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Segregation and Linkage Relationships of Allozymes in *Pinus Brutia* Ten.

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Abstract

Female gametophytes of *Pinus brutia* Ten. were analyzed to study Mendelian segregation and linkage among allozyme loci in a seed orchard composed of 28 clones. Isozyme variants of nine enzyme systems encoding 14 loci from megagametophytes were assayed by starch gel electrophoresis. While six of the 14 loci were monomorphic, the remaining eight were polymorphic. Analysis of observed segregation ratios of all polymorphic loci except for *Sdh1*, showed a good fit to the 1:1 ratios expected for Mendelian inheritance. Linkage rela-

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tionships of the eight polymorphic allozyme loci were analyzed by testing 23 of the 28 possible two-locus combinations. Two-locus segregations in three pairs (*Got2-Mdh1*, *Got2-Pgi2* and *Pgd3-Sdh1*) were significantly heterogeneous ($P \leq 0.05$). Relatively weak linkage was observed between only one pair of genes (*Adh2:Got2*). Recombination fraction for *Adh2:Got2* was found to be 0.24 (± 0.06).

Key words: linkage, allozyme, inheritance, seed orchard, *Pinus brutia*.

Introduction

Allozyme analyses are used in a wide area of forest genetics: genome mapping, mating system analysis, genetic variation and diversity studies, taxonomic determinations, and forest tree improvement programs (BROWN et al., 1975; NEALE and ADAMS, 1981; BOYLE and MORGENSTERN, 1985; STRAUSS and CONKLE, 1986; NIEBLING et al., 1987; SZMIDT and MUONA, 1989). Segregation distortion of allozymes can affect the evolution of natural populations and can cause biased estimates of allele frequencies, heterozygosities and mating system parameters (CHELIAK et al., 1984; STRAUSS and CONKLE, 1986). Therefore, the study of segregation distortion provides important information for use in population genetic studies. Furthermore, many studies in forest genetics require information from unlinked loci, especially those that include the interpretation of multilocus genetic structure (STRAUSS and CONKLE, 1986). In addition, information from the linkage of loci coding for quantitative characters can be a powerful tool in plant breeding (FAROOQ and AZAM, 2002).

In gymnosperms, linkage is easily analyzed by using the female gametophyte. When studying maternal gametes, crosses are not needed because of the haploid nature of this tissue, and their easily interpreted allozyme phenotypes (CHELIAK and PITEL, 1985; STRAUSS and CONKLE, 1986).

To our knowledge, no linkage information for *P. brutia* isozyme variants has been published to date. The main objectives of this study were to examine segregation distortion and to investigate the occurrence of linkage among allozyme loci of *P. brutia*.

Material and Methods

The genotypes of 28 *P. brutia* clones from a seed orchard were determined at 14 allozyme loci by KAYA et al. (2005). Eight of the 14 loci studied were polymorphic among the 28 clones. In the current study, to determine segregation and linkage relationship of allozyme loci in *P. brutia*, we obtained seeds from 25 of these genetically identified 28 clones, because these 25 clones were heterozygous at two or more of the eight polymorphic loci (KAYA et al., 2005). Megagametophytes of about 45 to 50 wind pollinated seeds were analyzed, using starch gel electrophoresis for each heterozygous locus in each of 25 clones. Analyses were performed on eight polymorphic loci: *Adh2*, *Got2*, *Mdh1*, *Mnr2*, *Pgd2*, *Pgd3*, *Pgi2* and *Sdh1*. The enzymes are listed in *Table 1* along with their enzyme commission numbers, abbreviations, gel buffers used, and numbers of loci scored. Details for electrophoresis procedures, including band pattern interpretation, have been presented in previous studies (KARA et al., 1997; KAYA, 2001; KAYA et al., 2005).

