Comparison of Spatial Genetic Structures in *Fagus crenata* and *F. japonica* by the Use of Microsatellite Markers

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(Received 10th November 2003)

Abstract

The spatial genetic structures of the tree species *Fagus crenata* and *F. japonica* were investigated by using 4 microsatellite markers. The study site was a 2-ha plot within a mixed population of these species. We used 2 different statistics, genetic relatedness, and the number of alleles in common (NAC), to study the extent of spatial genetic structure. Significant negative correlation between genetic relatedness and spatial distance was detected among all individuals in each species. However, this correlation was weak and likely resulted from extensive pollen dispersion caused by wind pollination. Spatial genetic clustering in *F. japonica* was stronger than in *F. crenata* over short distance classes. This result may be due to the different reproductive and breeding systems, which were the overlapping of individuals of kinship.

Key words: Fagus crenata, F. japonica, genetic structure, Japanese beech, NAC, Relatedness.

Introduction

Spatial genetic structure within forest tree populations has been investigated in many species and interpreted in terms of population history, level extent of gene flow, and selection (PERRY and KNOWLES, 1991; MERZEAU *et al.,* 1994; BREG and HAMRICK, 1995; SHAPCOTT, 1995; SHNABEL and HAMRICK, 1995; TANI *et al.,* 1998; UENO *et al.,* 2000). Knowledge of the genetic structure of a population is important for *in situ* conservation of natural populations (RITLAND, 1985; WILLIAMS and HAMRICK, 1996). Poor understanding of the spatial genetic structure could lead to misinterpretation of the importance of mating systems and gene flow mechanisms and result in implementing the wrong conservation program for the natural population.

The spatial genetic structure within a population is influenced by several factors, such as seed dispersal and mating system. Simulation studies based on isolation-by-distance models have demonstrated that strong spatial genetic structure tends to arise quickly and to persist across generations when gene dispersal is limited with respect to distance (TURNER *et al.,* 1982; SOKAL and WARTENBERG, 1983; BREG and HAMRICK, 1995). Recently, a simulation study by DOLIGEZ *et al.* (1998) showed that not only limited gene dispersal but also generation overlapping and self-incompatibility can influence spatial genetic structuring. In their study, generation overlapping resulted in strong spatial genetic structure (DOLIGEZ *et al.,* 1998). This effect of overlapping generations is presumably due to the increased kinship between mates that results from the combination of limited gene dispersal and mating of individuals with their ancestors (DOLIGEZ *et al.,* 1998). Furthermore, a long life span should enhance the effect of generation overlap and promote the development of spatial genetic structure. However, whether differences in the number of generations per lifetime affect population genetic structure has not yet been reported.

Fagus crenata and *F. japonica* are monoecious, long-lived, woody angiosperms (beech species) with outcrossing breeding systems based on wind pollination. The seeds are dispersed mainly by gravity but also secondary by animals (WATANABE, 1990; MIGUCHI, 1994). In a natural forest, *F. crenata* regenerates only by seedlings, and *F. japonica* regenerates from seeds and by production of spouts from the root collar. In this study, we investigated the genetic structures of *F. crenata* and *F. japonica.* The seed- and pollen-dispersal systems of both species are similar (KITAMURA and MURATA, 1980), but the lifetime of a generation is longer in *F. japonica* than in *F. crenata* (HARA, 1996). We studied individuals in a sympatric habitat of these species by using microsatellite markers to investigate how differences in ecological characters such as regeneration system and life span may affect their spatial genetic structures.

Materials and Methods

Materials

Fagus crenata and *F. japonica* are distributed in the cool temperate zone of the Japanese archipelago. *Fagus crenata* is found mainly in the heavy snowfall zone adjacent to the Sea of Japan and in scattered areas of the zone with little snow on the Pacific Ocean side. *Fagus japonica* is found only on the Pacific Ocean side, reaching less far north than *F. crenata* (SASAKI, 1970; NOZAKI and OKUTOMI, 1990). Both are allogamous, wind-pollinated species with gravity-dispersed seeds. *Fagus crenata* regenerates by seedlings that establish during recovery of canopy gaps in natural forests (NAKASHIZUKA and NUMATA, 1982; NAKASHIZUKA, 1983; YAMAMOTO, 1989). *Fagus japonica* regenerates both from seeds and by production of sprouts, characteristically forming stools arising from vigorous sprouting from the root collar (TOHYAMA, 1965; OHKUBO *et al.,* 1988; PETERS and OHKUBO, 1990; OHKUBO, 1992). Within a *F. japonica* stool, one or more of the sprouts may replace a canopy trunk that has died (OHKUBO, 1992), and the stools can reach substantial ages. Since new sprouts are formed on the outside of each stool, the stool gradually increases in size (OHKUBO *et al.,* 1996). The genetic homogeneity of such clusters of trunks has been demonstrated by isozyme analysis (KITAMURA *et al.,* 1992).

