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Linkage Relationships among Allozyme loci in Japanese Black Pine, Phinus thunbergii Parl.

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Summary

Linkage relationships among 32 polymorphic allozyme loci were investigated in Japanese black pine, *Pinus thunbergii* Parl. Haploid megagametophytes from 18 mother trees that possess doubly or more heterozygous loci were utilized for materials. Results of segregations in 221 instances among the possible 496 two-locus combinations of these loci were discussed by procedure of Akaike information criterion (AIC) in addition to conventional chi-square analysis. Segregation in most of the tested pairs revealed the independence in each locus. In fifteen pairwise combinations, however, linkage was recognized. *G2d* was always associated with *Pgm-1*. *Tzo-4* and *Lap-2* also showed almost complete linkage. *Me-1:Lap-2, Me-1:Tzo-4, Adh-3:Lap-1* and *Shd-1:Est-2* were linked closely with estimated recombination values of 0.13, 0.15, 0.26 and 0.27 respectively. *Mdh-1:Est-2, 6Pg-1:Est-4, Shd-2:6Pg-3, Got-2:Fm, Adh-3:Est-2* and *6Pg-3:Est-3* were linked with recombination values between 0.33 and 0.39. The remaining tested pairs were linked weakly with recombination values greater than 0.40. By AIC, linkages including nonrandom segregations in gametic genotype for specific loci were detected. Nineteen loci involved in these combinations were classified into six linkage groups, and a linkage map was made up on basis of the estimated recombination values and ordering of the loci in each group by three-point mapping.

Key words: Linkage, isozymes, allozymes, AIC, *Pinus thunbergii*, Japanese black pine.

Zusammenfassung

Die Kopplungsverhältnisse zwischen 32 polymorphen Allozym-Loci wurden bei *Pinus thunbergii* Parl. untersucht. Haploide Megagametophyten von 18 Sameneltern, die zwei oder mehrere heterozygote Loci aufwiesen, wurden verwendet. Die Segregation von 221 bei 496 möglichen Zwei-Locus Kombinationen wurden mittels des Akaike Informationskriteriums (AIC) und anhand der konventionellen χ^2-Analyse untersucht. Die Segregationsverhältnisse der meisten Paare zeigte die Unabhängigkeit der Loci an. Bei 15 paarweisen Kombinationen wurde jedoch Kopplung beobachtet. *G2d* war stets *Pgm-1* gekoppelt. *Tzo-4* und *Lap-2* zeigten meistens vollständige Kopplung. *Me-1:Lap-2, Me-1: Tzo-4, Adh-3:Lap-1* und *Shd-1: Est-2* waren mit einer geschätzten Rekombinationsrate von 0,13/0,15/0,26 bzw. 0,27 eng gekoppelt. *Mdh-1:Est-2, 6Pg-1:Est-4, Shd-2:6Pg-3, Got-2:Fm, Adh-3:Est-2* und *6Pg-3:Est-3* waren mit Rekombinationsraten zwischen 0,33 und 0,39 gekoppelt. Die übrigen der getesteten Paare waren mit Rekombinationsraten größer 0,40 nur schwach gekoppelt. Kopplung einschließlich nicht zufälliger Segregation der Parameter konnte bei einigen Loci beobachtet werden. Neunzehn Loci konnten in 6 Kopplungsgruppen eingeteilt werden. Die geschätzten Rekombinationsraten dienten zur Erstellung einer Kopplungskarte. Die Reihenfolge der Loci wurde anhand des "three point mapping" festgelegt.

Introduction

In breeding as well as genetic studies, it is very important to investigate the linkage relationship among loci and to make up the linkage map. It is necessary to clarify the relationship among loci as genetic markers when we study genetic structures and their dynamics in populations using isozymes and terpenes and so on. There are a few reports regarding linkage disequilibrium and epistatic selection in population (Mitton *et al.*, 1980; Roberds and Brotschol, 1985). If the linkage map was made up on easily detectable marker genes such as allozymes, it would be easy to locate newly-detected genes on the map. When economically important genes are found and recombined by crossing in the future, the selection in the early stage is possible by using marker genes closely linked to them. Such a technique for early selection is useful, especially in forest trees which have very long vegetative period and it takes long time to manifest a lot of characteristics. The information obtained from linkage maps of various species also proved useful for characterizing phylogenetic relationships among them (Cheliak and Pitel, 1985).

In coniferous species, linkage of genes which control isozymes can be revealed easily without making crosses by analysis of the independence of segregation in each locus

using the haploid megagametophyte tissue of seeds. Each megagametophyte has the same genetic constitution as the female gamete. This advantage expedited the progress of the linkage studies in forest tree genetics. On many allozyme loci of *Pinus ponderosa* (O'Malley *et al.*, 1979), *P. rigida* (Guries *et al.*, 1978), *P. sylvestries* (Rudin and Ekberg, 1978), *P. taeda* (Adams and Joly, 1980), *P. strobus* (Eckert *et al.*, 1981), *Abies balsamea* (Neale and Adams, 1981), *Picea glauca* (King and Dancik, 1983), *Pseudotsuga menziesii* (El-Kassaby *et al.*, 1982) and *Larix laricina* (Cheliak and Pitel, 1985), the linkage relationships have been investigated.

In this paper, the linkage relationships among 32 polymorphic allozyme loci encoding 19 enzyme systems in Japanese black pine were discussed. Akaike information criterion (AIC) method was adopted to decide the linkage in addition to chi-square test that has been generally used. A linkage map was established from the data of the pair-loci recognized as linkage.

