	12	6	:	6	0
	Ojika 101	*b/c*	12	6	:	6	0
	Minamitakaki 102	*c/o*	54	19	:	35	4.74 (0.04)
	Oki 102	*c/o*	110	50	:	60	0.91 (0.35)
	Kawanabe 15	*c/o*	83	36	:	47	1.46 (0.24)
Est-3	Minamimatsuura 117	*a/b*	96	46	:	50	0.17 (0.69)
	Motoyoshi 101	*a/c*	114	46	:	68	4.25 (0.05)
	Kawanabe 41	*a/c*	67	37	:	30	0.73 (0.40)
	Kimotsuke 8	*a/o*	124	59	:	65	0.29 (0.60)
	Oki 102	*b/c*	110	58	:	52	0.33 (0.58)
	Kawanabe 57	*b/c*	86	51	:	35	2.98 (0.10)
	Kimotsuke 13	*b/c*	15	5	:	10	1.67 (0.21)
	Sendai-sho 2	*b/c*	72	36	:	36	0
Est-4	Motoyoshi 101	*a/o*	108	52	:	56	0.15 (0.71)
	Oki 102	*a/o*	110	57	:	53	0.15 (0.71)
	Kawanabe 15	*a/o*	83	32	:	51	4.35 (0.04)
	Kawanabe 41	*a/o*	67	29	:	38	1.21 (0.28)
Amy	Kimotsuke 8	*a/b*	124	61	:	63	0.03 (0.86)
	Minamimatsuura 108	*a/c*	108	49	:	59	0.93 (0.34)
	Oki 103	*b/c*	114	53	:	61	0.56 (0.46)
	Minamimatsuura 101	*b/c*	60	37	:	23	3.27 (0.08)
	Kawanabe 76	*b/c*	36	17	:	19	0.11 (0.75)
	Asakuchi 101	*b/c*	78	39	:	39	0
	Kawanabe 57	*b/o*	72	42	:	30	2.00 (0.17)
Lap-1	Nishiuwa 101	*a/e*	12	3	:	9	3.00 (0.09)
	Tohhaku 101	*b/c*	12	6	:	6	0
	Minamimatsuura 101	*b/d*	60	31	:	29	0.07 (0.80)
	Minamimatsuura 111	*b/d*	78	41	:	37	0.21 (0.66)
	Minamata-sho 101	*b/d*	78	44	:	34	1.28 (0.27)
	Motoyoshi 101	*b/d*	114	64	:	50	1.72 (0.20)
	Kawanabe 41	*b/d*	67	37	:	30	0.73 (0.40)
	Kawanabe 57	*b/d*	86	45	:	41	0.19 (0.68)
	Kimotsuke 8	*b/d*	124	61	:	63	0.03 (0.86)
	Asakuchi 101	*b/e*	72	36	:	36	0
	Sendai-sho 2	*d/e*	89	39	:	50	1.36 (0.26)
Lap-2	Ojika 101	*a/o*	12	7	:	5	0.33 (0.57)
	Kawanabe 15	*b/c*	83	41	:	42	0.01 (0.92)
	Ohkawa 105	*b/c*	12	9	:	3	3.00 (0.09)
	Oki 102	*b/d*	110	54	:	56	0.04 (0.85)
	Minamitakaki 102	*b/d*	78	42	:	36	0.46 (0.51)
	Kawanabe 57	*b/d*	86	46	:	40	0.42 (0.53)
	Kawanabe 76	*b/d*	36	20	:	16	0.44 (0.51)
	Yatsuka 102	*b/o*	89	43	:	46	0.10 (0.76)
Fm	Oki 103	*a/b*	114	59	:	55	0.14 (0.72)
	Kawanabe 41	*a/b*	67	33	:	34	0.01 (0.91)
Pgi	Minamimatsuura 111	*a/b*	42	24	:	18	0.86 (0.37)
	Kawanabe 57	*a/c*	86	45	:	41	0.19 (0.68)
	Minamimatsuura 108	*b/c*	108	57	:	51	0.33 (0.57)
	Kanazawa-sho 102	*c/o*	12	8	:	4	1.33 (0.26)

