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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-m² 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 (KONOHARA, 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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of several ten to hundred meters form the neighboring natural stands. Completely isolated populations of endangered species or alpine plants are found worldwide, and studies on these fragmented populations have been widely published. However, whether the semi-fragments of common species found in Japan will be maintained in the future and the method by which they will be maintained remain unknown.

In terms of ecology, the forest fragmentation has been actively investigated with edge effects, though there is substantial discrepancy among recent studies about the existence and intensity of edge effects (MURCIA, 1995). For example, a tree census in two isolated stands in 1989 and 2000 was carried out to determine the structural changes in a small lucidophyllous tree population in Japan following fragmentation (KOMINAMI *et al.*, 2003). Although it was found that five tree species were lost over an 11-year period, no changes in structural or population characteristics were regarded as being due to forest fragmentation. However, such studies would not clearly detect the influence of forest fragmentation (if any) because they do not consider seed dispersal or genetic erosion, which can cause long-term changes.

On the other hand, rapid developments in molecular biological techniques such as allozyme and microsatellite analyses have greatly increased the power and scope of genetic research on habitat fragmentation. Theoretically, the fragmentation of populations should lead to an erosion of genetic variation and increased inter-population genetic divergence through: (1) increased random genetic drift, (2) increased inbreeding, (3) reduced inter-population gene flow, and (4) increased probability of local extinction of demes within meta-populations (YOUNG *et al.*, 1996). Forest fragmentation may have three main effects (ISAGI, 2004): (1) no detectable genetic changes may occur in the tree population because the trees have a long life span, and they retain the genetic diversity possessed prior to fragmentation; (2) it may have detectable adverse effects on pollination, seed dispersal, and the genetic traits of reproductive plants; or (3) it may change the foraging patterns of insects or animals so as to promote pollen and seed dispersal. In particular, the second effect, which is consistent with the mentioned theory of YOUNG *et al.* (1996), has been reported in many studies. For example, a microsatellite analysis by DAYANANDAN *et al.* (1999) revealed that the allelic richness was low in the sapling cohort of the isolated populations of *Carapa guianensis*. On the other hand, BACLES *et al.* (2004) found that remnants of *Sorbus aucuparia* maintained high levels of genetic diversity in isozyme and cpDNA markers such as the non-fragmented populations. However, the pollen-mediated gene flow has decreased due to forest fragmentation, and the high levels of genetic diversity within the remnants are maintained probably due to the help of effective seed dispersal by birds. The third effect has also been reported in some recent studies. For example, DICK (2001) reported that *Dinizia excelsa* inhabiting fragmented forests produced, on an average, over three times as many seeds as those produced by the trees in continuous forests. Furthermore, the microsatellite assays of seed arrays performed by DICK (2001) showed that genetic

diversity was maintained across habitats. A fragmented population of *Fraxinus excelsior* has also maintained genetic diversity at high levels than those reported for continuous populations (BACLES *et al.*, 2005). BACLES *et al.* (2005) suggested that the opening of the landscape facilitated airborne pollen movement, thereby alleviating the expected detrimental genetic effects of fragmentation. As described above, the potential effects of forest fragmentation are highly varied. A recent study suggested that approaches based on life-history traits potentially permit the prediction of the species' response to forest fragmentation (KOLB and DIEKMANN, 2004). However, further studies will be required in order to verify these approaches.

Quercus crispula Blume (Fagaceae) is a common tree species widespread in the cool-temperate deciduous forests of southern Sakhalin, the Kuril Islands, Japan, and Korea. It is an intermediately shade-tolerant species that is capable of sustained regeneration and has a life span of several hundred years; it attains a maximum height and diameter of 30 m and 1.5 m, respectively (WATANABE, 1994). *Q. crispula* is monoecious and a highly outcrossed, wind-pollinated species (WATANABE, 1994). Its seeds are dispersed by gravity or rodents (*Apodemus* spp. and *Sciurus* spp.) and birds (*Garrulus* spp. and *Nucifraga* spp.); it thus provides important food for animals. Almost all of these life-history traits appear advantageous in alleviating the genetic effects of forest fragmentation. Nevertheless, *Q. crispula* exhibits a relatively high level of inbreeding depression, and it was suggested that inbreeding individuals would have a high mortality rate within each patchy structure (UBUKATA *et al.*, 1999). Hence, despite its several advantageous life-history traits, even small isolations can seriously affect *Q. crispula* population through inbreeding within fragments. Moreover, very steep rocky slopes with poor soil are not suitable for artificial forests and *Q. crispula* populations. On such slopes, populations of this species will probably need to propagate the next generation under severe conditions, and effects of even slight fragmentation can have a serious impact on them.

