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ORIGINAL ARTICLE

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Microsatellite markers reveal high allelic variation in natural populations of *Cryptomeria japonica* near refugial areas of the last glacial period

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Abstract Using 11 microsatellite markers, we investigated the allelic variation and genetic structure of *Cryptomeria japonica*, across most of its natural distribution. The markers displayed high levels of polymorphism (average gene diversity = 0.77, average number of alleles = 24.0), in sharp contrast to the lower levels of polymorphism found in allozyme and cleaved amplified polymorphic sequence markers in previous studies. Little genetic differentiation was found among populations ($F_{ST} = 0.028$, $P < 0.001$), probably because the species is wind-pollinated and long-lived. No clear relationship between Nei's genetic distances and geographical locations of the populations were found using the principal coordinate and unweighted pair-group method with arithmetic averaging analyses. The lack of such trends might be due partly to microsatellite homoplasy arising from mutation blurring the genealogical record. However, there was a trend towards high allelic diversity in five populations (Ashitaka, Ashiu, Oki-Island, Yakushima-Island-1 and -2), which are very close to, or in, refugial areas of the last glacial period as defined by Tsukada based on pollen analysis data and current climatic divisions. We postulate that these refugial populations might have been less affected by genetic drift than the other populations due to their relatively large size.

Key words Allelic richness · Principal coordinate analysis · Sugi · Refugia · Colonization

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Introduction

Populations in glacial refugia are generally expected to harbor higher levels of genetic diversity than similar populations in areas that have been colonized since the retreat of the glaciers (here termed "colonized populations") because colonization often involves only a few individuals (Hewitt 1996). However, the ability of different kinds of genetic markers and analytical approaches to detect and resolve genetic diversity within or among populations differs markedly. At neutral loci, "bottlenecks" (transitory reductions in the effective population size) may theoretically have caused strong reductions in allelic richness, and a more limited decrease in gene diversity, since rare alleles are more readily affected by genetic drift than frequent alleles (Nei et al. 1975). Recent studies on European and North American tree species have shown that refugia and postglacial migration routes can be identified using DNA markers. Comps et al. (2001) reported evidence for a significant and steady decline in allelic richness during the postglacial recolonization of European beech using allozyme markers, but the expected reduction in gene diversity was not found. It has also been reported that allozyme variations in colonized populations decrease in other tree species (see, for instance, Tomaru et al. 1997).

Cryptomeria japonica is a wind-pollinated (anemophilous) species that produces abundant pollen every few years. Fossil pollen of this species has been analyzed in detail at many sites, allowing the identification of potential refugia and most probable routes of postglacial recolonization (Tsukada 1982). According to these studies, during the last glacial period, refugia of *C. japonica* occurred in several regions in Japan: the Izu Peninsula, along Wakasa Bay, Oki Island, Yaku Island, and probably from the Kii Peninsula to the southern part of Shikoku Island. In addition, there are two inferred recolonization pathways: one along the Sea of Japan side from the vicinity of Wakasa Bay, and the other along the Pacific side from the Izu Peninsula. Today's natural forests are widely distributed in moist temperate regions from Aomori Prefecture (40°42′N) to Yakushima Island

(30°15′N) (Hayashi 1960). However, the natural distribution is discontinuous and scattered in limited areas as a result of the extensive exploitation of this species during the past 1,000 years (Ohba 1993). Variations among natural forests of *C. japonica* in different regions have been investigated using morphological traits (needle length, needle curvature, and other features; Murai 1947) and diterpene components (Yasue et al. 1987). These studies have suggested that there are two main varieties: *C. japonica* "Omote-sugi" (on the Pacific Ocean side of Japan) and *C. japonica* var. *radicans* "Ura-sugi" (on the Sea of Japan side). This divergence is probably associated with the differences in their refugial origins in the last glacial period.

Genetic variation in natural populations of *Cryptomeria japonica* has been reported in studies based on allozymes (Tsumura and Ohba 1992, 1993; Tomaru et al. 1994) and cleaved amplified polymorphic sequence (CAPS) markers (Tsumura and Tomaru 1999). The studies cited found little genetic differentiation among populations, which is consistent with investigations of other conifers with a wide distribution. However, these studies did not find clear differences between the two main varieties, or between refugial and colonized populations, except between the Yakushima Island population and the other populations (Tsumura and Ohba 1993).