* Chi-square test wasn't carried out because the number of sample was too small.

three zones according to the segregation test (*Figure 1*). The slower three bands were appeared in zone 1. Two single- and one double-banded phenotypes were found in this zone, and each one was controlled by three alleles in *Adh-1* respectively. Four single-banded variants were observed in zone 3. They were controlled by four alleles in *Adh-3* respectively. In another zone, allelic variant was not recognized. All the individuals analyzed had Rf 33 band. This band seems to be encoded by manamorphic locus (*Adh-2*). The faint bands were omitted from genetic analysis as they were difficult to be identified in lots of family.

2) *Sorbitol dehydrogenase*

Four kinds of single-banded pattern were found for SoDH (*Figure 1*). As the segregations in three families fol-

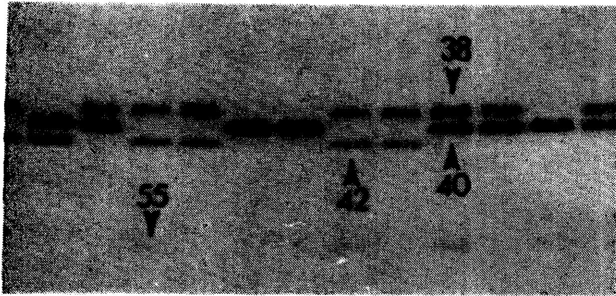

Figure 2. — Phenotypes showing segregation of allozymes at *Shd-1* and *Shd-2* in Oki 102 family.

lowed the expected Mendelian ratio, these variants appeared to be controlled by four alleles in *Sod* (*Table 3*).

3) Shikimate dehydrogenase

A total of nine bands appeared in the region below Rf 50 for ShDH. These bands were clustered into two zones that were overlapped (*Figure 1*). Three bands in zone 1 were controlled by *Shd-1* with three alleles, and eight bands in zone 2 were controlled by *Shd-2* with eight alleles. Migrational difference was not seen between $Shd\text{-}1^{b}$ and $Shd\text{-}2^{a}$, and also between $Shd\text{-}1^{c}$ and $Shd\text{-}2^{b}$ (*Figure 1*, *Figure 2*). In megagametophytes possessing two isozymes with the same electrophoretic migration, the bands showed extremely high activity as two isozymes were overlapped each other. The additional single band appeared in Rf 55 is mentioned in 6PGD.

4) Glycerate-2-dehydrogenase

One non-active and two double-banded patterns were observed for G2DH (*Figure 1*). These phenotypes were recorded as the variants in a single locus (*G2d*) with three alleles including null gene ($G2d^{0}$).

5) Malate dehydrogenase

Six bands could be recognized in between Rf 18 and Rf 29 (zone 1, zone 2) for MDH (*Figure 1*). Some bands detected between Rf 31 and Rf 40 were over-stained and had heavy tailing. As it was difficult to identify them correctly, they were omitted. Three isozyme patterns were found in zone 1. These phenotypes were controlled by three alleles including one null gene in *Mdh-1*. In zone 2, two double-banded phenotypes could be seen. These bands were controlled by two alleles in *Mdh-2*.

6) Malic enzyme

Two kinds of staining solution were adopted for detecting ME activity (*Table 1*). A-solution exhibited five active bands and none activity in zone 1. In B-solution, a total of seven bands appeared; four bands in zone 2, one in zone 3, and two in zone 4 (*Figure 1*). Six alleles in *Me-1* controlled five single-banded phenotypes and one no-banded phenotype in zone 1. Bands in zone 2 showed the same migration as those encoded by *Mdh-2*, but lower activity than those of MDH. Phenotypes between *Mdh-2* and this zone of ME showed agreement in all megagametophytes. The bands controlled by *Mdh-2* and the bands in this zone of ME consist of the same protein molecular. The enzyme protein transcribed and translated from the genes in *Mdh-2* seemed to have the activity of ME, too. Variation was not observed in zone 3. Two phenotypes appeared in zone 4. Segregation observed at this zone indicated a locus (*Me-3*) with two alleles (*Table 3*).