The objective of this study was to evaluate the effects of forest semi-fragmentation on *Q. crispula*. We examined the genetic diversity and differentiation of populations in this species in the Chichibu Mountains, central Japan. The genetic diversity in a semi-fragmented stand and the gene flow into the fragment from other sources were also studied. Here, we describe the results of these analyses and discuss their implications regarding the characteristics and management of the fragmented natural forests, particularly in Japan.

Materials and Methods

Study site and sampling

This study was performed in the University Forest in Chichibu, The University of Tokyo, (138°48' E, 35°56' N) located in the central area of Japan (Fig. 1). The mean annual temperature in the area is 10.9°C, the annual precipitation amounts to 1400–1500 mm and the maximum snow depth from winter to early spring is usually less than half a meter (UNIVERSITY FOREST IN CHICHIBU,

Figure 1. – Location of the sampled five populations of *Quercus crispula* in the Chichibu Mountains.

2002). The mountains are mostly covered with cool temperate second-growth forests dominated by species such as oak or beech, while man-made forests of species like *Cryptomeria japonica* or *Chamaecyparis obtusa*, occupy some areas (UNIVERSITY FOREST IN CHICHIBU, 2002). The major oak species in this region are *Q. crispula* and *Q. serrata*, the latter predominantly in warmer areas than *Q. crispula*. In some area, both species coexist and hybridize with each other. The slopes are typically very steep, especially in the lower portions of the valleys, due to severe erosion and the formation of a V-shaped valley by the Arakawa River. To elucidate the genetic diversity and differentiation in this region, leaf samples for genetic analysis were randomly collected in the summer of 2004 from five populations (Iriyama, Yatake, Ohchi, Bakemono, and Wasabi; Table 1). The number of individuals sampled per population ranged from 23 to 49. The collected samples were stored at -80°C until DNA extraction.

Table 1. – Topographic conditions for each of the five populations of *Quercus crispula* in the Chichibu Mountains.

population	altitude(m)	direction	slope($^{\circ}$)
Iriyama	1200-1400	SW	34
Yatake	1100-1300	E	27
Ohchi	700-1000	NW	21
Bakemono	1100-1400	SW	31
Wasabi	1100-1300	NE	39

To describe the gene flow into a fragmented stand from outside, we investigated a semi-fragmented stand and one plot in unfragmented stand located on the same, southwest-facing hillside named “Iriyama” (Fig. 2). The semi-fragmented stand had been connected with the neighboring natural forest until about 30 years ago. The semi-fragmented stand is composed of 35 mature oak trees (33 *Q. crispula* and 2 *Q. serrata*), in a triangular area of 2500 m², situated on a steep slope (mean gradient 31 $^{\circ}$). Such steep areas with poor soil escaped afforestation, although the surrounding forest has been changed into artificial conifer forests. The nearest natural forest to this stand is 50 m away, and it is one of the typical examples of semi-fragmented forest in Japan. The species composition of the stand was very similar to that of the neighboring natural forest because of the past connection. These forests were secondary deciduous forests dominated by oak (*Q. crispula*), beech (*Fagus crenata*), and maple (*Acer* spp.). The mean age of the oak trees is around 70 years, and therefore these trees had germinated before the fragmentation. No dwarf bamboo was present in the fragmented stand, although there were some colonies in the neighboring forest. We had already collected leaves of adults randomly in “Iriyama” including the semi-fragment and the neighboring natural forest to elucidate the genetic diversity and differentiation, but another time we collected leaves of all 35 adults in the fragmented plot. Moreover, leaves of nearly all seedlings germinated from May to July in this year were collected. The endocarps of hypogeal cotyledons were also sampled in the fragmented plot, where possible, since they are of maternal origin, allowing the seeds’ mother trees to be identified (GODOY and JORDANO, 2001; SUYAMA, 2004; GRIVET *et al.*, 2005). Till quite recently, many researchers had used seedlings to detect the parents with some assumptions or maximum-likelihood approach. But the new method with genetic analysis of endocarps was developed lately, and it is now the most effective approach to identify mother trees with high accuracy. As a result, we obtained leaves of 70 seedlings and endocarps of 52 seedlings. To compare the local genetic diversity in and gene flow into the fragmented plot with those in the unfragmented forest, we then collected leaves of all 72 adults in a unfragmented plot covering an area of 2500 m² on a relatively gentle slope (mean gradient 19 $^{\circ}$). Leaves of 238 seedlings were also collected for evaluating genetic diversity and 216 endocarps of them were collected for parentage analysis. The position of each individual was also recorded in the both plots. In addition, in the autumn of 2004 we collected 50 seeds randomly on the ground within 5 m in radius from two adults, designated Y126 and Y132, in the fragmented plot for supplementary assessment of pollen flow into the fragment from outside.