Like allozymes and CAPS markers, microsatellites have discrete and codominant alleles, but microsatellites have more alleles per locus and therefore allow higher resolution analysis. For instance, in a study by Chase et al. (1996), four polymorphic microsatellite loci provided greater resolution and sensitivity for the estimation of population genetic parameters than all of the allozyme markers analyzed previously in several detailed studies. Microsatellite variation might correlate directly with effective population size because populations with large effective sizes tend to retain high numbers of alleles originating from mutation, since the mutation rates of microsatellite loci are generally believed to be high compared to normal rates of point mutation (see, for instance, Jarne and Lagoda 1996). Microsatellite mutation rates of forest tree species such as conifers have not been investigated directly, and thus available information is limited to data on somatic mutation of *Pinus strobus* (Cloutier et al. 2003) and *C. japonica* (T. Takahashi et al. unpublished data). Both of these data sets suggest that microsatellite markers arising from somatic mutation tend to be stable. The influence of microsatellite mutation on the allelic composition of the markers used in the studies cited above may have been weak because of the longevity of *C. japonica*. If so, microsatellite variations of current natural populations might reflect the size and colonization history of past populations since the last glacial period.

Recently, microsatellite markers have been developed for *Cryptomeria japonica* in order to construct a genetic linkage map (Tani et al. 2003) and to investigate gene contamination in seed orchards (Moriguchi et al. 2003). In this paper, we discuss the allelic variation and population structure of *C. japonica*, as revealed by microsatellite markers, across most of its natural distribution. In addition we assess the availability and characteristics of microsatellite markers compared with the gene-coding markers (allozyme and CAPS markers; which have lower mutation rates than microsatellites) used in previous studies.

Materials and methods

Plant materials

Mature needles were collected from 757 trees representing 29 populations. The locations of the sampled populations covered most of the natural distribution of *Cryptomeria japonica* (Fig. 1). All of these trees were from natural populations that have become established since the last glacial period, in forests that were designated candidates for an in situ gene-conservation program introduced by the Japanese Forestry agency, with the exception of the Tateyama, Sado-Island and Ashiu populations. We allowed at least 50 m between sampled trees to avoid sampling half-sibs. We also collected samples from trees as large in size as possible in old-growth forest to avoid sampling young trees that might have been influenced by artificial plantations. The needles were stored at –30°C before DNA extraction.

According to suggestions by Tsukada (1982), based on pollen analysis and current climatic divisions, we divided the populations into three groups, defining populations in

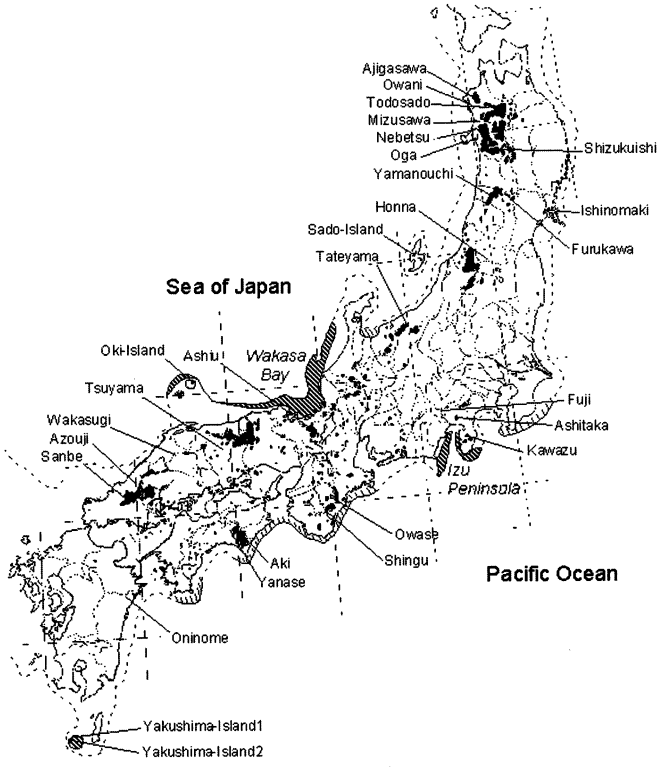

Fig. 1. Natural distribution (Hayashi 1960) and locations of 29 surveyed natural populations of *Cryptomeria japonica* in Japan. The *dotted line* indicates the coastline ca. 18,000 years ago. Areas shaded in *bold* or with *thin diagonal lines* show refugia and probable refugia, respectively, at that time (Tsukada 1982)