7) 6-Phosphogluconate dehydrogenase

A total of seven bands appeared. Segregation could be observed in overlapping two zones between Rf 30 and Rf 36 independently (*Figure 1*, *Figure 3*). Three single-banded phenotypes appeared in zone 1. This was interpreted as a single locus (*6Pg-1*) with three alleles. Four single-banded phenotypes appeared in zone 2. Segregation observed at this zone indicated a locus (*6Pg-2*) with four alleles. Migrational difference was not recognized between isozymes controlled by $6Pg\text{-}1^{b}$ and $6pg\text{-}2^{c}$. In zone 3, two phenotypes were observed. They were interpreted as being controlled by $6Pg\text{-}3^{a}$ of activity and $6Pg\text{-}3^{o}$ of non-activity. They appeared the same in ShDH and PGI, but the activity of this band was the highest in 6PGD. This protein seems to have three enzyme activities. The observed values of segregation in these zones agreed to the expected values (*Table 3*).

8) Glucose-6-phosphate dehydrogenase

Three kinds of phenotype were observed (*Figure 1*). Two bands named Rf 18H and Rf 18 had the same migration but were quite different in their activities (*Figure 4*). Three allozymes encoded by single locus (*G6p*) were determined as the result of segregations in three heterozygous families (*Table 3*).

9) Glutamate dehydrogenase

There were two phenotypes segregated under the Mendelian ratio in Oki 102 family (*Figure 1*, *Figure 5*). These two bands were controlled by two alleles of *Gdh*. Segregation of phenotypes was not recognized in other families, and all megagametophytes in them possessed Rf 15 band. Gdh^{b} encoded for Rf 15 band was quite rare.

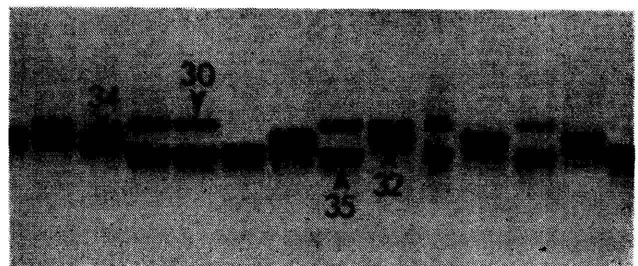

Figure 3. — Phenotypes showing segregation of allozymes at *6Pg-1* and *6Pg-2* in Kimotsuke 8 family.

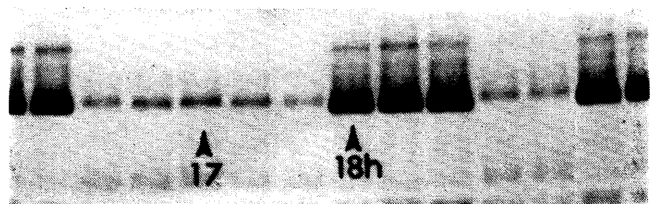

Figure 4. — Phenotypes showing segregation of allozymes at *G6p* in Oki 103 family.

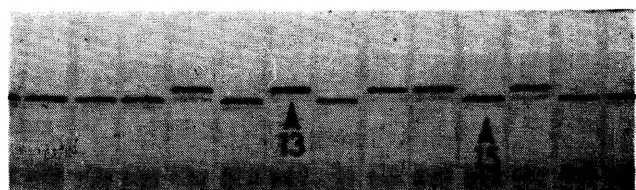

Figure 5. — Phenotypes showing segregation of allozymes at *Gdh* in Oki 102 family.