Materials and Methods

1. Materials and isozyme analysis

Ramets of 17 plus trees and ortet of one resistance clone, Sendai-sho 2 for the pine wood nematode were used as mother trees for the collection of wind-pollinated seeds. The megagametophytes from doubly or more heterozygous clones were analyzed to investigate the linkage relationship among the following 32 polymorphic loci in 19 enzyme systems: Alcohol dehydrogenase (*Adh-1, Adh-3*), Sorbitol dehydrogenase (*Sod*), Shikimate dehydrogenase (*Shd-1, Shd-2*), Glycerate dehydrogenase (*G2d*), Malate dehydrogenase (*Mdh-1, Mdh-2*), Malic enzyme (*Me-1, Me-3*), 6-Phosphogluconete dehydrogenase (*6Pg-1, 6Pg-2, 6Pg-3*), Glucose-6-phosphate dehydrogenase (*G6p*), Glutamate dehydrogenase (*Gdh*), Peroxidase (*Px*), Tetrazolium oxidase (*Tzo-3, Tzo-4*), Glutamate oxalomate transaminase (*Got-1, Got-2*), Glucoxinase (*Gk*), Phosphoglucomutase (*Pgm-1, Pgm-2*), Esterase (*Est-1, Est-2, Est-3, Est-4*), Amylase (*Amy*), Leucine aminopeptidase (*Lap-1, Lap-2*), Fumarase (*Fm*), Phosphoglucose isomerase (*Pgi*).

The same procedures as Shiraishi (in press) were used in tissue preparation and extract, polyacrylamide gel electrophoresis and detection of enzyme activities.

2. Statistical procedures

The following two statistical methods were adopted to judge linkage.

Chi-square test

This analysis systematized by Mather (1938) has been generally used for linkage study. The genotypes of megagametophytes from doubly heterozygous maternal parents for two loci being represented as *A* and *B*, were scored within each family. The chi-squares, χ_A^2 and χ_B^2 for gametic single locus segregation in *A* and *B* locus, and χ_L^2 for joint segregation, were calculated for the genotypic score. The χ_A^2 and χ_B^2 were used to test the goodness of fit to an expected 1:1 Mendelian ratio at five percent level. χ_L^2 was used to test the goodness of fit to an expected 1:1 ratio in two-locus joint segregation. When the pairing alleles in both or only one locus segregated according to the Mendelian ratio, and at the same time, the significant deviation in joint segregation recognized at five percent level, the two loci were determined to be linked each other.

Akaike Information Criterion procedure

Akaike information criterion (AIC) introduced by Akaike (1973 and 1976) was the statistic for lack of fit in the statistical models.

Let the f be the number of parameters without restraint in model and the l be the maximum likelihood estimate. AIC is:

$$AIC = (-2)\log_e l + 2f$$

The model scored smaller AIC estimate is evaluated as better one, and the model scored minimum AIC estimate (MAICE) is the best among the models assumed. Akaike adopted this statistic to the genetic analysis, and found that AIC could obtain clearer result in comparison with chi-square test.

Four kinds of gametic genotype, *A1B1, A1B2, A2B1* and *A2B2* from doubly heterozygous maternal parent (*A1B1/A2B2*) are segregated on 1:1:1:1 ratio under Mendelian law, if they are independent. Some distortion in segregation, however, could happen depending on the linkage, reduced or nonrandom production of gametes in *A* and/or *B* locus. Considering the three factors that influence for the single-locus segregation in each locus and the joint segregation, the nine models could be designed as shown in *Table 1:*

Model 0: This is non-restrictive model. In this model, the expected value of each genotype equals to the observed value.

Model 1: The pairing alleles segregate according to the Mendelian ratio in both *A* and *B* locus. The two loci are not linked.

Model 2: The pairing alleles segregate according to the Mendelian ratio in both *A* and *B* locus. The two loci are linked.

Table 1. — Definitions of three parameters, q_A, q_B and r, and the number of parameter (f) without imposed restriction in each AIC model.

Model	0	1	2	3	4	5	6	7	8
r	-	$=0.5$	<0.5	$=0.5$	<0.5	$=0.5$	<0.5	$=0.5$	<0.5
q_A	-	$=1$	$=1$	$\neq 1$	$\neq 1$	$=1$	$=1$	$\neq 1$	$\neq 1$
q_B	-	$=1$	$=1$	$=1$	$=1$	$\neq 1$	$\neq 1$	$\neq 1$	$\neq 1$
f	3	0	1	1	2	1	2	2	3

Table 2. — The expected probability ($p_{A.B.}$) of four genotypes (*A.B.*) in each AIC model.

