西日本におけるスギ(Cryptomeria japonica)天然林5集団のアロザイム変異

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Allozyme variation of five natural populations of *Cryptomeria japonica* in western Japan

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ABSTRACT

Genetic variation and geographical diversity of five natural populations of *Cryptomeria japonica* in western Japan were investigated for nine allozyme loci. Genetic variation of this species was somewhat lower than the other coniferous species. Most of the variation was attributable within population (98.16%) rather than between populations (1.84%). The G_{ST} value was only .0156 and genetic distances between populations were also small, averaging .0062. These results indicate that natural *Cryptomeria* forests in western Japan have maintained relatively middle genetic variation, comparing with the other conifer species, but these populations were not genetically differentiated each other.

1. INTRODUCTION

Cryptomeria japonica, sugi, is an endemic species in Japan and one of the most important tree species for Japanese forestry. The species is naturally distributed in areas of the cool temperate zone to the warm temperate zone (from north of Aomori Prefecture to Yaku Island in the south). Japanese *Cryptomeria* forests have been widely planted (4.39 million ha) and represent 44% of Japan's cultivated forests. Reports of genetic variation and geographical diversity in *Cryptomeria* are, however, limited. Yasue et al. (1987) reported two major groups of sugi differentiated geographically, one on the Japan Sea side and the other on the Pacific Ocean side of Japan. The groups were classified by the diterpene components which encode at least two loci. Other reports treated needle morphology (Murai, 1947; Toyama, 1960, 1961a, 1961b).

Many recent reports of genetic variation and diversity research using allozyme markers have been published. Because allozyme is neutral or nearly neutral for natural selection (Kimura, 1983) and many loci can be investigated. In conifers, most allozyme studies have been done with female gametophytes, a maternal haploid tissue. Female gametophytes also have relatively high concentrations of active enzymes in their extracts and lower contents of secondary metabolites such as phenolic and tannin-like compounds. Isozyme analysis with female gametophytes is difficult because *C. japonica* seeds are very small and the proportion

of empty seeds is high. Therefore in this study, isozyme analysis was made on needle tissue, and the inheritance of 10 previously identified enzyme systems is clarified (Tsumura et al., 1989, 1990). Nine loci encoding six enzyme systems were used to investigated *C. japonica* genetic variation and geographical diversity.

2. MATERIALS AND METHODS

Sample Collection

Five natural populations of *C. japonica* in western Japan were located in areas between the Izu Peninsula of Shizuoka Prefecture and Shimane Prefecture (Fig. 1). Needle tissue was collected individually as an electrophoretic sample from each population between February and March, 1988, and stored at −25°C until isozyme analysis was done. Sample size of five populations of Kawazu, Amagi, None, Yanase and Nichihara, were 147, 63, 93, 92 and 128, respectively.