Table 1. Characteristics of 11 microsatellite loci in *Cryptomeria japonica*

Locus	Repeat motif	PCR primer sequences (5′–3′)[a]	T_a (°C)[b]	Size range (bp)
Cjs0201	$(GA)_{29}$	F: CTCCTTGTAATCTTATTCCC R: GTATGAGCCTACACAAATACTC	58	84–186
Cjs0333	$(GA)_{26}$	F: AGGAGATTAGGATGGTGGG R: GGTTTGCCTCTTCTATGAG	60	194–302
Cjs0520	$(TG)_{18}$	F: TCCCTTTTGGTATTTTACAC R: ACTCAAATTGCGATAATCTC	50	169–215
Cjg0077	$(CT)_{10}$	F: CCTTGTACACTTATTTGTACCT R: AGGGAGGAGAAATAGACAT	60	99–181
CS1219	$(GT)_{10}$	F: AAGGTGTTGTTTTAAGGAGG R: CAGCCATCTATTATTTGTGC	50	93–115
CS1364	$(AC)_7$	F: TGATTATGGTCGGTGGTCTT R: GTGATGTGGTGTTATCTTGT	60	283–349
CS1525	$(CA)_{18}$	F: ATGAAGTGCCCTTGGTTTGT R: ATCGCCTCCTCTTTTATCCT	50	172–253
CS1579	$(TG)_{11}$	F: ACTCTAGCAGCATTTCTCAC R: CAGATTTTGTATGAGTGGTT	55	282–312
CS1906	$(TGA)_6$	F: AGTCATTCCCAGGCAGTGTC R: ATCCCTCCACCTCTCCTACC	60	332–360
CS2169	$(GA)_9$	F: GTAGAGGAGGGATATAGAGT R: TCCTTGTCCATCTCTCTTTA	56	136–174
CS2230	$(GA)_9$	F: AGACATAAAGAGGGAGGTAGAG R: TACTCTTGCTGACTGGTCCG	50	100–130

[a] *F* Forward, *R* reverse
[b] Annealing temperature

the vicinity of refugial areas of the last glacial period as refugia populations, populations in the vicinity of probable refugial areas in the last glacial period as probable refugia populations, and populations colonized after the last glacial as colonized populations (see Fig. 1, Table 1).

Laboratory analysis

Total DNA was extracted from these samples using a modified CTAB method (Tsumura et al. 1995). The genotypes of 11 microsatellite loci (Tani et al. 2003, 2004; Moriguchi et al. 2003) in each individual were determined. One of these 11 microsatellite loci (*Cjs0201*) was newly developed as a marker from a microsatellite-enriched library (Tani et al. 2004), and its sequence has been submitted to the DNA Data Bank of Japan (DDBJ). Of the 11 markers, 3 (*Cjs0333*, *Cjs0520*, and *CS2169*) were mapped to different linkage groups (Tani et al. 2003), but the locations of the other markers have not yet been mapped. PCR amplification was performed in 10-µl reaction volumes containing 3 ng genomic DNA, 1 × PCR buffer (200 mM Tris-HCl, 500 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer (the forward primer of each pair labeled with dye), and 0.25 U *Taq* polymerase in a GeneAmp Model 9700 PCR System (PE Applied Biosystems, Foster City, Calif.). PCR conditions included a 5-min denaturing step at 94°C, followed by 30–35 cycles of 94°C for 30 s, the annealing temperature for 30 s, and 72°C for 30 s. The specific annealing temperatures for the primer pairs are shown in Table 1. Finally, there was an elongation step at 72°C for 5 min. The genotypes were determined using an ABI 3100 Genetic Analyzer and Genotyper software ver. 3.7 (PE Applied Biosystems), and confirmed manually.

Statistical analysis

To estimate within-population variation, we used four parameters calculated from the allele frequencies of all loci analyzed: the number of alleles (N_a), observed heterozygosity (H_o), Nei's (1978) gene diversity (H_e), and allelic richness (for a minimum of six diploid individuals). Allelic richness was measured by the method of El Mousadik and Petit (1996), which is based on the rarefaction method of Hurlbert (1971). We also calculated the number of rare alleles (defined as alleles with a frequency <1% in the total population), and private alleles (unique to one population) per individual in each population. Nonparametric significance tests (the Kruskal-Wallis test with Bonferroni correction and the Mann-Whitney *U* test) were performed to examine differences in allelic diversity among defined groups.