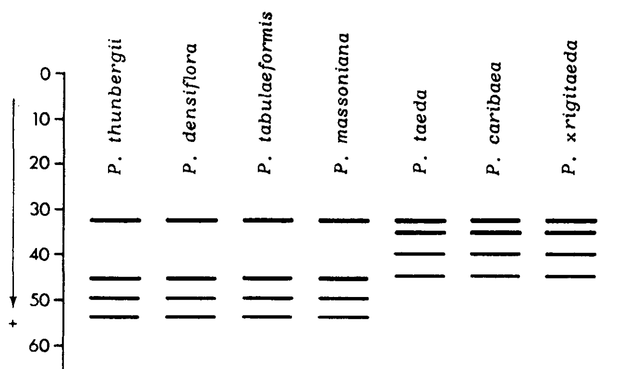

Figure 6. — Observed electrophoretic phenotypes in five species and one hybrid of genus *Pinus*.

10) Peroxidase

Two phenotypes, one had Rf 42 band and another didn't, were observed (*Figure 1*). One additional band with poor and inconsistent POD activity was sometimes observed in Rf 55. This band, however, was not scored in this survey. Segregations observed in four families indicated a locus with Px^a and Px^o (non-activity) (*Table 3*).

11) Tetrazolium oxidase

All the megagametophyte tissues for this analysis have four bands in the region below Rf 60, and no segregation could be observed. To verify how many loci control these bands, TZO isozyme of other five species and one hybrid belong to two subsections of genus *Pinus*, *P. densiflora*, *P. tabulaeformis*, *P. massoniana*, *P. taeda*, *P. caribaea*, and *P.* x *rigitaeda*, were tested (CRITCHFIELD and LITTLE 1966). Three species in subsect. *Sylvestres* had the same pattern as Japanese black pine, but three species in subsect. *Australes* had the different pattern from the Japanese black pine (*Figure 6*). As for Rf 32 band appeared in zone 1, all species of both subsections showed the same banding pattern. As for three bands in zone 2, ones in subsect. *Sylvestres* showed the same migration as Japanese black pine, but in subsect. *Australes*, the migration was small, and three bands were grouped to appear between Rf 35 and Rf 45. This led to believe that these bands appeared in zone 1 and zone 2 were controlled by at least two different loci, that is *Tzo-1* controlled the Rf 32 band, and *Tzo-2* controlled other three bands.

Another eight bands appeared between Rf 74 and Rf 87. Zone 3 and zone 4 could be interpreted as loci segregated separately. These bands were determined to be controlled by *Tzo-3* (two alleles) and *Tzo-4* (eight alleles). Both loci had null gene.

12) Glutamate oxaloacetate transaminase

Six bands appeared and segregated in three zones respectively (*Figure 1*). The segregation test in four families led that zone 1 was interpreted as a locus (*Got-1*) with two alleles, and zone 2 was as another locus (*Got-2*) with four alleles (*Table 3, Figure 7*). Allelic variants could not be recognized in zone 3, that assumed to be controlled by monomorphic locus (*Got-3*).

13) Glucokinase

Three double-banded phenotypes and non-active phenotype appeared (*Figure 1*). These active bands were interpreted as isozymes controlled by three alleles (Gk^a, Gk^b, Gk^c) and non-active phenotype was encoded by Gk^o.

