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Allozyme Variation in Natural Populations of *Abies firma* in University Forest in Chiba, University Forests, The University of Tokyo and in and around the Kanto Area.

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Introduction

The Japanese fir (*Abies firma* Sieb. et Zucc.) is one of the dominant endemic tree species in Japan, with a natural distribution ranging from Honshu Island to Shikoku Island and Kyushu Island. *A. firma* is a coniferous evergreen species that is frequently found in warm-temperate zone mixed forests with the hemlock evergreen species *Tsuga sieboldii* (SHIDEI, 1974); many ecological studies have examined these mixed forests (KABAYA, 1975; KAJI, 1975; OKANO and ARAGAMI, 1999). The area of *A. firma* forest has decreased as a result of logging, residential developments, and other forms of development. These forests should be conserved as a traditional Japanese landscape and as a genetic resource for the future.

Genetic diversity is essential for the sustainability of species and populations. Therefore, we need to know the current genetic condition of species and populations. Only one report on the genetic diversity of *A. firma* using mitochondrial DNA markers (TSUMURA and SUYAMA, 1998) has been published. More detailed investigations are required with respect to the conservation of *A. firma* populations using genetic approaches.

The University Forest in Chiba, and surrounding forests, contain typical natural forests dominated by *A. firma*; many ecological studies of these *Abies* forests have been made (KABAYA, 1975; KAJI, 1975; SAKAI and OHSAWA, 1993; OZAKI and OHSAWA, 1995). TSUMURA and SUYAMA (1998) found that the *A. firma* population in the University Forest in Chiba had just one haplotype, while the populations on Shikoku and Kyushu Islands have several haplotypes. Therefore, the University Forest population may have low genetic diversity, although the genetic diversity of a species evaluated using mitochondrial DNA polymorphisms cannot be compared directly with that of other species.

Many types of molecular marker are available for population genetics analyses, including isozymes, randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment-length polymorphism (AFLP), and microsatellite loci. For population genetic analyses, any marker should be codominant to estimate the exact allelic frequency. Of the molecular markers mentioned, allozymes, RFLP, and microsatellites are codominant markers. Recently, DNA techniques have improved greatly and many studies using DNA markers have been reported. Nevertheless, of these three types of molecular marker,

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allozyme analysis is still the fastest and least expensive, and it provides useful data for population genetic analysis (TSUMURA, 2001).

Therefore, we studied the genetic variation in natural populations of *A. firma* in the University Forest in Chiba, and in the Kanto area, to evaluate the genetic character of this species using allozyme markers.

Materials and Methods

1. Sample Collection

Current-year needles or winter buds were collected from 410 trees, representing two natural populations in the University Forests and three natural populations in and around the Kanto area (Fig. 1), during the winters of 2000 to 2003. We collected needles individually from mature trees. Samples were stored at –80°C before allozyme analysis.