In order to determine segregation distortion, the 1:1 ratio of variants in each polymorphic locus was tested for each segregating pair of allozymes by a Chi-square goodness-of-fit test. To find linkage relationships, 23 of the possible 28 two-locus combinations were found among the 25 clones sampled. Chi-square tests for linkage were performed following the procedure described in ADAMS and JOLLY (1980) for all segregating pairs of loci.

Table 1. – The assayed enzymes, their abbreviations, enzyme commission numbers, gel buffers used, and numbers of loci scored in *Pinus brutia*.

Enzyme's name	Abbreviation	E.C. No.	Buffer*	Numbers of loci scored
Aconitase	Aco	4.2.1.3	$MC_{6.1}$	1
Alcohol dehydrogenase	Adh	1.1.1.1	TBE	1
Glutamate dehydrogenase	Gdh	1.4.1.2	TC	1
Glutamate oxaloacetate-transaminase	Got	2.6.1.1	TC	3
Malate dehydrogenase	Mdh	1.1.1.37	$MC_{8.1}$	1
Menadione reductase	Mnr	1.6.99.2	TBE	2
6-Phosphogluconate dehydrogenase	6pgd	1.1.1.44	$MC_{6.1}$	2
Phosphoglucoisomerase	Pgi	5.3.1.9	$MC_{8.1}$	2
Shikimate dehydrogenase	Sdh	1.1.1.25	$MC_{6.1}$	1

* $MC_{6.1}$ = Morpholine Citrate (pH 6.1); $MC_{8.1}$ = Morpholine Citrate (pH 8.1); TBE = Tris-Borate-EDTA; TC = Tris-Citrate. Details on the gel buffers are reported in KARA *et al.,* 1997.

Table 2. – Observed single locus segregation of allozymes from heterozygous mother trees. Chi-square tests of heterogeneity of mother trees and goodness of fit to the 1:1 ratio.

Locus	Alleles				Heterogeneity		Segregation	
	1	2	3	Total	χ^2 (df)	*P*	χ^2 (df=1)	*P*
Adh2	26	24	-	50	-	-	0.10	0.780
Got2	365	344	-	710	16.0 (15)	0.37	0.60	0.430
Mdh1	274	271	-	546	21.0 (10)	0.02	0.02	0.900
Mnr1	120	147	-	267	0.80 (5)	0.98	2.70	0.100
Pgd2	210	216	-	426	12.0 (9)	0.22	0.10	0.770
Pgd2	20	-	14	34	-	-	1.10	0.300
Pgd3	109	125	-	234	1.20 (4)	0.88	1.10	0.300
Pgd3	-	57	57	114	2.60 (2)	0.28	0.00	1.000
Pgd3	143	-	143	286	11.0 (5)	0.04	0.00	1.000
Pgi2	263	272	-	536	19.0 (12)	0.09	0.20	0.700
Sdh1	**210**	**171**	**-**	**381**	**6.80 (8)**	**0.56**	**3.99**	**0.046**
Sdh1	-	117	99	216	5.50 (4)	0.24	1.50	0.220
Sdh1	43	-	46	89	0.30 (1)	0.61	0.10	0.750

The recombination fraction (r) was calculated by the binomial estimator: $r=\frac{k}{n}$, where k is the number of observations in the smaller class (repulsion) and n is the total number of observations. The standard error of r is given by $\sqrt{r(1-r)/n}$ (RUDIN and EKBERG, 1978). Recombination fractions (r) and their standard errors were estimated for all locus pairs.

Results and Discussion

In two previous studies (KAYA, 2001; KAYA et al., 2005), we found that 25 out of 28 orchard clones were heterozygous at two or more loci. In the current study we used these heterozygous clones for segregation and linkage analysis.