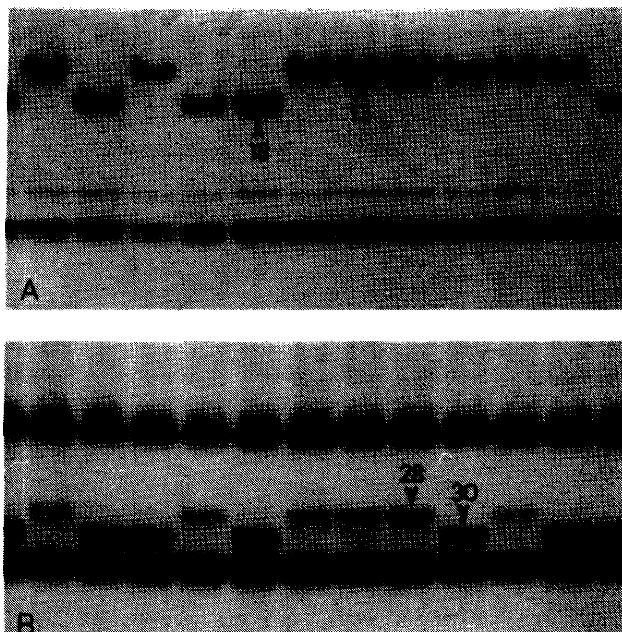

Figure 7. — Phenotypes showing segregation of allozymes at *Got-1* in Oki 103 family (A) and at *Got-2* in Oki 102 family (B).

14) Phosphoglucomutase

Two bands appeared in zone 1 and seven bands appeared in zone 2. A phenotype with non-activity was also recognized in zone 1 (*Figure 1*). These three phenotypes in zone 1 were determined to be controlled by a locus (*Pgm-1*) with three alleles including null gene. Five phenotypes appeared in zone 2 were four double-banded and one single-banded. They were determined to be controlled by another locus (*Pgm-2*) with five alleles.

15) Esterase

The bands stained intensely and segregated were shown in *Figure 1*. More than ten faint bands appeared on gel for Est were omitted for the test. The 10 dark bands were divided into four zones. Segregation could be observed at four zones controlled by polymorphic loci respectively; *Est-1* with three alleles, *Est-2* with four alleles, *Est-3* with four alleles, *Est-4* with two alleles. *Est-2*, *Est-3*, and *Est-4* had a null gene respectively ($Est\text{-}2^o$, $Est\text{-}3^o$, $Est\text{-}4^o$).

It has been noted that there are differences in substrate specificity between isozymes. Such differences have been reported in many other plant such as Lap in corn (SCANDALIOS, 1969), POD in rice (ENDO, 1972), Est in barley (KAHLER

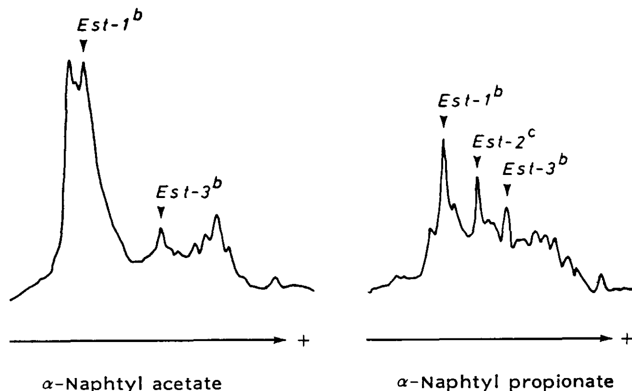

Figure 8. — Differences of activity in three loci against two kinds of chemical for substrate of esterase.

and ALLARD, 1970), and Est and Acid phosphatase in Japanese cypress (SHIRAISHI and KAMINAKA, 1981). The differences of substrate specificity were investigated in the isozymes controlled by four loci for Est in Japanese black pine using three families. The substrate used for the Est isozyme analysis was the compound of α-Naphthyl acetate and α-Naphthyl propionate (*Table 1*). The dye solutions including either α-Naphthyl acetate or α-Naphthyl propionate as substrate was also used so as to investigate the specificity of the isozymes for these two chemicals. A difference in substrate specificity was found between the isozymes encoded by these loci (*Figure 8*). The isozymes controlled by *Est-1* showed high activity for α-Naphthyl acetate. The isozymes controlled by *Est-2* was detected only for α-Naphthyl propionate. The difference in substrate specificity between loci was thus confirmed. To investigate the selectivity for much more kinds of chemical will help to account for how each isozyme functions.

16) Amylase

Three triple-banded phenotypes and non-active one were observed (*Figure 1*). Except for the non-activity phenotype, the middle band showed the most intense activity of the three bands in each phenotype. One locus (*Amy*) with four alleles was verified by testing observed segregation for deviations from the expected Mendelian ratio (*Table 3*).