Genetic analysis

DNA was extracted by a modified CTAB procedure (LIAN *et al.*, 2003). But the sampled endocarps had been buried under ground for more than half a year, and they included impurities. Therefore, the endocarp DNA often showed imperfect amplification in polymerase chain

Figure 2. – Location of the two plots of *Quercus crispula* in Iriyama with contour lines and the individual maps within each plot. The fragmented plot is a semi-fragment of natural forest from the neighbor natural forest more than 50 m away, while the unfragmented plot is located in the middle of an unfragmented natural forest. On the individual maps, large stars indicate the seedlings with the new alleles which were not found in any adults within each plot. Large circles and small squares indicate then adults and other seedlings, respectively.

reaction (PCR) or amplified multi-band which could be difficult to be genotyped. To cope with these problems, the resulting extract of endocarps was purified using a Wizard SV Gel and PCR Clean-Up System (Promega). Extract was amplified by PCR, using a multiplex PCR Kit (QIAGEN), with six nuclear microsatellite (SSR) primer pairs in one tube: QpZAG1/5, QpZAG9, QpZAG15, QpZAG16, and QpZAG110 (STEINKELLNER *et al.*, 1997) and MSQ13 (DOW *et al.*, 1995). The 5.0 µl amplification reaction mixtures included 2.5 µl of MasterMix solution (QIAGEN), 1.3 µl of RNase-free water, 0.5 µl of primer mix solution, and 0.7 µl of the extracted DNA. The used primer mix solution included six primer pairs of 0.5 pmol/µl. The reactions were performed with the following temperature program: 15 min denaturing at 95 °C followed by 30 cycles of 30 s denaturing at 94 °C, 90 s annealing at 57 °C and 60 s extension at 72 °C, with a final extension step of 60 °C for 30 min. For the endocarp DNA, the PCR cycles were rearranged from 30 to 40, to obtain sufficient amplification (to genotype). Finally, the PCR product was loaded into an ABI3100 Genetic Analyzer (Applied Biosystems) and amplified allele sizes were estimated using GeneMapper software (Applied Biosystems).

Statistical Analysis

Based on the derived genotypes, genetic diversity was estimated within each population by calculating the expected heterozygosity (H_e). Using rarefaction procedures to account for uneven sample sizes, allelic richness ($A_{[46]}$) (MOUSADIK and PETIT, 1996) was also calcu-

lated based on the smallest number of genes sampled in one population. The inbreeding index (F_{IS}) and their significance were also assessed with 1000 randomizations using the program FSTAT version 2.9.3.2. (GOUDET, 2001). The number of locus pairs showing significant genotypic disequilibrium (GD) was estimated with the same program. The genetic differentiation between populations was evaluated by calculating overall fixation index (F_{ST}) (WEIR and COCKERHAM, 1984). 95 % and 99 % confidence intervals were evaluated by 1000 bootstrap permutations, and the significance of genotypic population differentiation at each locus and over loci was tested by the log-likelihood (G)-based exact test (GOUDET *et al.*, 1996) using FSTAT. The pairwise fixation index (F_{ST}) was also calculated, and its significance was assessed with 1000 randomizations using the same program.

In addition, for both adult and seedling groups in the two plots, expected heterozygosity (H_e), allelic richness ($A_{[70]}$), inbreeding indices (F_{IS}), and the number of locus pairs showing significant genotypic disequilibrium (GD) were calculated using FSTAT. To detect gene flow from outside into the plots, we identified and counted new alleles that were present in the seedling group but not in the adult group. Additionally, the number of seedlings with these new alleles was counted.