Study site and sampling methods

The study site was located in the Forest Reserve near Mount Takahara (36° 53' N) at an altitude of ca. 900 m, on a south-east-facing slope ranging from 13° to 36° on the Pacific Ocean side of Japan (*Figure 1*). The 4-ha plot (100×400 m) was established for a long-term ecological survey (PETERS and OHKUBO, 1990). The 2-ha subplot in this 4-ha plot that was chosen for

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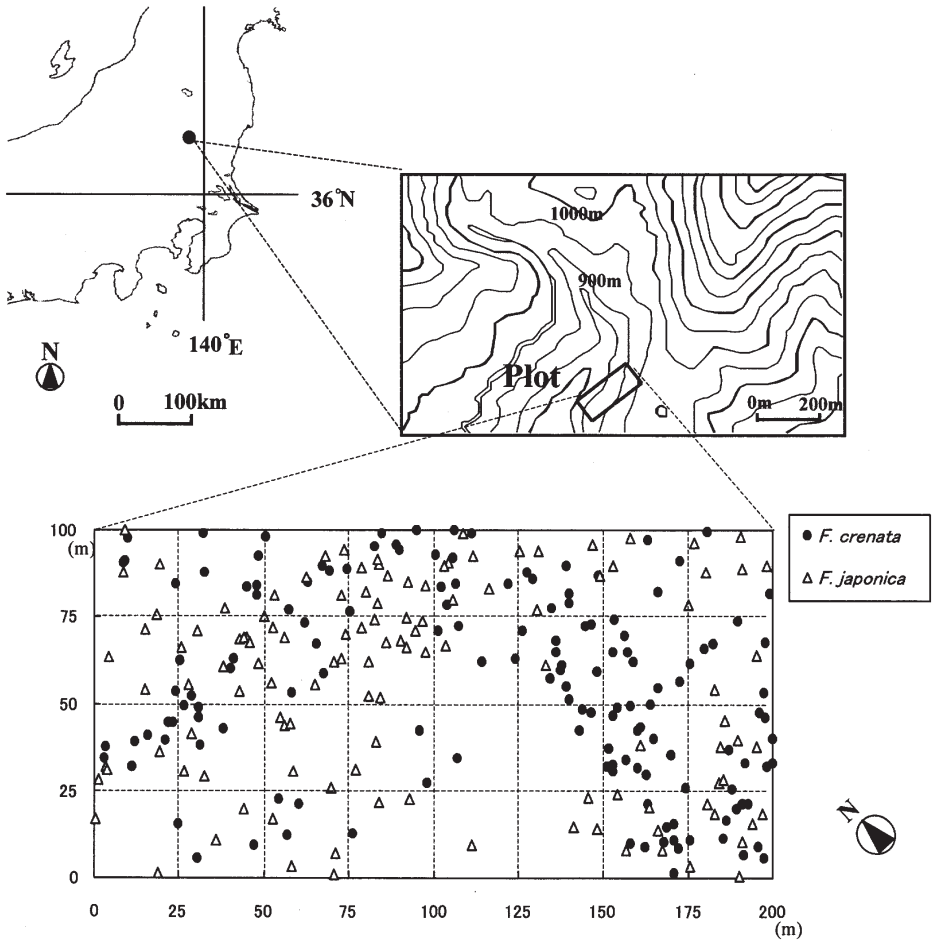

Figure 1. – Location of the 2-ha study site and distribution map of 147 *Fagus crenata* (DBH > 5 cm) and 127 *F. japonica* (DBH > 5 cm) individuals. Closed circles: *Fagus crenata* individuals; open triangles: *F. japonica* individuals.

observations on the genetic structure within *F. crenata* and *F. japonica* populations was located in an area containing a relatively high density of individuals of both species (*Figure 1*). Leaf samples were collected from 147 *F. crenata* (diameter at breast height, DBH > 5 cm) and 127 *F. japonica* (DBH > 5 cm) individuals and were stored at –30 °C prior to DNA extraction. Even large stools of *F. japonica* usually consist of single individuals, so we collected leaf samples from only one sprout of each stool to avoid resampling the same individuals.