17) Leucine aminopeptidase

Five kinds of band appeared in zone 1 were classified into five single-banded phenotypes (*Figure 1*). There appeared two bands named Rf 45 and Rf 45L that had the same migration but were quite different in activity. Five alleles controlling these phenotypes were identified. Five banding patterns appeared in zone 2 (*Figure 1*). These variants were controlled by *Lap-2* locus with five alleles. The variant that had a faint band (Rf 62L) was observed also in zone 2.

18) Fumarase

Two phenotypes appeared for FM (*Figure 1*). The segregation ratio of the haploid megagametophytes in two families was close to the expected 1:1 Mendelian ratio (*Table 3*). These two kinds of isozyme were found to be controlled by Fm^a and Fm^b respectively.

19) Phosphoglucose isomerase

Three kinds of band were observed except for the Rf 55 band that mentioned in 6PG. A total of four phenotypes were observed including a null type (*Figure 1*). They were determined to be controlled by a locus (*Pgi*) with four alleles.

The single locus segregations of allozymes in 37 loci were investigated in a total of 141 heterozygous families (*Table 3*). As the results, the significant deviations of the observed segregation ratio from the expected Mendelian ratio were found in eight (5.7%) of those families, Motoyoshi 101 (*Shd-2, Pgm-1* and *Est-3*), Minamimatsuura 117 (*Shd-2*), Asakuchi 101 (*Me-3*), Ohkawa 105 (*Tzo-4*), Minamitakaki 102 (*Est-2*), and Kawanabe 15 (*Est-4*). This can occur because of the following reasons: (1) It can be due to chance alone. (2) Some selection can be done between the two alleles of the family. (3) The embryonic lethal gene can exist around these loci and has linkage with them.

As the sample size was small in *Tzo-4* of Ohkawa 105 family, its segregation distortion was not discussed. There was one family that showed the segregations with significant distortion in each four locus, *Me-3, Est-2, Est-3,* and *Est-4*. Other families with the same genotype, however, fit to the Mendelian expectations in these loci satisfactorily. This result indicated that the selection was not governed between genes for these four loci. In *Shd-2,* besides two families mentioned above, Yatsuka 102 and Minamitakaki 102 had a little excess of $Shd\text{-}2^a$ and $Shd\text{-}2^g$. In addition to Motoyoshi 101 family that departed significantly at 5% level from the Mendelian expectation, in *Pgm-1* of Asakuchi 101 family, $Pgm\text{-}1^o$ exhibited lower frequency than $Pgm\text{-}1^b$. This result led the possibility of some selection against $Pgm\text{-}1^o$. Generally, isozyme has been reported to be neutral in the natural selection (ENDO, 1963). ADAMS and JOLY (1980) however, noted that there was a difference in survival between alleles in *Pinus taeda*. Further investigation is necessary in Japanese black pine to clarify the selection pressure against these alleles and the linkage with the embryonic recessive lethal or semilethal genes in these clones.

No variation could be observed in five of 37 loci, that is *Adh-2, Me-2, Tzo-1, Tzo-4,* and *Got-3* in this survey (*Table 2*). Extremely low acceptable mutation rate was expected in protein produced by these genes. Especially *Tzo-1* showed no difference between two subsections of *Diploxylon*. Other 32 loci have more than two alleles. Multiple alleles more than five were observed in *Shd-2, Tzo-4, Me-1, Pgm-2, Lap-1,* and *Lap-2.*

In ShDH and 6PGD, three sets of genes that were located in the different loci but coded for isozymes with the same migration were recognized. This may be explained that the genes from the different ancestor possessed the same electrophoretic character by chance. It is possible, however, that the sets are homoeo-alleles caused by duplications of chromosome, and have been maintained well.