Parentage analysis

We entered all possible genotypes for each seedling and adult into a database for all six of the markers. Before identifying the parent trees of seedlings in the

fragmented plot, we calculated total exclusion probability (CHAKRAVARTI and LI, 1983) using the CERVUS program (MARSHALL *et al.*, 1998) in order to estimate the resolution power of these six markers. We had the genotype information of endocarp which is useful to identify the parent trees of seedlings, but the number of successfully genotyped endocarps was limited (see the results). So we conducted simple parentage exclusion following the classic procedure of DOW and ASHLEY (1996) before using the genotype information of endocarp. First, if a seedling matched no adult in the forest fragment, its parent trees were assumed to be located outside of the fragment. Second, if a seedling matched only one adult in the fragment, the matching adult was assumed to be its maternal tree. This is because a seed developed from a female flower located outside the fragment with pollen from inside is considered to have been transported into the fragment with little probability. Third, if a seedling matched multiple adults in the forest, both of its parent trees were assumed to be present in it. We then detected adult trees that genotypically matched the 36 endocarps in order to confirm that seeds were dispersed into this stand. Because of the low quality of endocarp DNA (mentioned above), amplified multibands can be found with three markers (QpZAG9, QpZAG15, and QpZAG110). So the other three markers (QpZAG1/5, QpZAG16, and MSQ13) were used for this part of the investigation. For reference, the dispersal distances of pollen and seed were then calculated based on the positions of seedlings together with their putative parents within the plot. On the other hand, the mother trees of seedlings in the unfragmented plot were also identified with 172 genotyped endocarps at the three markers. If more than one trees genotypically matched endocarps, other three markers were also used exceptionally to identify the true mother tree. The seed dispersal distance was then calculated like the fragmented plot.

Based on the results of our analysis of embryonic tissues and endocarps from the seeds collected in the autumn, we gathered data on seeds whose endocarps genotypically matched either of the two target adults at the three markers. Then, whether their father trees existed in the fragmented plot or not was checked by simple exclusion with all six markers in order to assess the extent (if any) of pollen flow into the forest fragment from the outside.

Results

Genetic diversity and differentiation of Q. crispula populations in the Chichibu Mountains

Genetic diversity result based on the six nuclear microsatellites is summarized in Table 2. Each population had similar, high levels of genetic variation ($H_e = 0.752-0.792$, $A_{[46]} = 8.050-9.124$). The inbreeding coefficient was not deviated significantly from 0 in any populations, and no locus pairs showing significant genotypic disequilibrium were found. The F_{ST} value was 0.016 with confidence intervals of 0.011–0.021 (95%) and 0.010–0.023 (99%), which showed that the level of population differentiation was very low. Genetic differentiation among populations measured by pairwise F_{ST} is shown in Table 3 which indicates that Wasabi and Bake-

Table 2. – Sample number, expected heterozygosity, allelic richness, inbreeding coefficient and number of locus pairs showing significant genotypic disequilibrium in five *Quercus crispula* populations based on six SSR loci.

	<i>N</i>	<i>H_e</i>	<i>A_[46]</i>	<i>F_{IS}</i>	<i>GD</i>
Iriyama	49	0.792	9.124	0.029	0
Yatake	43	0.787	8.855	0.069	0
Ohchi	37	0.756	8.613	-0.023	0
Bakemono	23	0.753	8.508	-0.006	0
Wasabi	26	0.752	8.261	0.139	0

N: number of individuals, H_e : expected heterozygosity, $A_{[46]}$: allelic richness, F_{IS} : coefficient of inbreeding, *GD*: number of locus pairs showing significant genotypic disequilibrium.

Table 3. – Genetic differentiation measured by pairwise F_{ST} (WEIR and COCKERHAM, 1984) (above diagonal) and geographic distance (km: below diagonal) among five *Quercus crispula* populations.

	Iriyama	Yatake	Ohchi	Bakemono	Wasabi
Iriyama		*0.0059	*0.0190	^{NS} 0.0122	*0.0158
Yatake	1		*0.0206	^{NS} 0.0120	*0.0185
Ohchi	17	17		*0.0265	*0.0190
Bakemono	5	4	16		^{NS} 0.0192
Wasabi	5	5	16	1	

P-values determined using 1000 replicates.