Wright's (1951) *F*-statistics were calculated to measure the deviation from Hardy-Weinberg equilibrium at each locus in each population. Fixation indices, F_{IS} (used to estimate inbreeding within individuals in a population; the inbreeding coefficient), were calculated on the basis of Weir and Cockerham's (1984) *F* values, with FSTAT version 2.9.3.2 (Goudet 2000). The significance of positive or negative values of F_{IS} was calculated using 6,600 randomizations (the default value in FSTAT) for each locus and across loci for each population.

Genetic structure was assessed using two models: the infinite allele model (Kimura and Crow 1964), and the stepwise mutation model (SSM; Ohta and Kimura 1973; Kimura and Ohta 1978). Values of F_{ST} (Weir and Cockerham 1984), R_{ST} (Slatkin 1995), and hierarchical analysis of molecular variance (AMOVA) (Michalakis and Excoffier 1996) were calculated using the program ARLEQUIN, version 2.0

(Schneider et al. 1997), in which significance levels for the overall values were determined after 1,023 permutations. We also calculated R_{ST} values for all loci except *Cjs0333*, *CS1364*, *CS1525* [for which the allelic frequencies did not follow SMM (Ohta and Kimura 1973)].

Associations between populations were analyzed by principal coordinate (PCO) analysis based on estimates of pairwise genetic similarities, calculated using the similarity coefficient of Nei (1978). The association was revealed by the first two PCO axes, which were found to be representative of higher-order axes. PCO allows underlying trends in the raw data to be visualized in a process involving data reduction by representation of the accessions in a space formed by newly constructed dimensions. Nei's genetic distance (Nei 1978) was also calculated for each population pair, providing a basis for clustering populations by the unweighted pair-group method using arithmetic averages (UPGMA; Sokal and Seneath 1963).

Results

Genetic diversity at microsatellite loci

Levels of genetic diversity within each population were very high at all except two loci: *CS1906* and *CS2230*. N_a per locus ranged from 6 to 51, H_e varied from 0.36 to 0.97, and H_o from 0.21 to 0.95 (Table 2). The fixation indices (F_{IS}) of *CS1906* and *CS2230* in each population showed significant positive deviations from the Hardy-Weinberg equilibrium. However, the mean values of F_{IS} across populations for single loci varied from locus to locus (Table 2).

Genetic differentiation and structure of populations

To measure the degree of population differentiation, two theoretical models were used: the infinite alleles model (F_{ST}) and the stepwise mutation model (SMM; R_{ST}). The overall values of genetic differentiation for each model were similar, and each was significantly different from zero ($P < 0.001$): $F_{ST} = 0.028$, $R_{ST} = 0.032$, R_{ST} (excluding *Cjs0333*, *CS1364* and *CS1525*) = 0.027.

The first two axes of the PCO analysis explained 39.3% of the total variation (Fig. 2). There seems to be slight clustering into two main varieties, *Cryptomeria japonica* "Omote-sugi" (on the Pacific Ocean side) and *C. japonica* var. *radicans* "Ura-sugi" (on the Sea of Japan side; Fig. 2). However, there was no clear relationship between genetic distances and geographical location of the populations from a dendrogram of genetic distance of these populations based on UPGMA (data not shown). PCO analysis also showed that Fuji populations were separated from the other populations (Fig. 2).

Allelic diversity across natural populations

The mean values of the parameters used to estimate allelic diversity (i.e., allelic richness, and the numbers of rare and private alleles across populations) varied. In particular, there was a trend toward high allelic diversity in five populations (Ashitaka, Ashiu, Oki-Island, Yakushima-Island-1 and -2), which are very close to, or in, refugial areas of the last glacial period as defined by Tsukada (1982) based on pollen analysis data and current climate divisions. Three populations (Sanbe, Azouji and Oninome) in western Japan seem to have high allelic diversity. However, allelic diversity of the Shingu, Owase, Aki, Yanase populations, which are

Table 2. Overall genetic parameters for each of the 11 microsatellite loci used in this study