DNA extraction and microsatellite genotyping

Total DNA was extracted from leaves of each *F. crenata* and *F. japonica* individual by a modified CTAB method (TSUMURA *et al.,* 1996). The genotypes of 4 microsatellite loci (TANAKA *et al.,* 1999) for each individual of each species were determined. Microsatellite primer pairs from *F. crenata* could be used for *F. japonica* because they are closely related (TANAKA *et al.,* 1999). PCR amplification was performed in 10-µl reaction volumes containing 5 ng of genomic DNA, 1×PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer (the forward primer labeled with dye in each case), and 0.25 units of *Taq* polymerase, with the GeneAmp PCR System Model 9700 (PE Applied Biosystems). The annealing temperature and number of PCR cycles for the primer pairs (*mfc2, mfc5, mfc11,* and *mfc12*) were 64 °C, 58 °C, 56 °C, and 56 °C for 30 s; and 30, 35, 35, and 30 cycles, respectively. The denaturation and extension conditions were the same for all primer pairs: 94 °C and 72 °C for 30 s, respectively. The genotype was determined with an ABI 310 Genetic Analyzer and GeneScan software version 2.0.1 (PE Applied Biosystems).

Data analysis

For each locus, the allele frequencies and observed heterozygosity (H_O) were calculated with genotype data. The expected heterozygosity, H_E, and fixation index, $F = 1 - (H_E/H_O)$, were calculated according to WRIGHT (1965). Null allele frequencies were estimated from the deviation from the Hardy-Weinberg equilibrium, with the Cervus 1.0 program (MARSHALL *et al.,* 1998).

Measurements of genetic relatedness, as defined by QUELLER and GOODNIGHT (1989), were used to detect genetic structure in *F. crenata* and *F. japonica.* In general, genetic relatedness is the probability of 2 individuals sharing a gene that is identical by descent. The genetic relatedness between all possible pairs of the 147 *F. crenata* individuals and 127 *F. japonica* individuals was calculated with the Relatedness program, version 5.0.6. (GOODNIGHT, 1999), according to QUELLER and GOODNIGHT (1989). The spatial distance between all individual pairs was also calculated for each species. The correlation between genetic relatedness and spatial distances among all individuals in each species was tested by Mantel tests with the R Package program, version 4.0 (CASGRAIN and LEGENDRE, 2000). We used 5000 times randomization in the Mantel test, which is a realistic minimum for estimating a significance level of about 0.01 (MANLY, 1997). The spatial distances between individuals were divided into 12 distance classes, at 5-m intervals from 0 to 60

m. The mean genetic relatedness was calculated for each of the 12 distance classes by averaging the pairwise genetic relatedness values. The statistical test for correlation among individuals in each of the 12 distance classes was also the Mantel test.

Spatial genetic structure was also analyzed according to the number of alleles in common (NAC) (BREG and HAMRICK, 1995). The NAC method computes the average number of alleles in common per locus between pairs of individuals in a given distance class. The NAC was calculated for each distance class in both *F. crenata* and *F. japonica* with the Spatial Autocorrelation program version 1.1.1. (TAKAHASHI *et al.,* 2000). The mean NAC value was calculated for each distance class by averaging the pairwise NAC values (SURLES *et al.,* 1990). A grand mean of NAC values, which represents the value expected under the null hypothesis of spatial randomness of alleles, was obtained by averaging all pairs of NAC values. To evaluate the significance of excesses or deficits in NAC, variances of NAC values were calculated in each distance class by the bootstrap procedure. A NAC value that is significantly greater than the grand mean in a certain distance class implies that individuals in that distance class share more alleles than would be expected if the distribution were random (TAKAHASHI *et al.,* 2000).

Results

Genetic diversity

The genotypes of 4 microsatellite loci were determined for 147 individuals of *F. crenata* and 127 of *F. japonica*. These microsatellite loci were highly polymorphic in these species. The number of alleles per locus ranged from 13 to 35 in *F. crenata* and 7 to 24 in *F. japonica;* and the observed heterozygousity (H_O) varied from 0.451 to 0.932 per locus in *F. crenata* and from 0.627 to 0.857 in *F. japonica* (*Table 1*). The fixation index *F*, which measures deviation from Hardy-Weinberg equilibrium, was significantly different from zero for 2 loci (*mfc11, mfc12*) in *F. crenata*, and the estimated null allele frequencies of these 2 loci in *F. crenata* appeared to be high (*Table 1*). We detected at total of 124 alleles for 4 loci in the 2 species, and the number of species-specific alleles were 57 in *F. crenata* and 27 in *F. japonica* (*Table 2*). The overall gene differentiation between the 2 species was estimated, yielding G_{ST} (based on the infinite allele model; NEI, 1973) and R_{ST} (based on the stepwise mutation model; SLATKIN, 1995) values of 0.061 and 0.672, respectively.