Genotype	*A1B1*	*A1B2*	*A2B1*	*A2B2*
Ob.val.*	a	b	c	d
Model 0	a/n	b/n	c/n	d/n
Model 1	$1/4$	$1/4$	$1/4$	$1/4$
Model 2	$(1-r)/z_2$ $[(a+d)/2n]$	r/z_2 $[(b+c)/2n]$	r/z_2 $[(b+c)/2n]$	$(1-r)/z_2$ $[(a+d)/2n]$
Model 3	$1/z_3$ $[(a+b)/2n]$	$1/z_3$ $[(a+b)/2n]$	q_A/z_3 $[(c+d)/2n]$	q_A/z_3 $[(c+d)/2n]$
Model 4	$(1-r)/z_4$ $[(a+d)(a+b)/n^2]$	r/z_4 $[(b+c)(a+b)/n^2]$	$r\cdot q_A/z_4$ $[(b+c)(c+d)/n^2]$	$(1-r)\cdot q_A/z_4$ $[(a+d)(c+d)/n^2]$
Model 5	$1/z_5$ $[(a+c)/2n]$	q_B/z_5 $[(b+d)/2n]$	$1/z_5$ $[(a+c)/2n]$	q_B/z_5 $[(b+d)/2n]$
Model 6	$(1-r)/z_6$ $[(a+d)(a+c)/n^2]$	$r\cdot q_B/z_6$ $[(b+c)(b+d)/n^2]$	r/z_6 $[(b+c)(a+c)/n^2]$	$(1-r)\cdot q_B/z_6$ $[(a+d)(b+d)/n^2]$
Model 7	$1/z_7$ $[(a+b)(a+c)/n^2]$	q_B/z_7 $[(a+c)(b+d)/n^2]$	q_A/z_7 $[(c+d)(a+c)/n^2]$	$q_A\cdot q_B/z_7$ $[(c+d)(b+d)/n^2]$
Model 8	$(1-r)/z_8$ [?]	$r\cdot q_B/z_8$ [?]	$r\cdot q_A/z_8$ [?]	$(1-r)\cdot q_A\cdot q_B/z_8$ [?]

$n=a+b+c+d$; $z_2=2(1-r)+2r=2$; $z_3=2+2q_A$; $z_4=(1-r)+r+r\cdot q_A+(1-r)q_A$; $z_5=2+2q_B$; $z_6=(1-r)+r\cdot q_B+r+(1-r)q_B$; $z_7=1+q_B+q_A+q_A\cdot q_B$; $z_8=(1-r)+r\cdot q_B+r\cdot q_A+(1-r)\cdot q_A\cdot q_B$
[ ]; General solutions obtained by method of maximum likelihood except model 8 (shown with "?"). The approximate values were computed by Newton-Raphson method in model 8.
*) Observed value

Table 3. — The number of families (below the diagonal) and the total of megagametophytes (above the diagonal) examined in each particular pair of 32 polymorphic loci.

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1. *Adh-1*		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	83	–	–	65	–	83	–.	83	–	83	–	–	83	–	–
2. *Adh-3*	–		–	104	193	–	–	–	–	–	104	–	104	–	104	–	89	–	–	–	–	–	–	104	176	104	–	89	104	–	–
3. *S o d*	–	–		–	72	–	–	72	–	–	–	–	–	–	–	72	–	–	–	–	–	–	–	72	–	–	–	72	–	–	–
4. *Shd-1*	–	1	–		104	–	–	–	–	–	104	–	104	–	104	–	–	–	–	–	–	–	–	–	104	104	104	–	–	104	–	–
5. *Shd-2*	–	2	1	1		108	72	144	263	108	104	52	104	108	104	144	89	315	–	108	155	108	72	214	230	408	264	108	404	259	160	–
6. *G 2 d*	–	–	–	–	1		–	–	108	72	–	–	–	–	–	–	–	–	–	–	72	180	72	–	–	108	108	72	180	–	–	–
7. *Mdh-1*	–	–	–	–	1	–		72	–	–	–	–	–	–	–	72	–	–	–	–	–	–	–	54	54	–	–	–	–	72	–	–
8. *Mdh-2*	–	–	1	–	2	–	1		–	–	–	–	–	–	–	144	–	–	–	–	–	–	–	126	54	–	–	–	72	72	–	–
9. *M e-1*	–	–	–	–	3	1	–	–		–	–	–	–	–	–	–	–	173	–	–	173	108	90	54	90	198	108	–	191	83	–	–
10. *M e-3*	–	–	–	–	1	1	–	–	–		–	–	–	108	–	–	–	108	–	108	72	72	72	–	–	–	–	180	72	–	108	–
11. *6Pg-1*	–	1	–	1	1	–	–	–	–	–		124	104	124	104	–	–	–	124	–	–	–	–	124	228	228	104	124	124	104	–	–
12. *6Pg-2*	–	–	–	–	1	–	–	–	–	–	1		–	214	–	72	–	103	178	–	–	–	180	389	210	277	135	358	403	86	67	230
13. *6Pg-3*	–	1	–	1	1	–	–	–	–	–	1	–		–	104	–	–	–	–	–	–	–	–	–	104	104	104	–	–	104	–	–
14. *G 6 p*	–	–	–	–	1	–	–	–	–	1	1	2	–		–	36	–	144	178	108	–	–	–	214	124	124	68	304	160	–	108	36
15. *G d h*	–	1	–	1	1	–	–	–	–	–	1	–	1	–		–	–	–	–	–	–	–	–	–	104	104	104	–	–	104	–	–
16. *P x*	–	–	1	–	2	–	1	2	–	–	–	1	–	1	–		–	–	–	–	–	–	–	198	54	–	–	36	144	108	–	36
17. *Tzo-3*	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	72	–	–	89	–	–	–
18. *Tzo-4*	1	–	–	–	4	–	–	–	2	1	–	2	–	2	–	–	–		36	108	238	–	173	157	173	157	150	108	150	166	175	–
19. *Got-1*	–	–	–	–	–	–	–	–	–	–	1	1	–	1	–	–	–	1		–	–	–	–	178	124	124	–	124	124	–	–	–
20. *Got-2*	–	–	–	–	1	–	–	–	–	1	–	–	–	1	–	–	–	1	–		–	–	–	–	–	–	–	108	–	–	108	–
21. *G k*	1	–	–	–	2	1	–	–	2	1	–	–	–	–	–	–	–	3	–	–		72	227	54	155	90	65	72	155	148	–	–
22. *Pgm-1*	–	–	–	–	1	2	–	–	1	1	–	–	–	–	–	–	–	–	–	–	1		72	–	–	108	108	72	180	–	–	–
23. *Pgm-2*	1	–	–	–	1	1	–	–	1	1	–	2	–	–	–	–	–	2	–	–	3	1		126	173	90	83	234	126	83	–	108
24. *Est-1*	–	–	1	–	4	–	1	2	1	–	1	4	–	2	–	3	–	3	1	–	1	–	2		232	245	67	178	389	54	67	36
25. *Est-2*	1	1	–	1	3	–	1	1	1	–	2	2	1	1	1	1	–	2	1	–	2	–	2	3		404	255	196	210	327	–	86
26. *Est-3*	–	2	–	1	5	1	–	–	2	–	2	3	1	1	1	–	1	2	1	–	1	1	1	3	4		347	196	457	190	67	86
27. *Est-4*	1	1	–	1	3	1	–	–	1	–	1	2	1	1	1	–	–	2	–	–	1	1	1	1	3	4		–	243	255	67	68
28. *A m y*	–	–	–	–	1	1	–	–	–	2	1	4	–	3	–	1	–	1	1	1	1	1	3	2	2	2	–		322	108	108	180
29. *Lap-1*	–	1	1	–	5	2	–	1	2	1	1	5	–	2	–	2	1	2	1	–	2	2	2	5	2	5	3	4		169	67	122
30. *Lap-2*	1	1	–	1	3	–	1	1	1	–	1	1	1	–	1	2	–	2	–	–	2	–	1	1	4	2	3	2	2		–	86
31. *F m*	–	–	–	–	2	–	–	–	–	1	–	1	–	1	–	–	–	2	–	1	–	–	–	1	–	1	1	1	1	–		–
32. *P g i*	–	–	–	–	–	–	–	–	–	–	–	3	–	1	–	1	–	–	–	–	–	–	1	1	1	1	1	2	2	1	–	

