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Ecological and genetic effects of cutting in an *Alnus trabeculosa* Hand.-Mazz. (Betulaceae) population

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In order to assess the ecological and genetic effects of cutting, we compared two portions of *Alnus trabeculosa* population at Yuda (Iwate Prefecture, Japan): one that has been cut about 30 years ago and one that has remained uncut. These portions were compared in terms of the degree of sprouting, genetic variation and gene distribution using isozyme markers. First, we determined the multilocus genotype (MLG) of all ramets, then sorted them into individuals according to the distribution of the MLGs. The average ($\pm$SE) of largest distance between ramets in one individual was 2.1 ($\pm$0.18) m, which is consistent with the distance (2.0 ($\pm$0.20) m) obtained by tracing physical connections between ramets. We found no significant differences in genetic variation between the two portions, but there were significant differences in their degree of sprouting. Furthermore, there were striking differences in gene distribution: the cut portion showed greater clustering of individuals with identical genetic components, which may be due to regeneration in the gaps made by cutting, reflecting the location of the mother trees, and seed and pollen dispersal from them.
Heredity (2003) **91,** 331–336. doi:10.1038/sj.hdy.6800316

Introduction

Alnus trabeculosa Hand.-Mazz. is a deciduous, broad-leaved tree, which grows only in open swampy places, and reaches a height of 5 m. The species is sparsely distributed in the warm- and intermediate-temperate zones of Honshu and Kyushu (Japan), with northern-most limits in Yuda (Iwate Prefecture). It is also found in the southeastern part of continental China (Kurata, 1971). Since its habitat is dwindling and human activities are having an adverse influence on it, populations of the species are declining, and it is now classed as 'near threatened' (Environment Agency of Japan, 2000).

This species has the capacity to sprout, as well as to reproduce sexually via seeds (Handa *et al*, 1996). In accordance with this, some individuals in the Yuda population were observed to form sprouting clonal stands, and the extent of each individual could not be determined by simple visual inspection.

Genetic variation and, especially, family structure can result from several evolutionary processes, including isolation in small patches, limited pollen or seed dispersal, selection and human influences (Sokal and Menozzi, 1982). As a tool for analyzing family structure, spatial autocorrelation has been used in several studies of forest tree populations. For example, differences in genetic structure between populations with different age classes have been detected (Hamrick *et al*, 1993;

Epperson and Alvarez-Buylla, 1997; Parker *et al*, 2001); genetic structure found in younger classes disappeared in older classes as the population was thinned. Strong structuring has also been detected using spatial auto-correlation in a population in which vegetative repro-duction and limited dispersal of pollen and seeds were observed (Shapcott, 1995). It has also revealed striking differences in genetic structure between populations with differing histories of anthropogenic disturbance (Knowles *et al*, 1992; Takahashi *et al*, 2000).

Assessing subtle differences among evolutionary processes using spatial autocorrelation is unrealistic. Nevertheless, investigating family structure can help to describe the main evolutionary forces affecting the population under study (Sokal and Menozzi, 1982), and provide fundamental information needed for selecting natural populations for conservation, or breeding pro-grams. Family structure should also be taken into account in order to maximize diversity, and to avoid making erroneous estimates of population diversity (Epperson, 1989).

Our group has previously detected the existence of family structure in the Juo population of *A. trabeculosa* in Ibaraki, central Japan, using spatial autocorrelation (Miyamoto *et al*, 2000, 2002). We concluded that for both *in situ* and *ex situ* conservation of this species, it is important to take into consideration the existence of family structure. We also suggested that the species' mode of seed dispersal and asexual propagation may be involved in the formation of family structure.

According to the owner of this site, part of the Yuda population was cut down about 30 years ago (pers. comm.). There now appear to be differences between the

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cut and uncut portions. The height and diameter of the trees in the harvested portion are smaller than those in the uncut portion. However, it is not clear whether there are ecological and/or genetic differences between these two *A. trabeculosa* portions.

Therefore, in order to assess the influence of cutting, we compared two portions of the Yuda population. First, we obtained information about multilocus genotypes (Okuizumi, 1993) for all the ramets, using isozyme markers. When ramets with identical MLGs are clustered, they are assumed to belong to a single individual. In order to identify individuals which are composed of one or more ramets with identical MLGs, a statistical method was used. Finally, we evaluated the degree of sprouting (in terms of number of sprouts per individual and the largest distance between ramets in one individual) as well as the genetic variation in the two portions with different cutting histories, and compared the pattern of gene distribution between the two portions using spatial autocorrelation analysis.