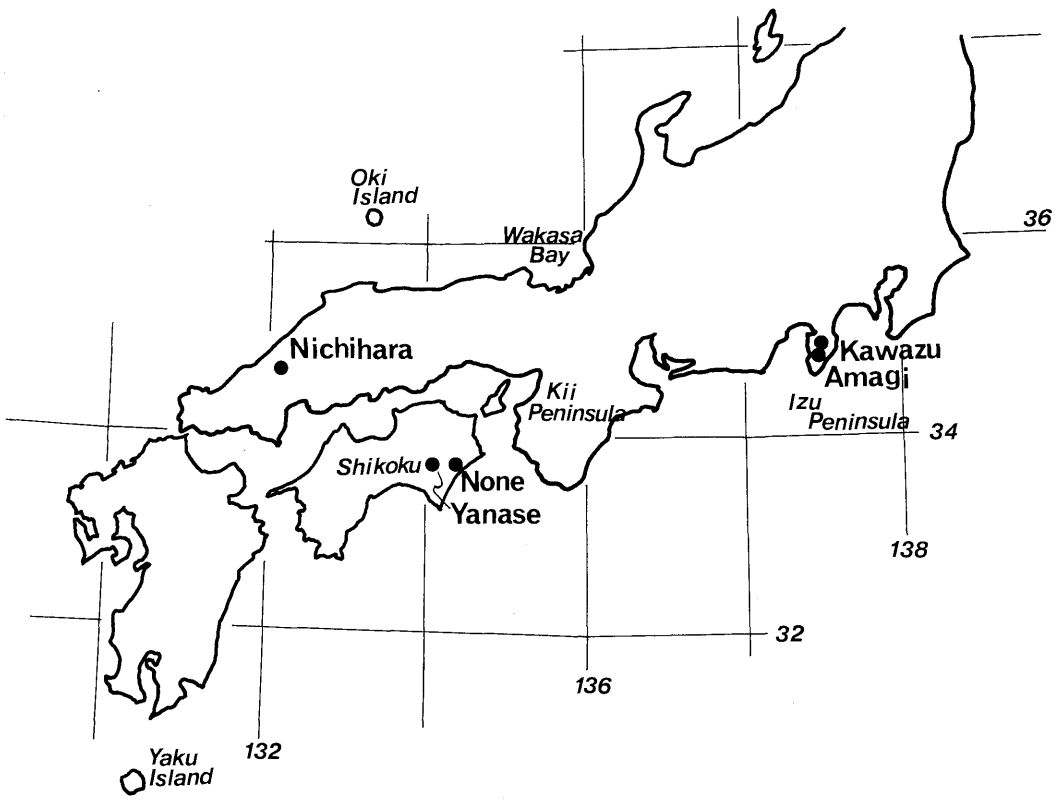

Fig. 1. Location of surveyed five natural populations of *Cryptomeria japonica* in western Japan.

Electrophoresis

The needle samples were prepared for electrophoresis using the following procedure (Tsumura et al., 1990). Needle samples of 100 mg from each tree were ground to a fine powder with a mortar and pestle under liquid nitrogen. Immediately after grinding, 100 mg of polyvinylpolypyrrolidone and 1ml of extraction buffer (93 mM Tris-HCl buffer, pH 7.5, 23.4% glycerol, 0.6% Tween 80, 11 mM DTT, 2.8 mM EDTA, 2.3 mM NAD, 1.6 mM NADP, 0.5% 2-mercaptoethanol, 0.08% BSA) were added. Individual samples of homogenate were then centrifuged at 15,000 rpm at 0°C for 60 minutes. For each enzyme detection, 10 μl of the supernatant were used for electrophoresis. Polyacrylamide vertical slab gel electrophoresis was done using Davis' (1964) and Ornstein's (1964) procedures. A running gel (7.5%) and spacer gel (3.75%) was used. Electrophoresis was carried out at 4°C, 12.3 mA/cm^2 for 150 minutes. Staining methods developed by Tsumura et al. (1990) were then used to analyze the six enzyme systems investigated in this study, shikimate dehydrogenase (ShDH; E.C. No. 1.1.1.25), 6-phosphogluconate dehydrogenase (6PGD; E.C. No. 1.1.1.44), diaphorase (DIA; E.C. No. 1.6.4.3), gultamate oxaloacetate (GOT; E.C. No. 2.6.1.1), phosphoglucomutase (PGM; E.C. No.2.7.5.1) and leucine aminopeptidase (LAP; E.C. No. 3.4.11.1) (Tsumura et al., 1989).

Statistical Analysis

Allele frequency in each population was estimated for the nine loci encoding the six enzyme systems. The following methods were used to quantify the amount of gene diversity within each population: 1) the proportion of polymorphic loci (*Pl*) (95% criterion), 2) the average number of alleles per locus (*Na*), 3) the effective number of alleles per locus (*Ne*) (Kimura and Crow, 1964), and 4) the average heterozygosity (expected heterozygosity averaged over all loci). Expected heterozygosity defined as $He=1-\sum x_i^2$, where $x_i=X_{ii}\times\sum X_{ij}/2$ and X_{ij} is the genotype frequency (Nei and Roychoudhury, 1974). Heterogeneity tests of allele frequency between populations followed the Workman and Niswander (1970) method.

Genetic diversity between populations was estimated by the following procedures: 1) F-statistics (F_{IS}) (Write, 1965) where F_{IS} represent the mean deviation of genotypic proportion from the Hardy-Weinberg expectation (defined as $F=1-H/2pq(1+1/(2N-1))$), where $H=$ the number of heterozygotes observed and $2pq(1+1/(2N-1))=$the number of heterozygotes expected). The mean fixation index over all subpopulations corresponds to the F_{IS} (Kirby, 1975). 2) Nei's gene diversity $H_T=H_S+D_{ST}$ and $G_{ST}=D_{ST}/H_T$ (Nei, 1973) where H_T is the gene diversity in total population, H_S is the average gene diversity between subpopulations, G_{ST} is the relative magnitude of gene differentiation within a subpopulation. 3) Nei's genetic distance (Nei, 1978) was calculated for each population pair and

was the basis for clustering populations by the unweighted pair-group method using arithmetic means (UPGMA) (Sokal and Sneath, 1963).