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Monoterpene Analysis of a Diallel Cross in Sitka Spruce

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Summary

The monoterpene composition of the cortical oleoresin of main stems and of branch apical shoots was analysed in the progeny of a complete diallel cross among seven trees of *Picea sitchensis*, and also in the parent trees and in half-sib open pollinated families. For the majority of the monoterpenes in each oleoresin system, practically all the significant variation was attributable to general combining ability, with the progenies reflecting the differences in parental means in additive combination with little significant deviation from this pattern due to specific combining ability or reciprocal effects. Non-additive variation was significant in three instances, in particular for α-pinene in main stem cortical oleoresin.

Key words: Picea sitchensis, Sitka spruce, oleoresin, monoterpenes, diallel cross, inheritance.

Zusammenfassung

Die Monoterpenzusammensetzung des Rindenharzes des Stammes und der Hauptäste wurde bei *Picea sitchensis* Nachkommenschaften eines vollständigen Kreuzungsdiallel (7 × 7) sowie bei den Kreuzungspartnern und deren Nachkommenschaften — entstanden aus freier Abblüte — analysiert. Für die Mehrheit der Monoterpene in jedem Harz-System ließ sich praktisch die gesamte Variation auf die generelle Kombinationseignung zurückführen, wobei die Nachkommenschaften die Unterschiede zwischen den Mittelwerten der Eltern bei additiver Kombination zeigten. Von diesem Muster gab es signifikante Abweichungen bedingt durch spezifische Kombinationseignung oder reziproke Effekte. Die nicht-additive Variation war in drei Fällen signifikant, besonders für α-Pinen im Rindenharz des Stammes.

Introduction

The monoterpene compositions of the volatile needle oil and of the cortical oleoresin of Sitka spruce (*Picea sitchensis* (BONG.) CARR.) show a high degree of variation from tree to tree (VON RUDLOFF, 1977; FORREST, 1980a,b). Part of this variation has been shown to be due to the origin of the populations (FORREST, 1980b), but there remains a high within-population variation. In order that monoterpene characteristics can be used as genotypic indicators in studies of population variability, it is essential to investigate the genotypic basis of this variation, and to determine the heritability of individual monoterpenes and their degree of freedom from environmental influence. The availability of the progeny of a complete diallel cross among seven trees of a Sitka spruce population provided the opportunity to carry out this form of analysis.

Heritability studies involving monoterpene data in conifers have been carried out on a number of species, but the majority have been concerned with pines rather than with spruces, and while there have been several reports on heritability estimates for individual monoterpenes, there appears to have been no biochemical work involving full diallel matings. Estimates of narrow-sense heritability, usually from regressions of progeny means on mid-parent values, and in some cases also of broad-sense heritability, have been made for the main monoterpenes in *Pinus monticola* DOUGL. (HANOVER, 1966, 1971), *P. elliottii* ENGELM. (SQUILLACE, 1971), *P. taeda* L. (ROCKWOOD, 1973), *P. pinaster* AIT. (BARADAT *et al.*, 1975), *P. silvestris* L. (HILTUNEN, 1976), and *P. virginiana* MILL.)MEIER and GOGGANS, 1978). Generally heritability values were found to be high or very high, indicating a high degree of isolation from environmental effects. In the spruces, the only analysis known to the authors involving controlled crossings is that of ESTEBAN *et al.* (1976) for *Picea abies* (L.) KARST., from which it was postulated that the relative concentrations of α-pinene, α- + β-pinene, and 3-carene were each controlled by one gene locus having one dominant and one recessive allele. Indirect evidence from monoterpene percentage frequency histograms was obtained for the mode of genetic control of the levels of the cortical oleoresin monoterpenes in *Picea glauca* (MOENCH) Voss; smooth distributions for the pinenes and limonene suggested multiple gene control, while skewed distributions with indications of bimodality for 3-carene, myrcene and β-phellandrene suggested control by few genes (WILKINSON et al. 1971). Data on monoterpene inheritance in Sitka spruce are lacking.

Materials and Methods

The seven parents of the diallel cross were part of a small stand planted in Laigh of Moray Forest (Grampian Region, E Scotland) in 1933. The crossing programme was