Locus	N_a[a]	H_o[b]	H_e[c]	F_{IS}[d]
Cjg0077	41	0.81	0.87	0.08
Cjs0201	51	0.95	0.97	0.01
Cjs0333	45	0.90	0.95	0.05
Cjs0520	20	0.62	0.77	0.19
CS1219	8	0.57	0.70	0.18
CS1364	9	0.67	0.79	0.15
CS1525	40	0.80	0.93	0.14
CS1579	16	0.79	0.85	0.06
CS1906	6	0.21	0.36	0.43
CS2169	17	0.59	0.71	0.17
CS2230	11	0.26	0.56	0.54
Mean	24	0.65	0.77	0.15

[a]Observed number of alleles per locus
[b]Observed heterozygosity
[c]Expected heterozygosity
[d]Fixation index

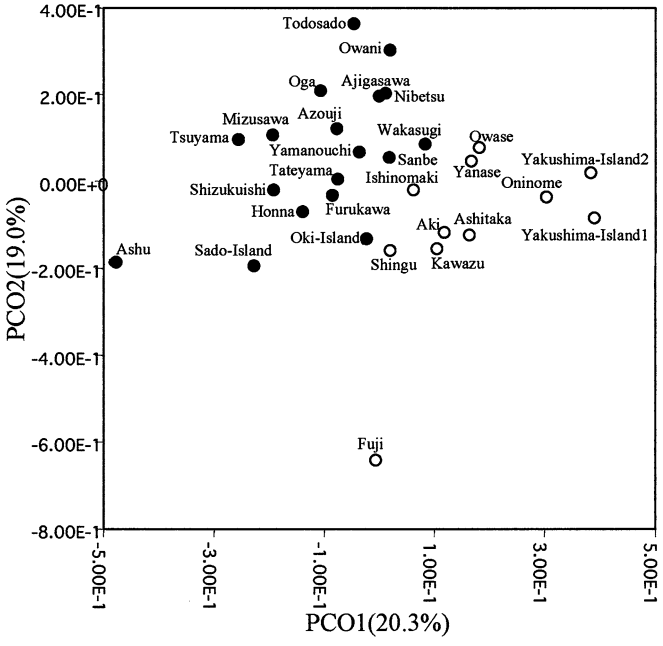

Fig. 2. Genetic similarity among 29 populations, as revealed by principal coordinate (PCO) analysis. *Open circles Cryptomeria japonica* "Omote-sugi" (on the Pacific Ocean side), *closed circles Cryptomeria japonica* var. *radicans* "Ura-sugi" (on the Sea of Japan side of Japan)

Table 3. Genetic diversity parameters for each population in this study

Population	n[a]	H_o[b]	H_e[c]	A[d]	Rare allele[e]	Private allele[f]	Region[g]
Ajigasawa	30	0.68	0.73	5.43	0.58	0.07	Colonized
Mizusawa	30	0.66	0.76	5.64	0.68	0.07	Colonized
Oga	30	0.63	0.75	5.55	0.34	0.03	Colonized
Nibetsu	30	0.65	0.74	5.67	0.52	0.03	Colonized
Todosado	26	0.59	0.69	4.93	0.36	0.00	Colonized
Owani	29	0.62	0.72	5.68	0.60	0.00	Colonized
Yamanouchi	29	0.62	0.74	5.51	0.41	0.00	Colonized
Shizukuishi	29	0.66	0.77	5.56	0.44	0.04	Colonized
Ishinomaki	31	0.64	0.76	5.89	0.68	0.07	Colonized
Furukawa	25	0.63	0.72	5.26	0.38	0.08	Colonized
Honna	28	0.65	0.77	5.46	0.45	0.04	Colonized
Sado-Island	30	0.70	0.78	5.76	0.55	0.00	Colonized
Tateyama	30	0.62	0.72	5.24	0.52	0.03	Colonized
Kawazu	23	0.68	0.76	5.67	0.51	0.05	Refugia
Ashitaka	30	0.75	0.81	6.29	0.78	0.07	Refugia
Fuji	11	0.67	0.74	5.29	1.06	0.19	Refugia
Shingu	23	0.63	0.74	5.50	0.50	0.05	Probable refugia
Owase	18	0.57	0.74	5.23	0.35	0.00	Probable refugia
Ashiu	30	0.80	0.80	6.08	0.94	0.30	Refugia
Tsuyama	18	0.66	0.73	5.43	0.75	0.12	Colonized
Sanbe	30	0.64	0.75	5.75	0.79	0.04	Colonized
Wakasugi	30	0.64	0.73	5.33	0.52	0.07	Colonized
Azouji	30	0.63	0.76	5.77	0.63	0.04	Colonized
Aki	16	0.63	0.70	5.11	0.27	0.00	Probable refugia
Yanase	20	0.70	0.70	5.22	0.57	0.00	Probable refugia
Oki-Island	30	0.69	0.76	5.84	0.88	0.24	Refugia
Oninome	20	0.62	0.71	5.72	0.83	0.06	Colonized
Yaku-Island1	27	0.59	0.69	5.68	0.96	0.19	Refugia
Yaku-Island2	24	0.59	0.72	5.63	0.74	0.17	Refugia