Nine pairs of *F. japonica* individuals had same genotypes for all loci. Therefore, an individual of each of them was excluded from further analysis, because they were probably clones.

Genetic structure

We detected significant negative correlation between the genetic relatedness and spatial distance among all individuals in *F. crenata* and *F. japonica*, but the r-values of the Mantel test in these species were not high ($r = -0.0998$, $p < 0.01$; $r = -0.0634$, $p < 0.01$, respectively). If there were a genetic structure within a population, a clear negative correlation with a high *r*-value would be expected. Therefore, the results indicate that genetic clustering exists for the species in our study site but was weak.

The means of the genetic relatedness within the *F. crenata* and *F. japonica* populations were positively significant in the first 2 distance classes and in the first 5 distance classes, respectively (*Figure 2*). The mean values of genetic relatedness in *F. japonica* are higher than those for *F. crenata* within short distances (less than 25 m) (*Figure 2*).

The grand mean of the NAC was 0.349 ($\pm 2.6 \times 10^{-5}$ SD) in *F. crenata* and 0.526 ($\pm 3.9 \times 10^{-5}$ SD) in *F. japonica.* The NACs were positively significant in the first 2 and 6 distance classes in *F. crenata* and *F. japonica*, respectively. The NACs in *F. japonica* were higher than in *F. crenata* for all distance classes (*Figure 3*).

Discussion

Genetic variability

The H_O and H_E values and number of species-specific allele were higher in *F. crenata* than in *F. japonica*, but the fixation index significantly derived from zero at 2 loci (*mfc11, mfc12*) in *F. crenata* (*Table 1, 2*). Homologous microsatellite loci are longer, i.e., contain more repeat units, in focal species than in related species (RUBINSZTEIN *et al.,* 1995; ELLEGREN *et al.,* 1997), which means that genetic diversity is usually higher in species using homologous microsatellite markers than in species using heterologous markers. In our study, the focal species, *F. crenata*, has longer repeats for all loci than *F. japonica*, and consequently *F. crenata* has higher heterozygosity (*Table 2*). This result was probably due to a biased selection of loci associated with the cloning procedure, which selected longer-than-average repeats during the library-screening stage of microsatellite development (ELLEGREN *et al.,* 1997). However, the microsatellite markers used in our study have sufficient polymorphism and resolution for studying the genetic structure of *F. japonica*, because we could detect 67 alleles in a total of 4 loci. Two estimates of gene differentiation between the 2 *Fagus* species were different, because the R_{ST} value is estimated from the microsatellite repeat number but the G_{ST} value is not (*Table 2*). Therefore, the R_{ST} value may be much more accurate for detecting genetic differentiation, if we can allow comparisons between species using a limited number of loci. In gener-

Table 1. – Characteristics of 4 polymorphic microsatellite loci in 147 individuals of *F. crenata* and 127 individuals of *F. japonica.* * and ** denote fixation index values that significantly differed from Hardy–Weinberg equilibrium at the 5% and 1% probability levels.

Species	Locus	A^a	$H_o{}^b$	$H_e{}^c$	F^d	Estimated Null allele frequency
F.crenata	*mfc2*	35	0.928	0.962	0.025	0.010
	mfc5	26	0.932	0.931	0.001	0.003
	mfc11	13	0.634	0.829	0.235*	0.130
	mfc12	23	0.451	0.784	0.425**	0.267
F.japonica	*mfc2*	22	0.841	0.913	0.079	0.037
	mfc5	24	0.857	0.903	0.046	0.024
	mfc11	7	0.627	0.701	0.074	0.049
	mfc12	14	0.677	0.771	0.122	0.066

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

Table 2. – Number of species-specific and shared alleles, mean length of detected alleles, and genetic differentiation of 2 *Fagus* species.