*: $P < 0.05$, NS: not significant.

mono are somewhat differentiated in spite of the short geographic distance between them.

Genetic diversity within two plots, and gene flow into these plots from outside sources

Individual maps of areas within the two plots are shown in Fig. 2, while the results of the genetic diversity analyses of the plots are shown in Table 4. The expected heterozygosity and allelic richness ranged from 0.719 to 0.779 and from 8.670 to 10.333, respectively. The inbreeding coefficients showed no significant deviation from zero.

In the fragmented plot, 14 alleles were found in 15 seedlings, but not in adults. The total exclusion probability with six markers was 0.9967 and 0.9998 for the first and second parents, respectively. Hybrid seedlings of *Q. crispula* and *Q. serrata* were not found in the samples. No putative parent trees were identified for eight of the 70 seedlings genotyped in the plot. Of the remaining 62 seedlings in the plot, maternal trees, but not paternal trees, were found for 29 seedlings; both parent trees were found for 33 seedlings. Extracting DNA from the endocarps was difficult due to its low quality for PCR; however, approximately 70%–80% of the endocarps were genotyped with respect to three markers

Table 4. – Sample number, expected heterozygosity, allelic richness, inbreeding coefficient and number of locus pairs showing significant genotypic disequilibrium in two *Quercus crispula* plots. The fragmented plot is a semi-fragment of natural forest from the neighbor natural forest more than 50 m away, while the unfragmented plot is located in the middle of an unfragmented natural forest. Both plots cover an area of 2500 m² respectively.

	<i>N</i>	<i>H_e</i>	<i>A</i> _[70]	<i>F</i> _{IS}	<i>GD</i>
Adults in the fragmented plot	35	0.779	10.333	0.083	0
Seedlings in the fragmented plot	70	0.755	9.663	0.025	0
Adults in the unfragmented plot	72	0.719	8.738	0.012	0
Seedlings in the unfragmented plot	238	0.745	8.670	0.026	0

N: number of individuals, *H_e*: expected heterozygosity, *A*_[70]: allelic richness, *F*_{IS}: coefficient of inbreeding, *GD*: number of locus pairs showing significant genotypic disequilibrium.

(QpZAG1/5, QpZAG16, and MSQ13). Of the 36 successfully genotyped endocarps in the fragmented plot, 32 endocarps matched with one adult in the plot (*Table 5*). Thus, four of the 36 seedlings were from sources outside the plot. The mean distance of pollen and seed dispersal within the plot were 29.7 m (SD: ± 18.6) and 16.8 m (SD: ± 11.1), respectively. In the unfragmented plot, 13 alleles were found in 21 seedlings, but not in adults. Of the 172 genotyped endocarps, 108 matched with only one adult, and 3 matched with two adults, respectively at the three markers in the plot. However, the true maternal trees of the three seedlings were also identified from multiple candidates with three other markers. Consequently, 61 of the 172 seedlings were from sources outside the plot (*Table 5*). The mean seed dispersal distance within the unfragmented plot was 10.38 (± 8.0) m.

In the autumn, we collected and genotyped 50 samples near each target tree, but only 16 and 17 seeds were identified as seeds of the two target adults (Y126 and Y132, respectively). Other seeds were come from adjacent adults in the fragmented plot. In addition, no father trees in this plot could be identified for ten of the 16 seeds dispersed from Y126 and twelve of the 17 seeds from Y132. Thus, the tendencies detected by the dis-

persed seeds analysis regarding pollen flow into the plot were similar to those found in the seedling analysis.

Discussion

Most previous studies of oak species have found high genetic diversity. Based on an analysis of six microsatellite markers, for example, in two forests in northern Britain, COTTRELL *et al.* (2003) found high expected heterozygosity values, ranging from 0.87 to 0.92 in *Q. robur* and *Q. petraea*. MARIETTE *et al.* (2002) assessed genetic diversity within populations of *Q. robur* and *Q. petraea* with six microsatellite marker, and the values of *H_e* ranged from 0.835 to 0.908. Considering these results, the populations of *Q. crispula* in the Chichibu Mountains have similar diversity to other oak stands. Addition to this, genetic differentiation among oak populations is low in general like the result in this study. For instance, BERG and HAMRICK (1992) derived low estimates of differentiation (*G*_{ST} = 0.032) among populations in a turkey oak (*Q. laevis*) forest by allozyme analysis. Similarly, SCHNABEL and HAMRICK (1990) showed that the variation among populations of *Q. macrocarpa* and *Q. gambelii* stands was low for both species (*G*_{ST} = 0.076–0.119). Probably because we studied in a smaller