3. RESULTS

Gene Diversity Within Populations

Allele frequencies for nine loci are shown in Table 1. Three loci, *Shd-1*, *Got-2* and *Pgm-2* were monomorphic in all populations (95% criterion). In at least one

Table 1. Allele frequencies for nine loci in five populations of *Cryptomeria japonica*

Locus	Allele	Population				
		Kawazu	Amagi	None	Yanase	Nichihara
Shd-1	*a*	.986	1.000	1.000	1.000	1.000
	b	.007				
	o	.007				
Shd-2	*a*	.068	.102	.043	.102	.207
	b	.929	.898	.957	.898	.793
	c	.003				
6Pg-1	*a*	.194	.134	.202	.229	.201
	b	.774	.854	.798	.763	.788
	c	.007				
	d	.007				
	e	.017	.012		.008	.011
6Pg-2	*a*	.014	.048	.006	.046	.092
	b	.986	.952	.994	.954	.908
Dia-3	*a*	.772	.807	.651	.660	.614
	b	.228	.193	.349	.340	.386
Got-1	*a*	.014	.011	.011	.043	
	b	.986	.989	.989	.921	1.000
	c				.035	
Got-2	*a*	.983	1.000	1.000	.996	1.000
	d	.017			.004	
Pgm-2	*a*	.003	.011			
	b	.997	.977	1.000	1.000	1.000
	c		.012			
Lap	*a*	.322	.091	.343	.355	.440
	b	.562	.716	.551	.594	.527
	c	.014	.023	.051	.004	.016
	d	.027	.159	.051	.031	.011
	e	.010	.011		.012	
	f	.034		.006	.004	
	g					.005
	h	.003				

of the populations the other six loci were polymorphic. The proportion of polymorphic loci ranged from 0.333 in None to 0.556 in Amagi; the mean was 0.474 (Table 2). The mean number of alleles per locus ranged from 2.00 in None and Yanase to 3.11 in Kawazu, the average being 2.38. The mean expected heterozygosity (*He*) of the Amagi population was the lowest (0.152) while that of Yanase was the highest 0.204. The mean *He* of the five populations was 0.178. The mean observed heterozygosity was 0.153. Fixation indexes (F_{IS}) estimated for the six polymorphic loci varied from 0.006 (Amagi) to 0.216 (Yanase) (Table 3). F_{IS} for three loci (*Dia-3*, *Got-1* and *Lap*) were statistically significant ($p<.001$).

Table 2. Proportion of polymorphic loci (*Pl*), the average number of allele per locus (*Na*), the effective number of allele per locus (*Ne*), and mean expected and observed heterozygosity (*He* and *Ho*) in five populations of *Cryptomeria japonica*

Population	*n*	*Pl* (%)	*Na*	*Ne*	*He*	*Ho*
Kawazu	147	44.4	3.11	1.21	.171	.138
Amagi	63	55.6	2.33	1.18	.152	.152
None	93	33.3	2.00	1.20	.163	.128
Yanase	92	44.4	2.00	1.26	.204	.187
Nichihara	128	44.4	2.44	1.24	.196	.159
Mean	104.5	47.4	2.38	1.22	.178	.153

Table 3. Fixation index (F_{IS}) of six polymorphic loci of five natural populations in *Cryptomeria japonica*

Locus	Kawazu	Amagi	None	Yanase	Nichihara	Mean
Shd-2	0.285	−0.114	—	0.315	0.005	0.049
6Pg-1	0.137	−0.156	0.164	0.158	−0.188	0.022
6Pg-2	—	—	—	—	−0.102	−0.102
Dia-3	0.098	0.198	0.125	0.391	0.381	0.252**
Got-1	—	—	—	0.203	—	0.203**
Lap	0.276	0.097	0.315	−0.022	0.094	0.185**
Mean	0.199	0.006	0.202	0.216	0.038	0.097