Segregation Analysis

To test the hypothesis of inheritance, Chi-square tests were calculated to determine the "goodness of fit" of segregating allozymes to the expected 1:1 ratio for megagametophytes from 25 heterozygous mother trees. Single-locus segregation data are presented in *Table 2*. No locus (except for *Sdh1*) shows deviation at the 0.05 significance level from the 1:1 Mendelian segregation of allozymes when the data for all heterozygous trees were pooled. Deviation from the expected 1:1 ratio in megagametophytes of heterozygous mother trees has been reported for several allozyme loci in several forest tree species such as *Pinus sylvestris*, *Pinus taeda*, *Picea abies* and *Pinus attenuata* (RUDIN and EKBERG, 1978; ADAMS and JOLY, 1980; NEALE and ADAMS, 1981; STRAUSS and CONKLE, 1986). Yet, segregation distortion was not reported for the *Sdh* locus in any *Pinus* species. However, in black spruce (*Picea mariana*), BOYLE and MORGENSTERN (1985) reported significant deviations at the *Sdh* locus and suggested that this locus should not be included in further analyses of black spruce populations sampled.

Several factors could contribute to segregation distortions. These factors include (1) tendency to score the common allele most often on gels that resolve poorly, (2) distortion genes linked to isozyme loci; linkage of embryonic lethal genes, (3) differential viability of gametes carrying different alleles (differential selection) or (4) chance sampling (FURNIER et al., 1986; STRAUSS and CONKLE, 1986; GEBUREK and WUEHLISCH, 1989). It is likely that the observed segregation distortion for the *Sdh1* locus in this study is due to chance sampling alone, since the trees that showed segregation distortion are the ones involving only "genotype" 1/2 for this locus (*Table 2*). Furthermore, only one out of nine heterozygous mother trees for the *Sdh1* locus deviated from the expected 1:1 mendelian segregation ratio.

Analyses of 25 clones for each of eight loci revealed significant segregation distortion only in seven cases among only six clones (*Table 3*). That is, only one clone (Clone 12) had significant distortion at more than one locus (at *Pgd3* and *Sdh1*). In four cases, the common (high frequency) alleles among the 28 clones surveyed were in excess (overrepresented). In the three remaining cases, segregation distortion was in favor of low frequency (uncommon) alleles (*Mdh1*, *Pgd3* and *Pgi2*). STRAUSS and CONKLE (1986) and ADAMS et al. (1990) indicated that overrepresentation of common alleles may, in part, explain their high frequency in populations. ADAMS et al.

Table 3. – Segregation data for clones with significant segregation distortion ($P \leq 0.05$).

Locus	Clone	Ratio*	Excess**
Got2	24	33:18	0.147
Mdh1	22	31:16	0.160
Mdh1	27	15:34	-0.194
Pgd3	12	20:35	-0.136
Pgi2	11	32:17	0.153
Pgi2	15	6:19	-0.260
Sdh1	12	32:18	0.140

* Ratio is common allele: uncommon allele.
** Excess = observed-expected frequency of the common allele.

Table 4. – Two-locus segregation patterns and Chi-square test results for linkage analysis.