—; not studied

Model 3: The pairing alleles in *B* locus segregate according to the Mendelian ratio, while, nonrandom segregation causes in *A* locus. The two loci are not linked.
Model 4: The pairing alleles in *B* locus segregate according to the Mendelian ratio, while, nonrandom segregation causes in *A* locus. The two loci are linked each other.
Model 5: The pairing alleles in *A* locus segregate according to the Mendelian ratio, while, nonrandom segregation causes in A locus. The two loci are not linked.
Model 6: The pairing alleles in *A* locus segregate according to the Mendelian ratio, while, nonrandom segregation causes in *B* locus. The two loci are linked each other.
Model 7: Nonrandom segregation causes in both *A* and *B* locus, and the two loci are not linked.
Model 8: Nonrandom segregation causes in both *A* and *B* locus, and the two loci are linked each other.

Let segregation ratios in *A* and *B* loci be $A1:A2 = 1:q_A$, $B1:B2 = 1:q_B$ respectively, and the recombination value between two loci be r. The definitions of the three parameters, q_A, q_B, and r and the number of parameter (f) without imposed restriction in each model were summarized as *Table 1.*

Assuming the discrete random variable $X = [x_1, x_2, ---, x_k]$ accords to the multinomial distribution of the parameter $P = [p_1, p_2, ---, p_k]$ independently and identically, the logarithmic likelihood function (l) could be defined as follows:

$$l = \log_e\{n!/(x_1!\cdot x_2!\cdot ---\cdot x_k!)\} + \Sigma x_i \cdot \log_e p_i$$

where: $n = x_1+x_2+ --- +x_k$

When the p was obtained so as the l being maximum by maximum likelihood method, the value of l is the maximum likelihood estimate ($\hat{l}$). As the first term in this equation is the constant, AIC can be compared only with second term. The maximum likelihood estimates ($\hat{l}$) for AIC of the nine models in this study were calculated by the following formula:

$$\hat{l} = a\cdot\log_e p_{A1B1} + b\cdot\log_e p_{A1B2} + c\cdot\log_e p_{A2B1} + d\cdot\log_e p_{A2B2}$$

$p_{A.B.}$ were expected probabilities of each genotype, and they were shown in *Table 2.* The estimates of $p_{A.B.}$ in each model were calculated from the expected probabilities by method of maximum likelihood. As in model 8, general solution of estimates could not be obtained, the approximate values were calculated using NEWTON-RAPHSON method that was able to solve the non-linear simultaneous equation (SAKANO, 1982).

Results and Discussion

Inheritance of isozyme variations in 19 enzyme systems was investigated in Japanese black pine, and 37 loci encoding them were found (SHIRAISHI, in press). More than six megagametophytes of wind-pollinated seeds from each 18 mother tree were analyzed and the genotypes in these loci were assumed previously. As no heterozygous mother trees were found in five, *Adh-2, Me-2, Tzo-1, Tzo-2,* and *Got-3,* of 37 allozyme loci, the association among remaining 32 loci were investigated. Two hundred and twenty-one combinations among possible 496 two-locus combinations of the 32 polymorphic loci were studied for linkage analyses. The number of families and the total number of megagametophytes analyzed for particular pair of loci are shown in *Table 3.*

Chi-square test for random joint segregation has been used to determine the linkage. Unbalanced segregation of a pair of alleles in only one locus doesn't affect the chi-square analysis for joint segregation to detect linkage (BAILEY, 1961). If both loci, however, are unbalanced, the chi-square analysis is not valid. Therefore, in addition to chi-square test, AIC analysis which can detect linkage even from the data with unbalanced segregations in both loci, was applied in this study.