**: Significant at 1% level

Genetic Differentiation Between Populations

Using the Workman and Niswander (1970) method, four polymorphic loci were subjected to the heterogeneity test. Three loci (*Shd-2*, *Dia-3* and *Lap*) showed significant differentiation between populations (Table 4). Virtually all populations had the same common allele, e.g., a common allele of the *Shd-1* locus was the "a" allele, of *Shd-2* was the "b", of *6Pg-1* was also the "b" etc. *Shd-2*^{a} allele frequency in Nichihara was larger than the other populations. *Dia-3*^{a} allele

Table 4. Heterogeneity test of allele frequencies in four polymorphic loci between five populations

Locus	Allele	Chi-square value	d.f.	Probability
Shd-2	*a*	33.21	4	<.01
	b	31.73	4	<.01
6-Pg-1	*a*	3.49	4	n.s.
	b	3.32	4	n.s.
Dia-3		22.55	4	<.01
Lap	*a*	33.08	4	<.01
	b	9.81	4	<.05
	c	12.31	4	<.05

d.f.: Degree of freedom
n.s.: Not significant

frequency in Amagi and Kawazu was significantly higher than the other populations. The allelic composition of *Lap* locus in the Amagi population differed from those in the other populations.

On the average, 98.16% of the gene diversity (H_S/H_T) was within populations and 1.84% was between populations. Genetic diversity in each locus was high within populations, e.g. *Dia-3* had a relatively higher gene diversity between populations but the value was only 4%. The other loci, therefore, were much less differentiated (Table 5). G_{ST} was only 0.0156 indicating that these five populations were not diversified in each other.

Estimates of the genetic distance between populations were very small, averaging 0.0062, ranging from 0.001 to 0016 (Table 6). There was no clear relation between genetic distances and geographical location of the populations from a dendrogram of genetic distance of these populations based on UPGMA.

Table 5. Gene diversity between five natural populations of *Cryptomeria japonica*

Locus	H_T	H_S	D_{ST}	G_{ST}	D_m
Shd-1	.0070	.0069	.0001	.0079	.0001
Shd-2	.1333	.1324	.0009	.0066	.0012
6Pg-1	.3316	.3298	.0018	.0054	.0024
6Pg-2	.0750	.0728	.0022	.0291	.0029
Dia-3	.3877	.3722	.0155	.0399	.0206
Got-1	.0548	.0535	.0013	.0237	.0017
Got-2	.0104	.0103	.0001	.0093	.0001
Pgm-2	.0095	.0094	.0001	.0070	.0001
Lap	.5532	.5466	.0066	.0087	.0119
Mean	.1736	.1704	.0032	.0156	.0042
S.E.	.0668	.0657	.0017	.0060	.0015

Table 6. Genetic identity (above diagonal) and genetic distance (below diagonal) between five populations of *Cryptomeria japonica* based upon data from 9 loci

Population	1	2	3	4	5
1. Kawazu		.996	.998	.992	.997
2. Amagi	.004		.990	.984	.991
3. None	.002	.010		.995	.999
4. Yanase	.008	.016	.005		.996
5. Nichihara	.003	.009	.001	.004	

4. DISCUSSION

Cryptomeria japonica forests investigated in western Japan showed slightly less genetic variation compared with other coniferous species when evaluating the proportion of polymorphic loci, the average number of alleles, and mean expected heterozygosity (Hamrick et al., 1981; Hamrick and Godt, 1989). Average heterozygosity is said to be the most effective index for comparing genetic variation. Because the proportion of polymorphic loci and the average number of allele are highly dependent on the number of loci examined and the sample size respectively (Nei, 1987). The average heterozygosities (*He*) in Yanase and Nichihara were higher than the those in the other three populations. One of the reasons of this can be considered that relatively larger area natural forests of *C. japonica* remain in the Yanase and Nichihara regions, while the other three populations are isolated with small areas of natural *C. japonica* forests by heavily logging.

Allele frequencies of three polymorphic loci, *Shd-1*, *Dia-3*, and *Lap*, were significantly different between populations. Reasons for these differences might include chance or widely different population location.