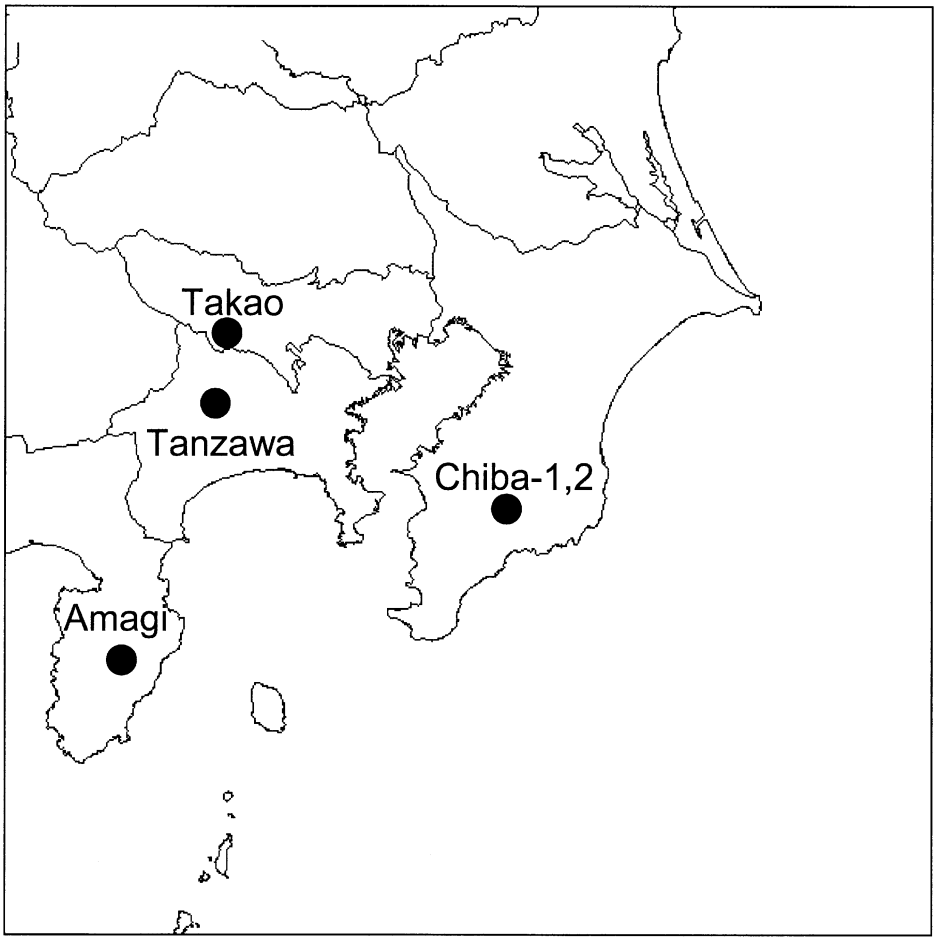

Fig. 1. The location of the five sampled populations.

2. Electrophoretic analysis of allozymes

One hundred mg of each needle were homogenized in 1,000 μl of extract buffer. Ten μl of the extraction supernatant was loaded onto a vertical polyacrylamide gel electrophoresis slab for each enzyme system. The procedures used for extraction, electrophoresis, and staining the enzymes were as reported by TSUMURA *et al*. (1990). Eight systems, whose inheritance the authors had clarified previously (SAITO *et al*., 2002), were analyzed: shikimate dehydrogenase (Shd; EC 1.1.1.25), glycerate-2-dehydrogenase (G2d; EC 1.1.1.29), 6-phosphogluconate dehydrogenase (6Pg; EC 1.1.1.44), glutamate oxaloacetate transaminase (Got; EC 2.6.1.1), phosphoglucomutase (Pgm; EC 2.7.5.1), leucine aminopeptidase (Lap; 3.4.11.1), alanine aminopeptidase (Aap; EC 3.4.11.1), and phosphoglucose isomerase (Pgi; 5.3.1.9).

3. Statistical analysis

The genetic diversity within populations was estimated using four parameters: (1) proportion of polymorphic loci (*Pl*, 99% criterion), (2) average number of alleles per locus (*A*), (3) allelic richness (*R*) (HURLBERT, 1971; MOUSADIK and PETIT, 1996), and (4) unbiased genetic diversity (*He*). Although most previous studies used effective number of alleles per locus (*Ne*), but in this study this parameter was not used because it is not independent of *He*. Allelic richness is a measure of the number of alleles, independent of sample size, which allows comparison of this quantity between different sample sizes (PETIT *et al*., 1998). To test the deviation of the observed genotype frequencies from Hardy-Weinberg equilibrium, we calculated the Fixation Index (F_{IS}) (WRIGHT, 1965).

Table 1. Levels of genetic variation for the eight allozyme loci within five populations of *Abies firma*

Population	Sample Size	Percentage of Ploymorphic loci (*Pl**)	No. of alleles per locus (*A*) (S.E.)	Arellic Richness (*R*) (S.E.)	Genetic diversity within population (*He*) (S.E.)	F_{IS}
Chiba-1	94.4	75.0	2.13 (0.23)	1.71 (0.17)	0.077 (0.026)	–0.042
Chiba-2	89.3	62.5	2.25 (0.31)	1.88 (0.25)	0.116 (0.041)	0.045
Takao	47.9	75.0	2.00 (0.27)	1.74 (0.19)	0.093 (0.046)	0.151
Tanzawa	63.8	75.0	1.75 (0.16)	1.67 (0.15)	0.147 (0.056)	0.045
Amagi	85.4	62.5	2.63 (0.32)	2.06 (0.29)	0.145 (0.051)	–0.005
Mean	76.1	70.0	2.15 (0.14)	1.81 (0.07)	0.116 (0.014)	0.039

* 99% criterion

Genetic differentiation among populations was estimated using three parameters: (1) the gene diversity, estimated using the equation $G_{ST}= (H_T–H_S)/H_T$ (NEI, 1987), where H_T is the gene diversity within the entire population, H_S is the average gene diversity within each population, and G_{ST} represents a measure of gene differentiation among populations; (2) NEI's standard genetic distance (NEI, 1972); and (3) the pairwise F_{ST} among populations (WEIR and COCKERHAM, 1984). These parameters, with the exception of NEI's genetic distance, were calculated using FSTAT ver. 2.9.3 (original version: GOUDET, 1995).