Table 5. – Number of found seedling, found endocarp, successfully genotyped endocarp and endocarp matched any adult in two *Quercus crispula* plots. The fragmented plot is a semi-fragment of natural forest from the neighbor natural forest more than 50 m away, while the unfragmented plot is located in the middle of an unfragmented natural forest. Both plots cover an area of 2500 m² respectively.

Number	Fragmented	Unfragmented
	plot	plot
Found seedling	70	238
Found endocarp	52	216
Successfully genotyped endocarp	36	172
Endocarp matched any adult in the plot	32	111
The percentage of seedlings originating within the plot [%]	89	65

geographical scale, the value of F_{ST} derived in our study was still lower than even the G_{ST} values in these previous studies. According to studies of pollen versus seed flow in oaks (PETIT *et al.*, 1993), most of the gene exchanges inferred from population differentiation can be attributed to pollen flow. Details of gene flow among populations are discussed below. Additionally, large effective population size is one of the reasons for the little differentiation among populations in oak species (MUIR *et al.*, 2004).

Similar levels of heterozygosity and allelic richness observed in the seedlings and adults in the two plots suggested that forest fragmentation did not exert serious effects to date, at least on the genetic diversity. Furthermore, sibs often grow close to each other in *Q. crispula* populations because of limited seed dispersal. Thereafter, the sibs would be engaged in frequent mating within each patchy structure (UBUKATA *et al.*, 1999). Considering the results of the above mentioned study and the fact that nearly all samples were collected in the two limited areas, high values of inbreeding coefficients were expected in these plots. However, the obtained F_{IS} values showed no significant deviation from zero; therefore, no evidence of inbreeding was detected. One of the possible explanations for this result is that inbreeding depression might have eliminated the individuals that were produced by sib matings (UBUKATA *et al.*, 1999). Further, slight decreases in the inbreeding coefficients from the seedling stage to the adult stage in the unfragmented plot or other forests (KAWAHARA *et al.*, 2004) support this hypothesis. Such decreases were not observed in the fragmented plot; this may be attributed to other factors. Fragmentation had never promoted inbreeding, at least in this plot. Although the density of *Q. crispula* in the fragmented plot was lower than that in the other plot, the difference was probably due to the geographic factors. Particularly, the low density of the adults that germinated in the fragmented plot prior to artificial fragmentation appears to be attributable to the steep slope. This is because the seeds from the adults do not settle down under most circumstances. In fact, they roll down steep slopes that have poor soil. Even a few seeds that were able to settle on the gentle slope would grow less easily. Although we could not clarify whether artificial fragmentation had a role in the low seedling density in the fragmented plot, genetic diversity had been maintained despite the small number of adults and seedlings.

In the fragmented plot, 14 alleles were found at the seedling stage, but not at the adult stage. Such calculations can underestimate the true gene flow; however, this result confirmed gene flow into the plot. This finding is consistent with the results of the previous studies. For example, MUIR *et al.* (2004) examined the gene flow among seven scattered forest fragments of *Q. petraea* and found evidence of extensive gene flow, particularly via pollens. STREIFF *et al.* (1999) conducted paternity analysis for a mixed oak stand of *Q. robur* and *Q. petraea* and found a high percentage (averaging 65% for *Q. robur* and 69% for *Q. petraea*) of offspring that were pollinated by male parents from sources outside their 5.76-ha study site. This result is similar to our

finding that the maternal parents of 62 of the 70 seedlings examined were present within the fragmented plot; however, the paternal parents of 23 of these seedlings did not appear to be present within the plot. In addition, no putative paternal trees within the plot were found for high ratios (10/16 and 12/17) of the seeds dispersed from two target adults. Thus, we can conclude that pollens can be frequently dispersed over a distance of 50 m. In fact, STREIFF *et al.* (1999) concluded that the mean dispersal distance of pollen was approximately 300 m. Recently, KOENIG and ASHLEY (2003) suggested that pollen flow might be limited to a much greater extent than that suspected previously. For example, the average effective distance of pollen movement was 65 m according to the data of 211 offspring from 21 adult valley oaks *Quercus lobata* (SORK *et al.*, 2002). However, the mean dispersal distance continues to be more than 50 m. Although our spatial scale of observation did not permit the estimation of the long-distance dispersal, we obtained a higher value for the mean dispersal distance of pollens than that of seeds within the fragmented plot. This result supports the existence of extensive gene flow, particularly via pollen.