Materials and methods

Studied population
We studied the population of *A. trabeculosa* at Ecchu-hata, Yuda, in Iwate Prefecture (285 m a.s.l.; 39°17′N, 140°43′E; average temperature $= 9.5°C$; warmth index ($\Sigma(T_m-5)$, when T_m is above 5, T_m: monthly mean temperature) $= 78.9°C$ per month; coldness index ($-\Sigma(5-T_m)$, when T_m is below 5) $= -23.2°C$ per month; precipitation $= 1605$ mm per year). This population, the northernmost population of the species in Japan, is located along a small river, which runs from west to east. The population is surrounded by a paddy field to the north, an artificial forest of *Cryptomeria japonica* (Japanese Cedar) to the south and a field to the west, where soil removed during the construction of the Akita Highway was dumped. This population is divided into two portions (western and eastern) that are 50 m apart. The western portion has many small trees and a few large ones, while, in contrast, the eastern portion is composed almost entirely of large trees. Trees in the western portion (portion A) are generally much smaller in height and diameter than those in the eastern portion (portion B), and were assumed to be immature. Judging from the distribution of genotypes among the ramets (see below), some plants in the investigated population seem to form sprouting, clonal stands. We defined genetically identical organisms derived from sexual propagation as 'individuals', and sprouts formed by asexual propagation belonging to a single individual as 'ramets'. We also surveyed the height and diameter at breast height (DBH) of all ramets taller than 1 m (532 ramets) and mapped them (Figure 1).

Isozyme analysis
Experimental samples (winter buds) were collected from all mapped ramets in the winter of 1998. Samples were stored at $-80°C$ until the enzymes were extracted. Isozyme analysis was based on the protocol described by Tsumura *et al* (1990). In *A. trabeculosa*, 12 putative polymorphic isozyme loci have already been reported, encoded by eight enzyme systems (Miyamoto *et al*, 2000): *Aap* (alanine aminopeptidase; AAP, E.C. 3.4.11.1), *Aco*
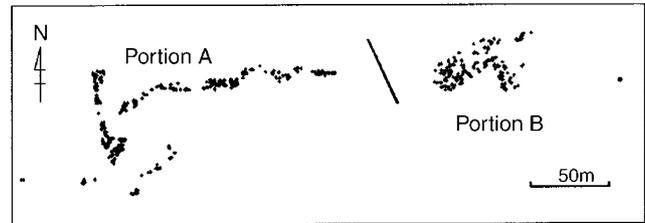

Figure 1 Map of all ramets analyzed in the Yuda population and the spatial relation between portions A and B.

(aconitase: ACO, E.C. 4.2.1.3), *Amy*-2 (amylase; AMY, E.C. 3.2.1), *Dia-1, Dia-2, Dia-3* (diaphorase; DIA, E.C. 1.6.4.3), *Got-1, Got*-2 (glutamate oxaloacetate transaminase; GOT, E.C. 2.6.1.1), *Lap* (leucine aminopeptidase; LAP, E.C. 3.4.11.1), *Pgi-1, Pgi-2* (phosphoglucoisomerase; PGI, E.C. 5.3.1.9) and *Shd* (shikimate dehydrogenase; ShDH, E.C. 1.1.1.25). These loci were used for the analysis.

Identification of individuals by the MLG of ramets
Information about the isozyme genotype at all 12 loci (multilocus genotype, MLG; Okuizumi, 1993) was obtained for all the ramets. When ramets with identical MLGs are clustered, they are assumed to belong to a single individual. In order to identify individuals, which are composed of ramets with identical MLGs located continuously, a statistical method was used. We made 20 distance classes of 1 m intervals (0–1, 1–2 m and so on). The number of joins of ramets with identical MLGs was counted in each distance class. For each MLG, the standard normal deviate (SND) of the observed number of joins from the expected number of joins (Sokal and Oden, 1978) was calculated using the formula:

$$SND = (\text{observed joins} - \text{expected joins})/\text{Variance}^{1/2}$$

The SND is assumed to be normally distributed, so it has critical values of ± 1.96 at the 5% level of significance. If ramets with identical MLGs are located closer to each other, than would be expected according to random chance, the SND is expected to decrease as the distance class increases. Therefore, we examined the largest distance classes where SND scored significant values for each MLG and adopted its value to define individual boundaries. We then sorted the ramets with identical MLGs into individuals according to these values. The SND values were calculated using the software developed by Takahashi (2001).

The spread and type of individuals
The spread of each individual was defined as the distance between its two most widely spaced ramets. We investigated the spread of each individual identified in the way stated above. Using *t*-tests, we then compared the differences in the spread between individuals belonging to the two portions. Moreover, we classified the individuals into two types: those with only one ramet (type 1) and those with two or more ramets (type 2). We also calculated the percentage of each type, the average number of ramets per individual of type 2, and evaluated the significance of the difference in the number of ramets per individual of type 2 between the two portions using a *t*-test.

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Physical analysis by root excavation

We dug down to the roots of each individual at two points and traced physical connections between ramets. In addition, we measured the largest distance between connected ramets belonging to each individual.