The relationships among the populations were visualized by constructing phylogenetic trees using the unweighted pair-group method with arithmetic means (UPGMA) (SNEATH and SOKAL, 1973), using NEI's standard genetic distances (1972) and bootstrap estimates to test the reliability of the trees using the program Population (LANGELLA, 2002).

Results

1. Within-population genetic variation

The levels of genetic variation and fixation index within the five *Abies firma* populations are shown in Table 1. The percentage of polymorphic loci (*Pl*) ranged from 62.5 to 75.0, with a mean of 70.0. The number of alleles per locus (*A*) ranged from 1.75 (Tanzawa) to 2.63 (Amagi), with a mean of 2.15. The allelic richness (*R*) ranged from 1.67 (Tanzawa) to 2.06 (Amagi), with a mean of 1.81. The within-population genetic diversity (*He*) ranged from 0.077 (Chiba-1) to 0.147 (Tanzawa), with a mean of 0.116. There was no clear tendency with respect to the level of genetic variation among populations, although *A* and *R* was highest in the Amagi population, and *He* in Amagi was relatively high. This suggests that the genetic variation in the Amagi population is slightly higher than in the other populations. The levels of genetic variation in the two populations in Chiba might be as high as in other populations in and around the Kanto area.

The fixation index (F_{IS}) ranged from –0.042 (Chiba-1) to 0.151 (Takao), with a mean of 0.039. The fixation index of each *A. firma* population did not deviate significantly from Hardy-Weinberg expectations. That is, these populations can be regarded as random mating populations.

2. Among-population genetic differentiation

The measures of genetic differentiation among the five populations are shown in Table 2. The mean H_T for the eight loci was 0.119 and 2.5% of the total variation was due to genetic differentiation among populations (G_{ST}). In other words, most of the genetic variation (97.5%) resided within populations. The pairwise F_{ST} among populations are shown in Table 3. The

Table 2. Distribtuion of genetic variation for the eight poplymorphic allozyme loci among five populations of *Abies firma*

Locus	Gene diversity within populations (H_S)	Total gene diversity (H_T)	Coefficient of genetic differentiation (G_{ST})
Shd	0.002	0.002	–0.001
G2d	0.014	0.015	0.004
6Pg	0.337	0.348	0.031
Got	0.165	0.172	0.041
Pgm	0.063	0.064	0.004
Lap	0.164	0.166	0.007
Aap	0.161	0.165	0.025
Pgi	0.019	0.019	–0.001
Overall	0.116	0.119	0.025
S.E.	0.040	0.042	

values range from 0.0033 to 0.0692, and the values of five pairs are significantly divided from 0. Four of the five significant pairs are for the Takao population. NEI's standard genetic distance ranged from 0.0019 to 0.0095. The smallest distance was observed between two populations in Chiba. The UPGMA phylogenetic tree of the five populations, based on NEI's standard genetic distance, is shown in Fig. 2. The populations formed two large clusters: one consisted of the two Chiba populations, and the other consisted of the Takao, Tanzawa and Amagi populations. The branches on the phylogenetic tree had high bootstrap support.

The relationship between F_{ST} or NEI's standard genetic distance and geographic distances was tested using the Mantel test, but there was no significant relationship. By contrast, the F_{ST} value suggests that the Takao population is slightly different from the others; in addition, the genetic distance and the cluster on the phylogenetic tree suggests that the two populations in Chiba are genetically most close each other and they can be thought of, genetically, as one group.