The total of 349 families were analyzed to discuss the linkage relationships in the available 221 two-locus combinations. Data from more than two families were obtained in 80 combinations of them, and only one family's data was used in remaining 141 combinations. From analyses of these data, linkage was recognized in 15 pairwise combinations among 19 loci (*Table 4* and *Table 5*).

In most of 141 two-locus pairs with single family, the chi-square analyses for joint segregations confirmed independence of the loci. In ten pairs, however, chi-square for joint segregation departed significantly from expected 1:1 ratio. Nine pairs except *Mdh-1:Est-2* exhibited Mendelian 1:1 ratio in both single locus segregation, and departed significantly from expected value only in joint segregation. In AIC analysis for these pairs, model 2 that the two loci

Table 4. — The combinations of two-loci (*A;B*) which recognized significant deviation from random joint segregation by chi-square analysis.

Combination A : B	Family	Sample size	Segregation classes A1B1:A1B2:A2B1:A2B2				Chi-square χ^2_A (p)	χ^2_B (p)	χ^2_L (p)
Adh-3:Est-2	Oki 102	104	28	21	20	35	0.35(0.56)	0.62(0.44)	4.65(0.04)
Adh-3:Lap-1	Sendai-sho 2	89	36	9	14	30	0.01(0.92)	1.36(0.25)	20.78(0.01)
Shd-1:Est-2	Oki 102	104	39	11	17	37	0.15(0.70)	0.62(0.44)	22.15(0.01)
Shd-2:6Pg-3	Oki 102	104	33	21	18	32	0.15(0.70)	0.04(0.85)	6.50(0.02)
Shd-2:Got-2	Oki 103	108	30	21	22	35	0.33(0.57)	0.15(0.71)	4.48(0.04)
Mdh-1:Est-2	Minamitakaki 102	54	25	8	10	11	2.67(0.11)	4.74(0.04)	6.00(0.02)
M e-1:Lap-2	Yatsuka 102	83	38	7	4	34	0.59(0.45)	0.01(0.92)	44.83(0.01)
6Pg-1:Est-4	Oki 102	104	34	20	18	32	0.15(0.70)	0	7.54(0.01)
6Pg-3:Est-3	Oki 102	104	33	20	21	30	0.04(0.85)	0.15(0.70)	4.65(0.04)
Got-2:F m	Oki 103	108	33	19	23	33	0.15(0.71)	0.15(0.71)	5.33(0.03)
Shd-2:Est-3	Minamimatsuura 117	72	27	19	12	14	5.56(0.03)	0.50(0.49)	1.39(0.25)
	Oki 102	104	32	22	18	32	0.15(0.70)	0.15(0.70)	5.54(0.03)
	Motoyoshi 101	108	18	22	27	41	7.26(0.01)	3.00(0.09)	0.93(0.34)
	Sendai-sho 2	72	22	18	14	18	0.89(0.36)	0	0.89(0.36)
	Kawanabe 41	52	14	14	8	16	0.31(0.59)	1.23(0.28)	1.23(0.28)
G 2 d:Pgm-1	Motoyoshi 101	108	64	0	0	44	3.70(0.06)	3.70(0.06)	108.00(0.01)
	Asakuchi 101	72	30	0	0	42	2.00(0.16)	2.00(0.16)	72.00(0.01)
M e-1:Tzo-4	Minamimatsuura 117	90	40	5	9	36	0	0.71(0.41)	42.71(0.01)
	Yatsuka 102	83	38	7	4	34	0.59(0.45)	0.01(0.92)	44.83(0.01)
Tzo-4:Lap-2	Yatsuka 102	83	42	0	0	41	0.01(0.92)	0.01(0.92)	83.00(0.01)
	Kawanabe 15	83	41	1	0	41	0.01(0.92)	0.01(0.92)	79.05(0.01)
Pgm-2:A m y	Minamimatsuura 101	54	19	6	13	16	0.30(0.59)	1.85(0.18)	4.74(0.04)
	Asakuchi 101	72	20	17	16	19	0.06(0.82)	0	0.50(0.49)
	Minamimatsuura 108	108	29	23	20	36	0.15(0.71)	0.93(0.34)	4.48(0.04)

Remarks; assumed that the largest linkage class is the parental type (*A1B1; A2B2*)

Table 5. — **Results of AIC analysis in the two-locus combinations recognized as linkage.**