Inter-population seed dispersal has not been investigated as intensively as pollen dispersal. However, the contribution of the former to the total gene flow can be twice that of the latter. Therefore, it is necessary to understand the process of seed dispersal (HAMILTON, 1999). DOW and ASHLEY (1996) detected parent trees of 100 *Q. macrocarpa* saplings in a 200 x 250 m stand by simple parentage exclusion. Of the 100 saplings, 32 saplings had both parent trees, while 62 saplings had only maternal trees in the stand. This result showed that almost all seeds had their origins in the stand; however, it might be due to the assumption that the seedling matched only one adult in the stand was assumed to have its maternal tree there. Similarly, in fragmented populations of the tropical tree *Symphonia globulifera*, ALDRICH *et al.* (1998) examined the demographic consequences of forest fragmentation by analyzing only leaf samples, and they suggested that the number of unique seedling genotypes per ha of fragmented forest greatly exceeded the corresponding frequency in large forests, probably due to the concentration of seeds in remnant forest fragments by foraging bats. However, there was no material evidence available for this study, similar to the endocarps. In this respect, our endocarp analysis proved that four of 36 seeds were certainly transported from sources outside the fragmented plot, although the number of samples was small because of the technical difficulties involved in extracting DNA from these tissues. Approximately 10% of seedlings originated from sources outside the plot, and this result was consistent with that obtained by simple parentage exclusion for 70 seedlings. The ratio of approximately 10% is sufficient to neutralize the negative effect of genetic drift caused by forest fragmentation; however, it was lower than the ratio ($61/172 = 35\%$) of the unfragmented plot.

The seeds from sources outside the fragmented plot were transported over a distance of more than 50 m.

Seed-dispersing animals such as *Apodemus speciosus*, *A. argenteus*, *Sciurus lis*, *Garrulus glandarius*, *Nucifraga caryocatactes*, and *Parus varius* have been identified in Chichibu Mountains (ISHIDA, 2000; SEKIJIMA *et al.*, 2001). We have not specifically identified the seed transporter; however, rodents generally transport acorns over distances less than 50 m. For example, MIYAKI and KIKUZAWA (1988) reported that most acorns of *Q. crispula* disseminated by mice (*Apodemus speciosus* and *A. argenteus*) traveled distances of approximately 30–40 m away from their mother trees. MIGUCHI and SUZUKI (1991) also found that the mean dispersal distance and the maximum dispersal distance were 13 m and 43 m, respectively for the acorns of *Q. crispula*, *Q. serrata*, and *Q. variabilis* that were transported by rodents. In contrast to rodents, birds can disperse seeds over longer distances. According to ISHIDA and SATO (2002), for example, jays (*Garrulus grandarius*) dispersed acorns of *Q. crispula* up to 500–1500 m from their origin in the Chichibu Mountains. BERG and HAMRICK (1992) studied the regional genetic variation in turkey oak (*Q. laevis*) and found that bird dispersal of acorns over distances on a scale of several kilometers could effectively enhance gene flow by wind-borne pollen; this was done by preventing inter-population differentiation among the fragmented populations. Hence, birds were the most probable candidates for the transport of *Q. crispula* seeds into the fragmented plot. In a mosaic of small fragments of mixed oak-autochthonous pine woodlands coexisting with several afforestation stands, European jays transported most of the acorns outside the oak stands, thereby decreasing the risk of intraspecific kleptobiosis (acorn pilferer) and secondary predation (GOMEZ, 2003). In our field observations, we also found many oak seedlings in the artificial forests. In this respect, the artificial forest that surrounds the fragmented plot may be a beneficial rather than a harmful habitat for birds. Irrespective of the abovementioned hypothesis, the results of the pollen and seed analyses suggest that both wind- and seed-dispersal played pivotal roles in inter-population gene flow into the plot, presumably accounting for the relatively low inbreeding coefficient of seedlings in this plot and probably preventing inbreeding depression in this oak population.