Table 3. Pairwise F_{ST} (below diagonal) and geographic distance in kilometer (above diagonal) for five populations of *Abies firma*

Population	Chiba-1	Chiba-2	Takao	Tanzawa	Amagi
Chiba-1	—	3.6	98.8	98.1	122.8
Chiba-2	0.0135	—	101.7	100.6	123.7
Takao	0.0417*	0.0328*	—	16.8	78.8
Tanzawa	0.0692	0.0379	0.0364*	—	62.1
Amagi	0.0434*	0.0145	0.0204*	0.0033	—

* significantly devided from 0 ($p<0.05$)

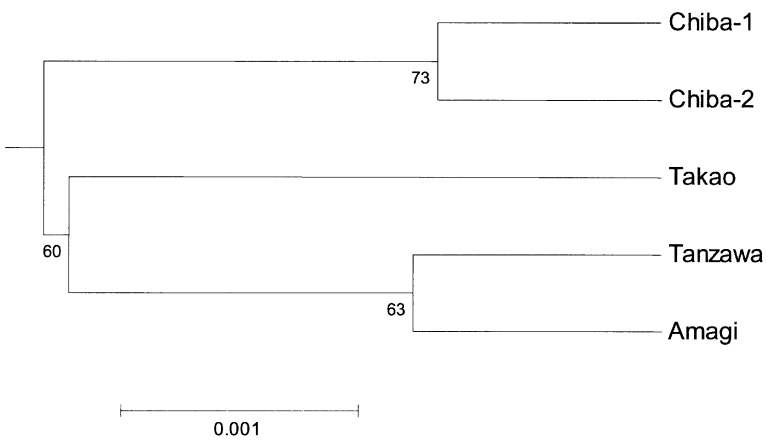

Fig. 2. A genetic distance phylogenetic tree for five populations of *Abies firma* using the UPGMA method based on NEI's standard genetic distance (NEI, 1972). Numbers within the phylogenetic tree represent bootstrap numbers based on 1000 replicates.

Discussion

1. Within-population genetic variation

Long-lived woody species have more genetic diversity within their populations than other life forms (HAMRICK *et al*., 1992). The average *Pl*, *A* and *He* within long-lived woody species was 49.3 (S.E. 1.8), 1.76 (0.04) and 0.148 (0.006). *Pl* and *A* for *A. firma* were 70.0 and 2.15 respectively and higher than the average for long-lived woody species, while *He* (0.116) was lower. Because *Pl* and *A* are tend to be influenced by sample size but not *He*, the populations of *A. firma* in this study might have low genetic diversity as compared to other long-lived woody species.

Variations within populations of woody species were significantly different among the categories of geographic range and regional distribution (HAMRICK *et al*., 1992). Comparing with other *Abies* species, *He* for *A. firma* is lower than that for *A. sachalinensis* (Mean=0.157; NAGASAKA *et al*., 1997), which is a boreal-temperate species, and than two temperate widespread species, *A. cephalonica* (0.239) and *A. bornmuelleriana* (0.201) (FADY and CONKLE, 1993). Comparing with other Japanese conifer species, the genetic variation of *A. firma* is also lower than that of *Pinus pumila* (0.225; TANI *et al*., 1996), which is boreal-temperate species, two temperate species, C*hamaecyparis obtuse* (0.202; UCHIDA *et al*., 1997) and *P. thunbergii* (0.240; MIYATA and UBUKATA, 1994), and than *Picea koyamae* (0.178; KATSUKI *et al*., 2004) which is distributed scarcely and is threatened to extinction. But it is higher than that of *A. mariesii* (0.054; SUYAMA *et al*., 1997), which is a boreal-temperate Japanese *Abies* species, even though the genetic diversity in populations of boreal-temperate tree is higher than those of species from lower latitudes (HAMRICK *et al*., 1992). It may be occurred that the distribution of *A. mariesii* is limited only in highland areas that are isolated, while *A. firma* is widespread. Moreover, SUYAMA *et al*. (1997) suggested that *A. mariesii* might have been an endemic to a small area of Honshu during the last glacial period. This result suggests that the whole genetic diversity of *A. firma* had not been limited as small as *A. mariesii* during that period. In any case, these results showed that the level of genetic diversity within these *A. firma* populations in this study is low comparing with other long-lived woody species, *Abies* specie and Japanese conifer species.

The low genetic diversities of *A. firma* populations in this study do not directly mean the low genetic diversity of this species. The low diversity might be result from the limited sampling area. During the last glacial maximum, *A. firma* were growing principally in southwestern Japan (TSUKADA, 1983). The eastern populations may have low genetic diversity associated with genetic drift through the distributional shift from the past center of distribution. These objective populations may have low genetic diversity than southwestern *A. firma* populations. The low genetic diversity of *A. firma* population in this area coincides with the result using mitochondrial DNA polymorphism by TSUMURA and SUYAMA (1998). This cline of genetic diversity of populations was shown in *Chamaecyparis obtsusa*, Japanese temperate conifer (UCHIDA *et al*., 1997). Therefore, the genetic variation of this species would be underestimated in this study. More extensive investigations into the populations located covering the distribution of this species should be made for the evaluation of genetic diversity of *Abies firma* as a species.