Combination	Family	Model								
		0	1	2	3	4	5	6	7	8
Adh-3:Est-2	Oki 102	288.9	288.4	285.7*	290.0	287.3	289.7	287.0	291.4	288.9
Adh-3:Lap-1	Sendai-sho 2	229.5	246.8	227.1*	248.8	229.1	247.4	227.7	249.4	229.5
Shd-1:Est-2	Oki 102	270.0	288.4	267.3*	290.2	269.2	289.7	268.7	291.6	270.0
Shd-2:6Pg-3	Oki 102	287.5	288.4	283.8*	290.2	285.6	290.3	285.7	292.2	287.5
Shd-2:Got-2	Oki 103	300.5	299.4	296.9*	301.1	298.6	301.3	298.8	303.0	300.5
Mdh-1:Est-2	Minamitakaki 102	143.8	149.7	145.6	149.0	144.9	146.9	142.8*	146.2	143.8
Me-1:Lap-2	Yatsuka 102	185.0	230.1	182.0*	231.5	183.4	232.1	184.0	233.5	185.0
6Pg-1:Est-4	Oki 102	286.6	288.4	282.7*	290.2	284.6	290.4	284.7	292.2	286.6
6Pg-3:Est-3	Oki 102	289.5	288.4	285.7*	290.3	287.6	290.2	287.5	292.2	289.5
Got-2:Fm	Oki 103	299.7	299.4	296.1*	301.3	297.9	301.3	297.9	303.1	299.7
Shd-2:Est-3	Minamimatsuura 117	198.5	199.6	200.2	196.0*	196.6	201.1	201.7	197.5	198.5
	Oki 102	288.4	288.4	284.8*	290.2	286.6	290.2	286.6	292.0	288.4
	Motoyoshi 101	294.8	299.4	300.5	294.1	295.2	298.4	299.5	293.1*	294.8
	Sendai-sho 2	203.8	199.6*	200.7	200.7	201.9	201.6	202.7	202.7	203.8
	Kawanabe 41	147.2	144.2*	144.9	145.9	146.6	144.9	145.7	146.6	147.2
G2d:Pgm-1	Motoyoshi 101	152.0	299.4	151.7	297.7	150.0*	297.7	150.0*	296.0	152.0
	Asakuchi 101	103.8	199.6	101.8*	199.6	101.8*	199.6	101.8*	201.6	103.8
Me-1:Tzo-4	Minamimatsuura 117	207.2	249.5	204.6*	251.5	206.6	250.8	205.9	252.8	207.2
	Yatsuka 102	185.0	230.1	182.0*	231.5	183.4	232.1	184.0	233.5	185.0
Tzo-4:Lap-2	Yatsuka 102	121.1	230.1	117.1*	232.1	119.1	232.1	119.1	234.1	121.1
	Kawanabe 15	130.5	230.1	127.9*	232.1	129.9	232.1	129.9	234.1	130.5
Pgm-2:Amy	Minamimatsuura 101	148.0	149.7	146.9*	151.4	148.6	149.9	147.1	151.6	148.0
	Asakuchi 101	205.1	199.6*	201.1	201.6	203.1	201.6	203.1	203.6	205.1
	Minamimatsuura 108	300.0	299.4	296.9*	301.3	298.8	300.5	298.0	302.4	300.0

*) MAICE

assumed to be linked, scored the minimum value (MAICE), and was selected as the best one. In the combination between *Mdh-1* and *Est-2* of Minamitakaki 102 family, chi-square for segregation of a pair of alleles in *Est-2* deviated significantly from random segregation. The linkage, however, was found by the significant departure from the expected value in joint segregation. AIC exhibited MAICE in model 6. So this model which took linkage into account with a nonrandom segregation in one locus was selected, and these two loci were confirmed to be linked. By using AIC analysis, it was possible to get more information on a sort of gametic selection which results in an unequal production of gametes with respective genes.

Among 80 two-locus pairwise combinations with more than two families, five linkage pairs were observed in the total of nine families. All the testing families in the two pairs, *Me-1:Tzo-4* and *Tzo-4:Lap-2*, exhibited the large chi-square value for the joint segregation, and model 2 of AIC was selected as the best model. Tight linkage was recognized in these two pairs. In the combination between *G2d:Pgm-1*, the chi-squares of two families exhibited significant distortion for independence in two loci, and no significant distortion for the single locus segregation in each locus. In AIC analysis, the models (Model 4, 6 in Motoyoski 101 and Model 2, 4, 6 in Asakuchi 101) that the two loci assumed to be linked were adopted. Three families were investigated for the relationship between *Pgm-2:Amy*. In two of them, Minamimatsuura 101 and Minamimatsuura 108, the linkage was observed as the results of chi-square and AIC analysis. In Asakuchi 101, the linkage wasn't confirmed. As two of three families exhibited the linkage, these two loci seemed to be linked weakly. In *Shd-2:Est-3* combination, only one of the five testing families, Oki 102 family exhibited the significant linkage. As there were the linkages between *Shd-2:6Pg-3*, and between *6Pg-3:Est-3* as mentioned above, *Shd-2* and *Est-3* seemed to be linked very weakly.

As mentioned above, linkage was recognized between *Adh-3* and *Lap-1* in this study. Also in *Pinus sylvestris*, the linkages were observed between the two loci (ADH-MA, ADH-MB) of alcohol dehydrogenase and the one (LAP-B) of leucine aminopeptidase (Rudin and Ekberg 1978). The difference in electrophoretical procedures between Rudin's and this study prevented the accurate identification of loci. However, it seemed that *Adh-3* and *Lap-1* in *P. thunbergii* were homologous to ADH-MA or ADH-MB, and LAP-B in *P. sylvestris*, respectively.

The fifteen pairwise combinations recognized as linkage involved 19 loci. These loci were classified into following six linkage groups.

group A: *Shd-2, 6Pg-3, Got-2, Est-3, Fm*
group B: *Adh-3, Shd-1, Mdh-1, Est-2, Lap-1*
group C: *Me-1, Tzo-4, Lap-2*
group D: *G2d, Pgm-1*
group E: *6Pg-1, Est-4*
group F: *Pgm-2, Amy*

Table 6. — **Estimated recombination value between the loci that belong to each linkage group.**