In conclusion, slight genetic divergence was observed between the examined populations of *Q. crispula* in Chichibu Mountains. The frequent gene flow among populations and/or large effective population size are the possible explanations for the slight genetic divergence. The parentage analysis in the fragmented plot showed that pollen dispersal by wind appears to promote inter-population gene flow. Seed dispersal (mainly by birds) may also contribute to inter-population gene flow, although the ratio of seed flow from sources outside the fragmented plot was lower than that of the unfragmented plot. Therefore, we observed that semi-fragmentation has not affected genetic diversity and there was no evidence of promoted inbreeding in the fragmented plot. From a practical perspective, the effects of forest semi-fragmentation, such as isolation by distances of ca. 50 m, do not pose a grave problem for oaks in the near future. Additionally, the fragmented forests are worth

conserving. Simultaneously, the transporters of genes, particularly birds, are important for conserving the fragmented populations. Therefore, seed-dispersing animals and birds should also be preserved. However, genetic diversity is slowly lost over subsequent generations, and it may take decades for the trees to lose their genetic diversity (LOWE *et al.*, 2005). The decrease in seed flow in the isolated plot after fragmentation can be a serious issue for the regeneration of *Q. crispula* in the future. Therefore, more detailed studies will be required to assess the long-term effect of forest fragmentation. In Japan, many semi-fragmented stands are scattered, particularly on partially steep slopes. Genetic connectivity among these semi-fragments will be particularly important in these areas. The Green Corridor project has been initiated to conserve such connectivity (MIURA, 1999).

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Date of Shoot Collection, Genotype, and Original Shoot Position Affect Early Rooting of Dormant Hardwood Cuttings of *Populus*

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Abstract

Identifying superior combinations among date of dormant-season shoot collection, genotype, and original shoot position can increase the rooting potential of *Populus* cuttings. Thus, the objectives of our study were to: 1) evaluate variation among clones in early rooting from hardwood cuttings processed every three weeks from shoots collected throughout the dormant season and 2) evaluate variation among genomic groups in early rooting of the same cuttings while testing for differences among three parental shoot positions (apical, middle, basal). We tested 22 clones belonging to six genomic groups (*[P. trichocarpa* Torr. & Gray × *P. deltoides* Bartr. ex Marsh] × *P. deltoides* 'BC', *P. deltoides* 'D', *P. deltoides* × *P. maximowiczii* A. Henry 'DM', *P. deltoides* × *P. nigra* L. 'DN', *P. nigra* × *P. maximowiczii* 'NM', *P. trichocarpa* 'T'). Cuttings, 20 cm long, were processed from shoots collected every three weeks beginning 1 Dec. 2003 until 9 Apr. 2004 from stool beds established at Hugo Sauer Nursery in Rhinelander, Wisconsin, USA (45.6°N, 89.4°W). We measured number of roots and root dry weight from harvested cuttings after 14 days of growth. The interaction between date of shoot collection and clone governed both traits ($P < 0.0001$). In general, clones exhibited the best rooting when cuttings were processed from shoots collected on or after 23 Feb. 2004.

The interaction between date of shoot collection, genomic group, and shoot position governed number of roots ($P = 0.0348$) and root dry weight ($P = 0.0262$). There was broad variation in number of roots and root dry weight of apical, middle, and basal cuttings within and among genomic groups across dates of shoot collection, with 15 Mar. 2004 being an important date because differences among positions began to develop or changed relative to earlier dates. Thus, for increased plantation establishment potential with similar genotypes, we recommend collecting stool shoots no sooner than the end of February and matching cuttings of specific shoot positions to each genomic group.

Key words: *Populus* genomic groups, hybrid poplar, adventitious rooting, genotype × environment interaction, whip, stool bed.

Introduction

Evaluation of genetic variation among and within populations of genotypes belonging to the genus *Populus* has been conducted for over half a century in North America (PALLARDY and KOZLOWSKI, 1979; FARMER, 1970; ALLEN and MCCOMB, 1956). The potential of selecting promising genotypes for numerous end-uses (including bioenergy, fiber, phytoremediation, and agroforestry) has prompted breeding of intra- and inter-species hybrids, along with gathering of open-pollinated collec-

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