The fixation indexes were significant in three of six polymorphic loci (Table 3), taking positive values in all loci investigated. These indicate that the homozygous individuals exceeded expectations in each locus and that inbreeding may have been promoted by family colonization within the population or by isolation of the population. The outcrossing rate was estimated using the formula $F=(1-t)(1+t)$, where t is an outcrossing rate (Nei and Syakudo, 1958). Here, t averaged 0.974. Although description of *C. japonica* mating systems is limited, by using a marker of plastid mutant which is inherited through pollen, Furukoshi (1978) estimated the selfing rate in a seed orchard at between 20% and 30%. This value is considered too high compared with the actual rate in natural populations since when using isozyme markers, the selfing rate of many coniferous species is reported to be less than 10% for natural populations or seed orchards (Barrett et al., 1987; Brown, 1989). The *C. japonica* selfing rate, also markedly smaller in this study, was estimated at 2.6%.

Gene diversity between the five natural populations showed no divergence.

G_{ST}, a good measure of the relative degree of gene differentiation among populations, was only 0.0156. Thus, 98.16% of the genetic variation exists within populations and only 1.84% between populations, indicating that the five natural populations were highly similar. Absolute gene differentiation (Dm) showing the average minimum genetic distance between populations, was also small at 0.0042. The average of genetic distance between populations was only 0.0062. These genetic distances were unrelated to the geographical distances between populations.

Other conifer also showed no divergence within species (Yeh et al., 1986; Fins and Seeb, 1986; Wheeler and Guries, 1982; Yeh and El-Kassaby, 1980; Gullberg et al., 1985; Dancik and Yeh, 1983; Guries and Ledig, 1982). Meanwhile, Hamrick (1983) indicated that conifers are one of the most genetically variable species groups and are also characterized by relatively low levels of geographical allozyme variation.

The relatively low level of allozymic differentiation of populations must in be part attributable to the mating system of conifers (Hamrick, 1983). Because conifers are wind pollinated, outcrossed, and have wind-borne seeds, all of these characteristics enhance the potential for gene flow (Mitton, 1983). Hamrick and Godt (1989) also demonstrated that outcrossed, wind-pollinated species have a much higher estimated number of migrants per generation than selfed or animal-pollinated species. Therefore, species with the potential for long-range gene movement should have the highest level of variation within populations and less variation among populations.

Using pollen analysis, Tsukada (1982, 1986) reported the migration and glacial refugia of *C. japonica* in Japan for the past 20,000 years. During the last glacial period refugia were located on the Izu Peninsula, Oki Island, Yaku Island, along Wakasa Bay and probably from the Kii Peninsula to Sikoku. He also speculated that during the last 15,000 years *C. japonica* has expanded in both northernly (along the Pacific and the Japanese Sea sides) and southernly directions (starting from near Wakasa Bay, the Izu Peninsula, and possibly the Kii Peninsula). If this speculation is correct, *C. japonica* forests of Wakasa Bay and Izu Peninsula regions should have high genetic variation because these forests were founder populations. In this study, two of the five populations were located on the Izu Peninsula. One of them, the Kawazu population, was found to have many alleles (27 total), 14 of which were rare. An allele of less than 5% frequency within a population is considered rare. The total number of alleles for the other populations ranged from 17 to 21, with from three to eight being rare. However, sample size of the Kawazu population was largest than those of the other populations and the number of alleles depends heavily on the sample size. Population heterozygosities on the Izu peninsula also were low compared with those of other areas. The results of the genetic distance did not support the estimate of pollen analysis (Tsukada, 1982, 1986).

Thus, with regard to original genetic variation, sample populations appeared to deviate from original populations. Presently, however, natural Japanese *C. japonica* forests are limited, being found only in conservation forests and limited areas in the higher mountains.

From these considerations, we offer two possible reasons, first the natural *C. japonica* forests may have lost somepart their original genetic variation, because most natural forests have been logged. If this is true, then it will be impossible to investigate the natural population's original genetic variation and the genetic diversity between populations using allozyme markers. Second, Tsukada (1982) mentioned that about 20,000 years ago, the *C. japonica* distribution was limited in the refugia. Actually, however, it might have already been widely distributed in Japan, but individual trees may have been so few or the existing trees could not produce male flowers due to the cool climate like glacial period that pollen analysis did not effectively detect the pollen in the soil. Furthermore, because of the longevity of *C. japonica* (several hundred years or more), 20,000 years is a relatively short time in term of generations. Thus, genetic diversity between the five surveyed populations is considered to be small and the G_{ST} value is only 1.56% because of *C. japonica*'s longevity, pollen flow and nature of seed dispersal.

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