Combination	Family	Sample size	No. of rec.*	Recombination value $r \pm S_r$	$R \pm S_R$
Group A					
Shd-2:6Pg-3	Oki 102	104	39	0.38±0.05	
Shd-2:Got-2	Oki 103	108	43	0.40±0.05	
Shd-2:Est-3	Minamimatsuura 117	72	31	0.43±0.06	0.43±0.02
	Oki 102	104	40	0.39±0.05	
	Motoyoshi 101	108	49	0.45±0.05	
	Sendai-sho 2	72	32	0.44±0.06	
	Kawanabe 41	52	22	0.42±0.07	
Shd-2:Fm	Oki 103	108	53	0.49±0.05	0.48±0.04
	Kawanabe 41	52	24	0.46±0.07	
6Pg-3:Est-3	Oki 102	104	41	0.39±0.05	
Got-2:Fm	Oki 103	108	42	0.39±0.05	
Est-3:Fm	Kawanabe 41	67	33	0.49±0.06	
Group B					
Adh-3:Shd-1	Oki 102	104	43	0.41±0.05	
Adh-3:Est-2	Oki 102	104	41	0.39±0.05	
Adh-3:Lap-1	Sendai-sho 2	89	23	0.26±0.05	
Shd-1:Est-2	Oki 102	104	28	0.27±0.04	
Mdh-1:Est-2	Minamitakaki 102	54	18	0.33±0.06	
Est-2:Lap-1	Kawanabe 57	86	37	0.43±0.05	0.46±0.03
	Kimotsuki 8	124	59	0.48±0.05	
Group C					
Me-1:Tzo-4	Minamimatsuura 117	90	14	0.16±0.04	0.15±0.03
	Yatsuka 102	83	11	0.13±0.04	
Me-1:Lap-2	Yatsuka 102	83	11	0.13±0.04	
Tzo-4:Lap-2	Yatsuka 102	83	0	0.00±0.00	0.01±0.01
	Kawanabe 15	83	1	0.01±0.01	
Group D					
G2d:Pgm-1	Motoyoshi 101	108	0	0.00±0.00	0.00±0.00
	Asakuchi 101	72	0	0.00±0.00	
Group E					
6Pg-1:Est-4	Oki 102	104	38	0.37±0.05	
Group F					
Pgm-2:Amy	Minamimatsuura 101	54	19	0.35±0.07	0.41±0.03
	Asakuchi 101	72	33	0.46±0.06	
	Minamimatsuura 108	108	43	0.40±0.05	

*) Number of recombinant type

The segregation data and estimated recombination values of available two-locus combinations in each group were shown in *Table 6*. The estimated recombination value (r) and associated standard error (S_r) were calculated as follows:

$$r = NR/n$$
$$S_r = \sqrt{r(1-r)/n}$$

where: NR and n are the number of recombinant types (*A1B2* plus *A2B1*) and the total number of megagametophytes analyzed respectively.

Map distance is generally used to express the distance between the loci on the chromosome. It is the percentage of the frequency of crossing over between the linked two loci. Frequency of crossing over is almost the same as the recombination value when the two loci were linked tightly. However, the discrepancy between the frequency of crossing over and recombination value becomes greater as the distance becomes greater. This gap is caused by missed counting of multiple crossing over, and recombination value is always estimated smaller than the frequency of crossing over. KOSAMBI's formula (1944) has been utilized in many kinds of organism for estimating the map distance from the recombination value (BHAT, 1948 and 1950). JENSEN and JØRGENSEN'S method (1975) using maximum likelihood estimate has been applied to adjust the map distance among loci. It was reported, however, that the location of loci on the chromosome affected the interference among loci (BAILEY, 1961). Therefore, it is necessary to gather the information where the set of loci located on the chromosome, i.e. the distance from centromere or chromosome terminus. There are, however, no available information in this species. Estimated recombination values were utilized without any transformation as relative distance between loci in this study.

In the two-locus combinations with the data from more than two families, the heterogeneity for the joint segregations among families was checked by chi-square test. As no significant departure was indicated in all linkage combinations, mean recombination value (R) and associated standard error (S_R) were calculated as follows:

$$R = \Sigma NR_i / \Sigma n_i$$
$$S_R = \sqrt{R(1-R)/\Sigma n_i}$$

where: NR_i and n_i are the number of recombinant types (*A1B2* plus *A2B1*) and the total number of megagametophytes analyzed in ith family.

Three linkage groups, A, B and C were composed of three or more loci and three remaining groups contained single pair combination. The placement of the loci were decided by three-point mapping (OHMURA, 1982) in group A, B, and C.

Group A

In seven of ten possible two-locus pairs of the five loci, data from six families (12 families in total) were available. Oki 103 was triple heterozygous clone in *Shd-2*, *Got-2*, and *Fm*. The estimated recombination values among these loci were 0.40 ± 0.05 in *Shd-2:Got-2*, 0.49 ± 0.05 in *Shd-2:Fm*, and 0.39 ± 0.05 in *Got-2:Fm*. As the recombination value was the largest between *Shd-2:Fm* and these two loci seemed to be linked most loosely, it was found these three loci were arranged as *Shd-2*, *Got-2* and *Fm* in order. Oki 102 had three heterozygous loci, *Shd-2*, *6Pg-3* and *Est-3*. The recombination values among the three loci were 0.38 ± 0.05 between *Shd-2* and *6Pg-3*, 0.39 ± 0.05 between *Shd-2* and *Est-3*, and 0.39 ± 0.05 between *6Pg-3* and *Est-3*. They were very similar each other. Kawanabe 41 possessed three heterozygous loci, *Shd-2*, *Est-3*, and *Fm*. The recombination value of each pair was 0.42 ± 0.07 in *Shd-2:Est-3*, 0.46 ± 0.07 in *Shd-2:Fm* and 0.49 ± 0.06 in *Est-3:Fm*. Respective recombination values were very large. As estimated recombination values contain some possible errors, in the case that they are on close together or large, enough attention should be paid to determine the linear arrangement of loci by three-point mapping. It was relinquished to decide the ordering of *6Pg-3* and *Est-3* in relation to *Shd-2*, *Got-2* and *Fm*. Additional families possessing suitable genotypes must be examined to clarify where *6Pg-3* and *Est-3* are located in this group.