Locus	Range(bp)	Mean allele length(bp)		Number of species-specific alleles		Number of share alleles	G_{ST}	R_{ST}
		F. crenata	*F. japonica*	*F. crenata*	*F. japonica*			
mfc2	125-221	178.1	137.5	27	14	8	0.029	0.751
mfc5	256-324	290.5	282.4	4	2	22	0.021	0.139
mfc11	313-349	319.4	319.3	8	2	5	0.082	-0.004
mfc12	228-336	297.6	266.3	18	9	5	0.119	0.797
all				57	27	40	0.061	0.672

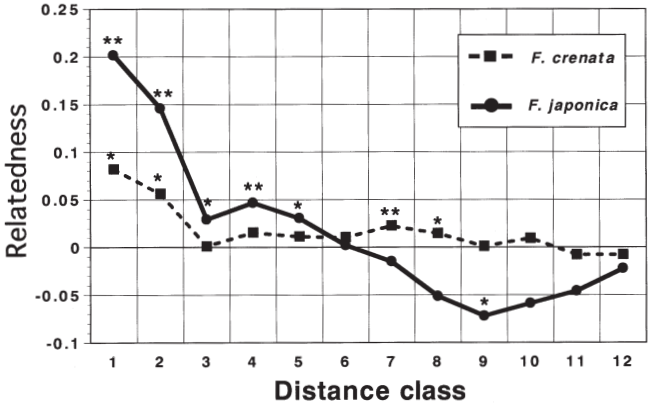

Figure 2. – Relationships between genetic relatedness and physical distance for 2 *Fagus* species. Distances were divided into 5-m-wide classes from 0–5 m (class 1) to 55–60 m (class 12). * and ** denote significant spatial autocorrelation for a distance class at the 5% and 1% probability levels, respectively.

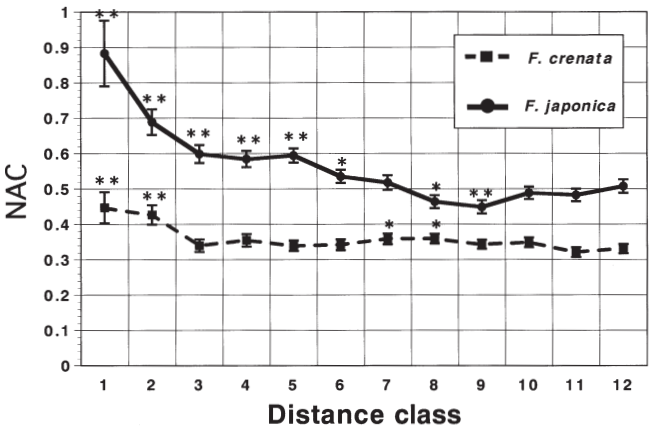

Figure 3. – NAC values for 12 distance classes in *F. crenata* and *F. japonica*, which were defined as in *Figure 2*. * and ** denote NAC values that significantly differed from the grand mean NAC values at the 5% and 1% probability levels, respectively. The error bars show the standard deviations.

al, a deviation from Hardy-Weinberg equilibrium can arise from nonrandom mating, such as selfing or assortive mating. However, the mean fixation index previously derived from allozyme data did not significantly differ from Hardy-Weinberg equilibrium in *F. crenata* (TOMARU *et al.,* 1997; TAKAHASHI *et al.,* 2000), and self-incompatibility has also been reported in this species (KOUNO and MUKOUDA, 1985). Because *Fagus* spp. is an allogamous species, its outcrossing rate is high (ROSSI *et al.,* 1996). Therefore, the deviation from Hardy-Weinberg equilibrium may be caused by more than just selfing or biparental inbreeding. For a microsatellite locus, a misamplified microsatellite allele, called a "null allele", frequently occurs during PCR amplification (CALLEN *et al.,* 1993). The expected null allele frequencies were high for those loci (*mfc11, mfc12*) in *F. crenata* (*Table 1*), indicating some possibility that null alleles exist in these loci of *F. crenata*.

Genetic structure

We detected spatial genetic clustering both in *F. crenata* and *F. japonica*, but it was weak. Because these are allogamous, wind-pollinated species, the main reasons for weak clustering were extensive pollen dispersal and high outcrossing. TOMARU *et al.* (1997) found some gene differentiation (G_{ST} = 0.039) among 17 populations of *F. crenata* by using isozyme markers; this suggests that pollen flow is not restricted, which would suppress population differentiation. Seeds of *F. crenata* are abundantly dispersed up to 10 m around a mother tree, but usually not dispersed beyond 30 m (YANAGIYA *et al.,* 1969; MAEDA, 1988). Although no data is available on seed dispersal in *F. japonica*, the seed dispersal distances of the 2 species should be about the same, because the seed size and form are similar (KITAMURA and MURATA, 1980). Almost all seeds of these species are considered to be dispersed close to their mother tree. Thus, the weak spatial genetic clustering in these species should be influenced by extensive pollen flow. However, if there were only a limited number of generations after foundation of the population and the population had not yet reached equilibrium, the founding effect may lead to some detectable genetic structure. *Fagus* populations have experienced cycles of colonization and extinction in response to cycles of glacial and warm periods and human disturbances (TSUKADA, 1988). The population history, especially the number of generations, also affects to the spatial genetic structure.