Locus pair	**Allelic combination**				**Heterogeneity**[#]		**Segregation at locus 1**		**Segregation at locus 2**		**Joint segregation**		**Recombination Fractions (Se)**[§]
Locus1-Locus2	**11**	**12**	**21**	**22**	**χ^2 (df)**[¥]	**P**	**χ^2 df=1)**	**P**	**χ^2 (df=1)**	**P**	**χ^2 (df=1)**	**P**	
Adh2-Got2	19	7	5	19	-	-	0.08	0.78	0.08	0.78	**13.52**	**<0.01**	0.24 (0.06)
Adh2-Pgd3	11	15	13	11	-	-	0.08	0.78	0.08	0.78	0.72	0.40	0.44 (0.07)
Adh2-Pgi2	14	4	9	7	-	-	0.12	0.74	4.24	0.04	1.88	0.17	0.38 (0.08)
Got2-Mdh1	80	75	78	67	**29.18 (15)**	**0.02**	0.33	0.56	0.85	0.36	0.12	0.73	0.49 (0.07)
Got2-Mnr2	31	29	25	34	4.18 (6)	0.65	0.01	0.93	0.41	0.52	1.02	0.31	0.45 (0.10)
Got2-Pgd2	58	62	55	51	15.71 (15)	0.40	0.87	0.35	0.00	1.00	0.28	0.59	0.48 (0.08)
Got2-Pgi2	74	75	70	74	**32.3 (18)**	**0.02**	0.09	0.77	0.09	0.77	0.03	0.86	0.49 (0.07)
Got2-Sdh1	104	81	92	81	19.3 (21)	0.56	0.40	0.53	3.23	0.07	0.40	0.53	0.48 (0.07)
Mdh1-Mnr2	27	26	22	25	1.64 (3)	0.65	0.36	0.55	0.04	0.84	0.16	0.69	0.48 (0.07)
Mdh1-Pgd2	40	50	45	57	11.88 (9)	0.22	0.75	0.39	2.52	0.11	0.02	0.89	0.49 (0.07)
Mdh1-Pgd3	53	55	66	67	14.52 (12)	0.27	2.59	0.27	0.04	0.85	>0.01	0.95	0.50 (0.07)
Mdh1-Pgi2	86	83	75	80	17.09 (18)	0.52	0.61	0.44	0.01	0.91	0.20	0.66	0.49 (0.07)
Mdh1-Sdh1	77	76	69	52	7.62 (15)	0.93	3.74	0.05	1.18	0.28	0.93	0.33	0.47 (0.07)
Mnr2-Pgd2	27	28	33	34	3.86 (6)	0.70	1.18	0.28	0.03	0.86	0.00	1.00	0.50 (0.07)
Mnr2-Pgd3	27	27	29	47	7.38 (6)	0.29	3.72	0.05	2.49	0.11	2.49	0.11	0.43 (0.07)
Mnr2-Pgi2	13	21	12	26	3.08 (3)	0.38	0.22	0.64	6.72	0.01	0.50	0.48	0.46 (0.07)
Mnr2-Sdh1	35	23	35	27	3.97 (6)	0.68	0.13	0.72	3.33	0.07	0.13	0.72	0.48 (0.08)
Pgd2-Pgd3	40	56	42	61	12.01 (9)	0.21	0.25	0.62	6.16	0.01	0.05	0.83	0.49 (0.07)
Pgd2-Pgi2	24	22	26	23	3.92 (3)	0.27	0.10	0.76	0.26	0.61	0.01	0.92	0.50 (0.07)
Pgd2-Sdh1	56	41	56	51	15.95 (12)	0.19	0.49	0.48	1.96	0.16	0.49	0.48	0.48 (0.08)
Pgd3-Pgi2	60	65	67	64	12.51 (15)	0.64	0.14	0.71	0.02	0.90	0.25	0.62	0.48 (0.09)
Pgd3-Sdh1	77	43	78	66	**28.00 (15)**	**0.02**	2.18	0.14	8.02	0.01	1.83	0.18	0.46 (0.07)
Pgi2-Sdh1	62	73	81	57	17.68 (18)	0.48	0.03	0.85	0.62	0.43	**4.5**	**0.03**	0.44 (0.08)

[#] Heterogeneities of two-locus segregations among clones are computed with the expected numbers for each class based on the observed overall segregations, not on the theoretical 1:1:1:1 segregation.
[¥] df = degrees of freedom.
[§] Se = Standart error.

(1990) also indicated that overrepresentation of uncommon alleles may explain the maintenance of these alleles in populations. It appears that such overrepresentations may be due to differential selection of the respective alleles (STRAUSS and CONKLE, 1986).

Linkage Analysis

Eight loci were polymorphic, hence available to study linkage. Of the 28 possible paired combinations of loci, 23 pairs were studied in at least one clone (*Table 4*). Significant segregation distortion at only one locus of a pair was observed at six pairs (*Adh2-Pgi2*, *Mdh1-Sdh1*, *Mnr2-Pgd3*, *Mnr2-Pgi2*, *Pgd2-Pgd3* and *Pgd3-Sdh1*). However, no significant segregation distortions at both loci of a pair were observed. Distortion at one locus does not disturb linkage estimates (RUDIN and EKBERG, 1978; STRAUSS and CONKLE, 1986).