[a]Sample size
[b]Average observed heterozygosity
[c]Average expected heterozygosity
[d]Average allelic richness
[e]Number of rare alleles per individual
[f]Number of private alleles per individual
[g]Location according to vicinity of refugia, probable refugia, and colonized areas defined by Tsukada (1982)

located in western Japan near probable refugial areas defined by Tsukada (1982), are relatively low.

Differences in allelic diversity amongst the three groups of *Cryptomeria japonica* populations (refugia, probable refugia and colonized populations, following proposals by Tsukada 1982), were found to be significant according to a Kruskal-Wallis test ($P < 0.05$). More specifically, the allelic richness values, and the numbers of rare and private alleles between refugia and probable refugia populations differed significantly ($P < 0.05$). In addition, although allelic richness values were not significantly different between refugia and colonized populations ($P > 0.05$), there were significant differences in the numbers of rare and private alleles between refugia populations and colonized populations ($P < 0.05$). There were no significant differences in mean gene diversity (H_e) between the three groups (Kruskal-Wallis test, $P > 0.05$). Furthermore, when we compared the allelic diversity of *C. japonica* between eastern populations and western populations, there were no statistically significant differences in their values (Mann-Whitney *U*-test, $P > 0.05$).

Discussion

Microsatellite markers in *Cryptomeria japonica*

In the present study, microsatellite markers displayed high levels of polymorphism (average gene diversity $H_e = 0.770$, average $N_a = 24.0$). This contrasts sharply with the low levels of polymorphism previously found in allozyme (average $H_e = 0.189$, average $N_a = 2.3$) and CAPS markers (average $H_e = 0.281$, average $N_a = 1.9$) in *Cryptomeria japonica* (Tomaru et al. 1994; Tsumura and Tomaru 1999). Our results indicate that microsatellite markers are much more informative than allozyme and CAPS markers in *C. japonica*, owing to their high polymorphism. The latter is attributable to their high mutation rates, which vary from 10^{-6} to 10^{-3} events per locus per generation (Hancock 1999), whereas mutation rates of allozyme loci are of the order of 10^{-6} events per locus per generation (see, for instance, Mukai and Cockerham 1977).

The average gene diversity over all loci in all populations exceeded the observed values, leading to positive mean

values of F_{IS} (Table 2). As a general explanation for these findings, a small excess of homozygotes may be due to nonrandom mating. Nevertheless, the mean values of F_{IS} across populations found here varied from locus to locus (Table 2). Therefore, since inbreeding can be expected to affect all loci equally, there are likely to be additional causes of the deviations between observed and expected values, such as selection and subdivisions in the population structure. In addition, missamplification of microsatellite alleles, leading to the generation of "null alleles," frequently occurs (Callen et al. 1993), and if null alleles occur at some loci, the gene diversity values may exceed the observed values.

Genetic structure of populations

Natural forests of *Cryptomeria japonica* show weak, but statistically significant, genetic differentiation among populations, as indicated by the average F_{ST} and R_{ST} values of 0.028 and 0.031, respectively, which are similar to G_{ST} values found in allozyme and CAPS marker studies (Tsumura and Ohba 1992, 1993; Tomaru et al. 1994; Tsumura and Tomaru 1999). The weak genetic differentiation between populations of *C. japonica* can be explained by the facts that *C. japonica* is a wind-pollinated, allogamous species with wind-dispersed seeds, and that long-lived woody species tend to have low levels of genetic variation among populations (Hamrick et al. 1992).