TAKAHASHI *et al.* (2000) detected genetic clustering in a natural stand of *F. crenata*, because the proportions of positively significant Moran's *I* values and like positively significant joins values were higher over a short distance than over longer distances, but evidence was not clear in the NAC correlogram. They also examined the genetic structure within the northernmost marginal population of *F. crenata* using Moran's *I*, NAC and standard normal deviate (SND) and found the genetic clustering in the population was weaker and less clear than in previously studied populations because the small number of generations since it was founded (TAKAHASHI *et al.,* 2002). KAWANO and KITAMURA (1997) found genetic correlation among mother trees, juveniles, and seedlings in *F. crenata* according to the Moran's *I* and R_{ij} values (which indicates coancestry). The limited genetic structure that we detected in *F. crenata* is consistent with these results, in that the extent of genetic clustering (as evident from positively significant NAC values and relatedness values) was higher for short than for longer distances (*Figure 2, 3*). MERZEAU *et al.* (1994) also detected a weak genetic structure in 1 of 3 *Fagus sylvatica* stands that they investigated, but the genetic structure in the 2 other stands appeared not to be stable. Because they may be due to a limited number of generations, an effective gene flow less limited or fertility difference or phenological incompatibilities between individuals (MERZEAU *et al.,* 1994). STREIFF *et al.* (1998) detected weak genetic structures in populations of *Quercus petraea* and *Q. robur*, which are wind-pollinated and produce gravity-dispersed seeds-the same reproductive characters as in *Fagus*. They suggested that a main reason for the weak genetic structures in *Q. petraea* and *Q. robur* was extensive pollen flow (STREIFF *et al.,* 1998).

There is some possibility that the genetic structures in both *Fagus* species may be weakened by secondary seed dispersal by animals. *Fagus crenata* seed can be dispersed by animals such as birds (*Nucifraga caryocatactes japonicus*) and wood mice (*Garrulus gladnarius pallidifrons*) (WATANABE, 1990; MIGUCHI, 1994), although the dispersal distance and amounts of seeds dispersed were not reported. Seed dispersal by animals may not strongly affect the genetic structure in those species, compared to pollen flow.

Despite the weak spatial genetic structures, we found a difference in the spatial genetic structures between *F. crenata* and *F. japonica* in the short-distance classes. The results concerning both genetic relatedness and NAC analysis showed that spatial genetic structure in *F. japonica* was stronger than in *F. crenata* (*Figure 2, 3*). Differences in the strength of genetic

structure in these species could be caused by different extents of overlap between generations. Regeneration of *F. crenata* depends mainly on the growth of seedlings or saplings under canopy gaps (e.g., NAKASHIZUKA, 1983). In contrast, *F. japonica* forms stools by vigorous sprouting and its stools help to maintain a clone (OHKUBO, 1992). A *F. japonica* stool can attain a substantial age: the lifetime of *F. japonica* is about 1000 years (HARA, 1996), whereas the lifetime of *F. crenata* is only about 200–300 years. *Fagus japonica's* longevity may also be inferred from the size of stools. In our study site, the average and maximum sizes of stools of *F. japonica* are 160 cm and 460 cm, respectively, in contrast to the maximum DBH size of *F. crenata* of 83.5 cm. The overlapping of generations in *F. japonica* is expected to be longer than in *F. crenata*; thus, different lifetimes might be one of the reasons for the difference in genetic structure between these species.

Acknowledgements

We are grateful to Dr. MAKOTO TAKAHASHI and Dr. SANEYOSHI UENO for valuable suggestions and technical support. We thank Mr. YOSHINARI MORIGUHI and Mr. TAKASHI SUGAYA for their help in sample collection, and Mr. TETSUYA IGARASHI, Dr. KAORU NIIYAMA, Dr. KEIKO KITAMURA, Dr. ATSUSHI ISHIDA, Dr. HIROSHI YOSHIMARU, Dr. HIROYOSHI IWATA, Dr. NAOKI TANI, and Dr. ASAKO MATSUMOTO for their kind suggestions.

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