The data from more than two families were obtained in *Shd-2:Est-3* and *Shd-2:Fm* pairs. The mean recombination values in these pairs were calculated as 0.43 ± 0.02 and 0.48 ± 0.04 respectively.

Group B

This group contained five loci. As no data for *Mdh-1* could be obtained except the association with *Est-2*, the ordering of *Mdh-1* in relation to *Adh-3*, *Shd-1* and *Lap-1* could not be determined. The arrangement of other four loci except *Mdh-1* was discussed. Among four families examined, Oki 102 was triple heterozygous clone in *Adh-3*, *Shd-1* and *Est-2*. The recombination values in each pair of these loci were 0.41 ± 0.05 (*Adh-3:Shd-1*), 0.39 ± 0.05 (*Adh-3:Est-2*) and 0.27 ± 0.04 (*Shd-1:Est-2*). As the largest value was exhibited between *Adh-3* and *Shd-1*, these three loci seemed to be arranged in the order of *Adh-3*, Est-2 and *Shd-1*. No families possessing heterozygous *Lap-1* and other two loci were available to decide the placement of *Lap-1*. The recombination values among *Lap-1*, *Adh-3* and *Est-2*, were 0.39 ± 0.05 in *Adh-3:Est-2* of Oki 102, 0.26 ± 0.05 in *Adh-3:Lap-1* of sendai-sho 2 family, and 0.43 ± 0.05 and 0.48 ± 0.05 in *Est-2:Lap-1* of Kawanabe 57 and Kimotsuki 8 respectively. As the recombination value between *Adh-3:Lap-1* was the smallest and between *Est-2:Lap-1* was the largest, these three loci seemed to be located in the order of *LAP-1*, *ADH-3* and *Est-2*. It needs to gain further data from the suitable family possessing the triple heterozygous loci including *Lap-1* to achieve accurate mapping. The mean recombination value in *Est-2:Lap-1* was 0.46 ± 0.03.

Group C

The data on all possible two-locus pairs of the three loci, *Me-1*, *Tzo-4* and *Lap-2* were obtained. The recombination values estimated from Yatsuka 102 family whose maternal parent was triple heterozygous in these loci, were 0.13 ± 0.04 in *Me-1:Tzo-4* and *Me-1:Lap-2*, and 0.00 ± 0.00 in *Tzo-4:Lap-2*. As no recombinant types were found in 83 megagametophytes analyzed in *Tzo-4:Lap-2* combination, the accurate placement of these loci could not be determined. By analyzing additional samples and detecting the recombinant class, the ordering of the loci will be decided. The mean recombination values were 0.15 ± 0.03 in *Me-1:Tzo-4* and 0.01 ± 0.01 in *Tzo-4:Lap-2*. The three loci in this linkage group located on close each other, especially *Tzo-4* and *Lap-2* were linked almost completely.

Group D

G2d and *Pgm-1* belong to this group. No recombinants were detected in the total of 180 megagametophytes from

two families, Motoyoshi 101 and Asakuchi 101 families. They were linked completely.

Group E

The recombination value between *6Pg-1* and *Est-4* calculated from the data of only one family, and it was 0.37 ± 0.05.

Group F

The slight linkage was recognized between *Pgm-2* and *Amy*. The mean recombination value calculated from the data of three families was 0.41 ± 0.03.

Conclusions

The linkage relationships were studied among 32 polymorphic allozyme loci in Japanese black pine. A total of 349 families in 221 two-locus pairs were examined. Linkage was detected in 15 pairwise combinations using chi-square and AIC analyses. *G2d* and *Pgm-1* were linked completely. *Lap-2* and *Tzo-4* were almost completely with an estimated recombination value of 0.01 ± 0.01, and they were also linked tightly to *Me-1* with recombination values of 0.13 ± 0.04 and 0.15 ± 0.03. *Adh-3:Lap-1* and *Shd-1:Est-2* were linked with recombination values of 0.26 ± 0.05 and 0.27 ± 0.04 respectively. Remaining nine pairwise combinations were linked weakly with recombination values greater than 0.30. The nineteen loci involved in these linkage combinations were classified into six linkage groups. On the basis of the estimated recombination value and ordering of the loci in each group, a linkage map shown in *Figure 1* was made up.

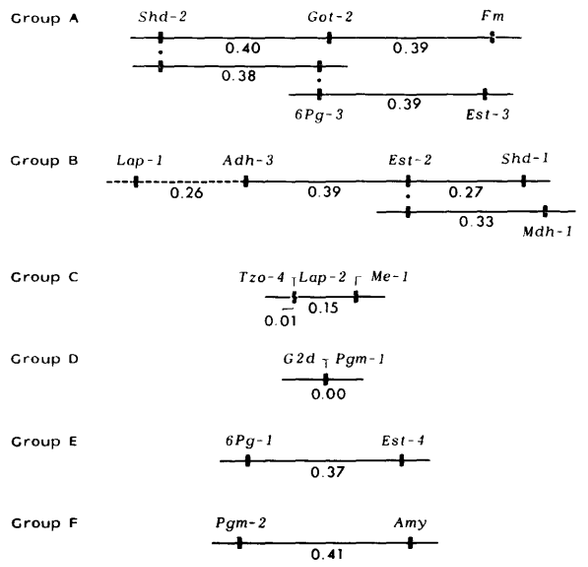

Figure 1. — A schematic linkage map of Japanese black pine, *Pinus thunbergii*.

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