No clear geographical trends among populations were found in the PCO and UPGMA analyses. Although microsatellite loci often exhibit a large N_a, this high diversity can also complicate the use of microsatellite data in evolutionary studies, such as the estimation of differentiation (Hedrick 1999) and genetic similarity between populations. One of the problems associated with microsatellite loci is their potential to demonstrate size homoplasy; a condition in which alleles that are identical in state (IIS) are not identical by descent (IBD) (Estoup and Cornuet 1999; Estoup et al. 2002). Homoplasy results from a mutation causing a proportion of alleles to either converge or diverge in state, blurring the genealogical record provided by IBD markers, and thus restricting our ability to infer the evolutionary processes that shape patterns of ancestry (Adams et al. 2004). We believe that the unclear results from PCO and UPGMA analyses might be due partly to microsatellite homoplasy. PCO analysis also showed that the Fuji population is separated from the other populations (Fig. 2). Although small, this population has unique alleles, presumably either through chance or because it has relatively high allelic diversity because of its location near to the refugial area of the last glacial period around Izu peninsula.

High allelic diversity in populations near refugial areas of the last glacial period

Values of the microsatellite allelic diversity parameters, i.e., allelic richness, and the numbers of rare and private alleles, were higher for the refugia populations than corresponding values for the other populations, but the expected H_e values of the refugia populations were similar to those of the other populations. These findings are consistent with data related to European beech reported by Comps et al. (2001). In several studies, allozyme variation of colonized populations has been found to be lower than that of refugia populations (see, for instance, Tomaru et al. 1997). We postulated that refugia populations may have been less affected by genetic drift than the other populations due to their relatively large size, and that key characteristics of the species, especially its longevity (and thus the low number of generations since the last glacial period), may have helped maintain their relatively high levels of diversity.

In particular, there is a large area of natural *Cryptomeria japonica* forest on Yakushima Island, where natural forests of *C. japonica* grow at altitudes of 600–1,800 m, and some huge individuals are more than 1,000 years old. Furthermore, large *C. japonica* trees on Yakushima Island have been logged since the Edo-era, about 300 years ago, but logging was less intense there than on the mainland, because of the isolation of Yakushima Island from the mainland, and its steep mountains. Therefore, even now this natural forest covers about 8,000 ha at the center of the island and relatively high allelic diversity has been maintained within it.

Small populations that might be derived from main refugia of the last glacial period, such as the Ashitaka and Oki Island populations, still have high genetic diversity in terms of allelic richness, and rare and/or private allele frequencies. Today, natural forests of *Cryptomeria japonica* have declined in size because of the extensive exploitation of the species during the past 1,000 years (Ohba 1993), except on Yakushima Island. However, evidence from fossil pollen data (see, for instance, Tsukada 1982) and buried *C. japonica* forests, which have been found near Ashitaka Mountain (Takeda 1931) and Oki Island (Takahara et al. 2001), indicate that there were large natural forests of *C. japonica* nearby in the past. The current forest reserve areas of Fuji, Kawazu, Ashitaka and Oki Island, which were derived from main refugia, are very small (1.0, 0.8, 4.7 and 14.0 ha, respectively). However, those populations represent very important genetic resources because of their high genetic diversity and direct descent from refugia populations.

Allelic diversity of populations located near probable refugia (the Shingu, Owase, Aki, Yanase populations), which are located only on the Pacific Ocean side in western Japan, is low. From fossil pollen data, Takahara (1998) suggested that, in western Japan, especially on Sikoku Island, the expansion of natural forests of *Cryptomeria japonica* along the Pacific Ocean side might have been much slower than along the Japan Sea side because of the rapid expansion of evergreen broadleaf forest in the postglacial age. If there were refugia in those regions, their areas may have been small, and the chance of genetic drift greater than in the refugia populations.

We were able to show, using microsatellite markers, that the allelic diversity of refugia populations is higher than that of probable refugia and colonized populations. Today's natural forest of *Cryptomeria japonica* is discontinuous, scat-

tered in limited areas. On the other hand, *C. japonica* has been widely planted throughout Japan over an area of 4.5 million ha, accounting for 44% of all Japanese artificial forest. If the genetic diversity within populations of the artificial forests is the same as that of natural *C. japonica* forests, the genetic diversity of current natural forests of *C. japonica* should be maintained in the future. However, artificial forests may have less genetic diversity, because most of these forests in western Japan consist of limited numbers of clones that have been historically used as cultivar clones. Even in eastern Japan, the planted seedlings originate from seed orchards consisting of a limited number of mother trees. The genetic variation of natural *C. japonica* forests might be affected by pollen contamination from artificial forests in the future. Therefore, current natural forests of *C. japonica* are very valuable and important both for gene conservation and for future breeding programs. Information obtained from this study could be useful for conservation of the genetic resources of this species.

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