Two-locus segregations in three pairs (*Got2-Mdh1*, *Got2-Pgi2* and *Pgd3-Sdh1*) were significantly heterogeneous ($P \leq 0.05$) (*Table 4*). These two-locus heterogeneities were due to heterogeneity in segregation ratios at any one of the two loci, not due to heterogeneity of joint segregations. Heterogeneity can be due to significant differences at deficiencies or excesses of various allozymes among the clones, although some clones segregate according to the expected ratios. The data for the *Got2-Mdh1* locus pair, for example, have been obtained from six clones; and mainly clones 8 and 27 caused the heterogeneity. This heterogeneity can be due to sampling error or differential viability of gametes carrying different alleles of an allozyme locus (SZMIDT and MUONA, 1989; CHELIAK and PITEL, 1985). Also, if a locus is linked to a lethal allele of another locus, one can expect deviation from the 1:1:1:1 ratio (SZMIDT and MUONA, 1989; CHELIAK and PITEL, 1984). In some clones given to heterogeneity, deficiencies or excesses of certain alleles can be stable for that clone. This case indicates that the allozyme gene itself, or at least a small part of the chromosome containing that gene, is the target of selection (ADAMS and JOLY, 1980).

Linkage was only accepted if joint segregation was significant at the $P \leq 0.05$ level and no more than one of the segregating loci showed a deviation from the 1:1 ratio at this ($P \leq 0.05$) level. Such a significant joint segregation ($P \leq 0.05$) was detected at only two out of 23 locus pairs tested: *Adh2-Got2* and *Pgi2-Sdh1* (*Table 4*). The data for linkage study at the *Adh2-Got2* locus pair is from only a single clone, for the other locus pair from seven clones (*Table 5*). Furthermore, the estimated recombination fractions of *Adh2-Got2* and *Pgi2-Sdh1* were 0.24 ($S_r = 0.06$) and 0.44 ($S_r = 0.08$), respectively. *Table 5*, where the data are evaluated separately for each clones at *Pgi2-Sdh1* locus pair, show that there is no significant linkage between *Pgi2* and *Sdh1* loci, although there appears to be significant joint segregation between these loci when the data are pooled. The estimated recombination fraction values also suggest that there is a weak linkage for only *Adh2-Got2* locus pair. Although there is not enough evidence about linkage between the *Adh2-Got2* locus pair in previous studies for most of the *Pinus* species, SZMIDT and MUONA (1989) and NIEBLING et al. (1987) mapped *Got2* and *Adh2* loci into the same linkage group in *Pinus sylvestris*.

Table 5. – Two-locus segregation patterns of seven clones for *Pgi2-Sdh1* locus pair.

Locus pair	Clone No	Allelic combination				Segregation at locus 1		Segregation at locus 2		Joint segregation	
Locus1-Locus2		11	12	21	22	χ^2 (df=1)*	P	χ^2 (df=1)*	P	χ^2 (df=1)*	P
Pgi2-Sdh1	5	7	11	14	9	0.61	0.43	0.02	0.88	1.98	0.16
	11	16	15	6	8	6.42	0.01	0.02	0.88	0.20	0.65
	14	6	5	10	5	0.62	0.43	1.38	0.24	0.62	0.43
	17	7	11	9	11	0.11	0.75	0.95	0.33	0.11	0.75
	19	10	11	15	6	0.00	1.00	1.52	0.22	2.38	0.12
	24	10	13	18	8	0.18	0.67	1.00	0.32	3.45	0.06
	28	6	7	9	10	1.13	0.29	0.13	0.72	0.00	1.00
	Pooled	62	73	81	57	0.03	0.85	0.62	0.43	**4.5**	**0.03**

* df = degrees of freedom

Conclusion

The segregation of allozymes in megagametophytes of heterozygous individuals reveals that seven out of the eight polymorphic loci studied did not deviate significantly from the Mendelian independent segregation rule.