2. Among-population genetic differentiation

The among-population genetic variation (G_{ST}) of seven *Abies* species was 0.063 (S.E. 0.019) and that of 121 gymnosperms species was 0.073 (S.E. 0.010) (HAMRICK *et al*., 1992). The G_{ST} for *A. firma* was 0.025 and was lower than 0.144 for *A. mariesii* (SUYAMA *et al*., 1997), 0.17 for *P. pumila* (TANI *et al*., 1996), 0.073 for *P. thunbergii* (MIYATA and UBUKATA, 1994), 0.045 for *Chamaecyparis obtusa* (UCHIDA *et al*., 1997) but higher than 0.0156 for *Cryptomeria japonica* (TSUMRA, 1991). The G_{ST} for *A. firma* was low, as compared to other *Abies* species and conifer species. These results suggest that these five *A. firma* populations are differentiated minimally. The low between-population values of NEI's genetic distance (0.0019 to 0.0095) also support this result. NEI's genetic distance for seven *A. nebrodensis* populations ranged from 0.002 to 0.151 (VICARIO *et al*., 1995) and those for 11 *A. mariesii* populations ranged from 0.000 to 0.0324 (SUYAMA *et al*., 1997). However *P. pumila*, which shows high genetic differentiation among populations (G_{ST}=0.170) (TANI *et al*., 1996), also had small genetic distances between two populations located near, in the same range of *A. firma* populations in this study. The low genetic distance of these five *A. firma* populations is reasonable. It might be result from the high gene flow of *A. firma* populations, which is a wind-pollinated species, and the narrow range of sampling populations.

In this study, we clarified the genetic variation of *Abies firma* population in the University Forest in Chiba and in and around the Kanto area. These forests studied are scarcely genetically differentiated, so they could be consider one group of genetic resources of *A. firma*. The *Abies* forest in University Forest in Chiba should be conserved as one of typical genetic resources in Kanto area, where deforestation has been carrying out.

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Summary

Genetic variation at eight allozyme loci was examined from five natural populations of *Abies firma*, two populations in University Forest in Chiba, University Forests, The University of Tokyo and three populations in and around the Kanto area (Takao, Tanzawa and Amagi). Within-population genetic variation (*He*) ranged 0.077 to 0.147 (Mean 0.116), was low compared to other long-lived woody species or Japanese coniferous species and the level was slightly low comparing with other *Abies* species. The genetic variation in the Amagi population is slightly higher than in the other populations and the levels of genetic variation in the two populations in Chiba might be as high as in other populations in and around the Kanto area. Most of the genetic variation is found within populations (G_{ST} = 0.025) and the populations are genetically differentiated minimally. A genetic structure was suggested, that is the two Chiba populations can be thought of, genetically, as one group among five populations, and Takao population is most different from others. It could be thought that the populations in the University Forest in Chiba exhibit genetic variation of *A. firma* populations characteristically found in and around the Kanto area.

Key words: *Abies firma*, Allozyme, Genetic variation, The University Forest in Chiba

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東京大学千葉演習林および関東周辺における モミ天然林のアロザイム変異

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要　　旨

東京大学千葉演習林内２箇所および関東地方周辺３箇所（高尾，丹沢，天城）の合計５箇所のモミ天然林集団について８アロザイム遺伝子座の遺伝的変異を調べた。各集団内の遺伝的変異*He*は0.077-0.147（平均 0.116）であり，他の長命の樹木種や日本産針葉樹木と比較して低く，他のモミ属樹木とのみ比較してもやや低かった。集団内変異が最も高かったのは天城集団であり，千葉演習林内の２集団は他集団と同程度の遺伝的変異を保持していた。遺伝的分化係数G_{ST}は0.025で，遺伝的変異の大部分が集団内にあり，５つの集団間はほとんど分化していなかった。その中でも特に千葉演習林内の２集団は遺伝的に１つのまとまりと考えることができ，他方，高尾集団は他の集団と遺伝的に最も異なるという構造が示唆された。千葉演習林におけるモミ天然林集団は関東周辺のモミ天然林集団に典型的な遺伝的変異を持つと考えられた。

キーワード： モミ・アロザイム・遺伝的変異・千葉演習林