The results obtained from linkage analysis showed that one of the 23 locus-pairs (*Adh2-Got2*) was weakly linked and that two-locus segregations in three pairs (*Got2-Mdh1*, *Got2-Pgi2* and *Pgd3-Sdh1*) were significantly heterogeneous. In many population genetic analyses of forest trees (for example mating system parameters) an assumption of independence is required, and unlinked loci provide more efficient and more accurate estimates for obtaining certain parameters (SHAW et al., 1981). Loci that are linked are clearly not independent and, therefore, should not be used for such estimates. In case of linked loci, one locus of the related pair must be discarded. Because there is no other evidence of linkage for the *Adh2-Got2* locus pair in *Pinus* species, the weak linkage observed in this study needs to be verified by increasing sample size, before taking into account each of these two loci for various population genetic analyses.

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Genetic Diversity and Gene Flow of *Quercus crispula* in a Semi-Fragmented Forest Together With Neighboring Forests

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Abstract

The genetic diversity and differentiation in *Quercus crispula* populations in the Chichibu Mountains, central Japan, were evaluated using six microsatellite markers. Gene flow into a 2500-m^2 semi-fragment of a natural forest from the neighboring natural forest at a distance of more than 50m was also evaluated using parentage analysis. All five populations in the mountains had similar levels of genetic diversity (H_e = 0.752–0.792), and the level of population differentiation was low (F_{ST} = 0.016). The semi-fragmented stand showed similar genetic diversity with the neighboring unfragmented forests, and Hardy-Weinberg disequilibrium was not found (F_{IS} = 0.083 in adults, 0.025 in seedlings). In the semi-fragment, 70 seedlings were examined; according to the parentage analysis, eight of the 70 seedlings (ca. 11%) had neither of the parent trees in this fragment. These seeds must have been transported from a distance of more than 50 m; therefore, there could be a possibility that the seeds were dispersed by birds. A similar trend of seed flow into the fragment was also confirmed by genotyping endocarps of hypogeal cotyledons, while more frequent seed flow was found in the neighboring unfragmented forest. Of the remaining 62 seedlings, the maternal trees (but not the paternal trees) of 29 seedlings and both parent trees of 33 seedlings were detected in the semi-fragment. These results indicate that the gene flow among the populations occurs frequently via pollen dispersal and occasionally via seed dispersal and that, at least the current levels of genetic diversity have been maintained in such fragmented forests.

Key words: forest fragmentation, microsatellites, parentage analysis, *Quercus crispula*.

Introduction

Since many years, forest fragmentation has resulted from human activities and has been affecting many ecosystems worldwide, including Japan. A geographic information system (GIS) analysis revealed that 53% of forest patches were smaller than 1ha even in rural area (MIYAMOTO and SHIMADA, 2001). In this country, semi-fragmented natural stands located close to neighboring natural stands are found more frequently rather than those far away from other natural stands. This is partly due to the construction of roads and buildings in the natural forests as seen in other countries. Many trunk roads with a width of several ten meters run through the natural forests. In addition, many mountains are occupied by various small stands due to cutting or planting activities of private small-scale forest owners (KONOHIRA, 1996). Approximately 41% of all forests in Japan are man-made (ANON, 1994), and this percentage is much higher than that in other countries. The unique geographical feature of Japan is one of the reasons for the large number of semi-fragmented natural stands. Of the total Japanese land area, 75% is classified as mountainous or hilly, and 60% of the forest area is characterized as having a slope of 20° or more (HANDA, 1988). Many areas consist of very steep rocky slopes. Consequently, natural stands are often found on partially steep slopes; these are inappropriate for afforestation. Therefore, these semi-fragments are at short distances

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