Genetic variation

To estimate genetic variation, the following genetic parameters (Hamrick and Godt, 1989) were calculated for all individuals of portions A and B: the percentage of polymorphic loci (Pl; we regarded the loci where frequency of the most frequent allele is less than 0.99 as polymorphic loci), the mean number of alleles per locus (N_a), the effective number of alleles per locus (N_e), the observed heterozygosity (H_o) and the expected heterozygosity (H_e). The N_e values were derived from $1/\Sigma p_i^2$,' (Kimura and Crow, 1964) and H_e values by averaging h_e over 12 loci, where $h_e = 2n(1-\Sigma p_i^2)/(2n-1)$ (Nei, 1987), p_i is the allele frequency of the ith allele at a given locus and n is the sample size.

Gene distribution analysis in each portion

Spatial genetic structure within the two portions was assessed by estimating coancestry (Cockerham, 1969) between all possible pairs of individuals at different interplant distances. For each individual containing more than two ramets, we assumed that one ramet was at its center of gravity.

In order to estimate coancestry, genotypes for individuals were first expressed as allele frequencies of 1.0, 0.5 or 0.0, depending on whether their genotype included 2, 1 or 0 copies of the respective alleles (Heywood, 1991). Estimates of coancestry ($\hat{\rho}_{ij}$) were then calculated as

$$\hat{\rho}_{ij} = \frac{\sum_{ij}(p_i-\bar{p})(p_j-\bar{p})}{k\bar{p}(1-\bar{p})} + \frac{2}{(8k+1)^{0.5}} \quad (i<j)$$

where p_i and p_j are the frequencies of same alleles at a locus for individuals i and j, $\bar{p}$ is the mean frequency for that allele and $k=n(n-1)/2$, which gives the number of possible pairs between n individuals located in each distance class (Loiselle *et al*, 1995). The second term adjusts for bias associated with a finite sample size, which causes the estimate of coancestry to be zero for a population in Hardy–Weinberg equilibrium. The correlation between pairs of plants in their frequencies of same alleles at a given locus ($\hat{\rho}_{ij}$) in an outbreeding population ($F_{IS}=0$) has expected values of 0.125 and 0.0625 for half-sibs and first cousins, respectively. A combined multilocus estimate of coancestry can be obtained by weighting the values for each locus by its polymorphic

index, $\Sigma p_i(1-p_i)$. Weights for individual loci were also adjusted for differences in sample size due to missing genotypes (Loiselle *et al*, 1995). Multilocus coancestry estimates have a sound foundation in population genetics theory and provide a natural means of summarizing data over alleles at a locus and over loci to provide a more powerful test for spatial genetic structure (Heywood, 1991). Therefore, multilocus estimates of coancestry were calculated at 5 m interplant-distance increments for each subpopulation. Tests of significance for estimated values of $\hat{\rho}_{ij}$ were performed using randomization procedures to generate populations under the null hypothesis of no spatial structure. In each subpopulation, intact multilocus genotypes were randomly drawn, with replacement, from the sampled data and assigned to points occupied by plants. New $\hat{\rho}_{ij}$ values, were then calculated. This randomization procedure was repeated 399 times for each subpopulation, giving (together with the originally sampled data) 400 $\hat{\rho}_{ij}$ values, from which 95 and 99% confidence intervals were constructed. The procedure tests the significance of the correlation in multilocus genotypes at each distance interval (Slatkin and Arter, 1991). We used software developed by Nason (1997, 1998) for the autocorrelation analyses of coancestry.

Results

Size analysis in each portion

The means and standard deviations of the height and DBH values of ramets in the Yuda population were 5.7 $\pm$2.7 m and 8.1$\pm$4.2 cm, respectively. The corresponding values were 4.3$\pm$1.7 m and 6.2$\pm$2.9 cm, respectively, for ramets in portion A, compared to 8.1$\pm$2.4 m and 11.6$\pm$4.1 cm in portion B (Table 1; Figure 2). Thus, ramets in portion B were approximately twice as tall and twice as wide as those in portion A, and differences between the ramets in the two portions in both height and diameter at breast height were significant ($p<0.01$ and $p<0.01$, respectively).

Isozyme analysis

Variation was detected at five of the 12 putative isozyme loci tested (*Aap, Amy-2, Got-2, Lap* and *Shd*).

Identification of individuals by the MLGs of ramets

Individuals were identified based on the results of the MLG analysis and evaluation of the distance classes for which the MLGs had significant SND values. A total of 532 ramets were sorted into 225 individuals by 57 MLGs. In all, 134 and 91 individuals were recognized from portions A and B, respectively.

Table 1 Height (m), DBH (cm), number and percentage of ramets belonging to individuals of types 1 and 2 in portions A and B, and the total population of *A. trabeculosa* at Yuda

	Height (m)	DBH (cm)	Type 1	Type 2[a]	Number of ramets per individual of type 2	Total[a]	Percentage of type 1	Percentage of type 2
Portion A	4.3$\pm$1.7	6.2$\pm$2.9	66	283/68	4.2	349/134	49%	51%
Portion B	8.1$\pm$2.4	11.6$\pm$4.1	47	136/44	3.1	183/91	52%	48%
Total	5.7$\pm$2.7	8.1$\pm$4.2	113	419/112	3.7	532/225		

[a]Number of ramets/number of individuals.

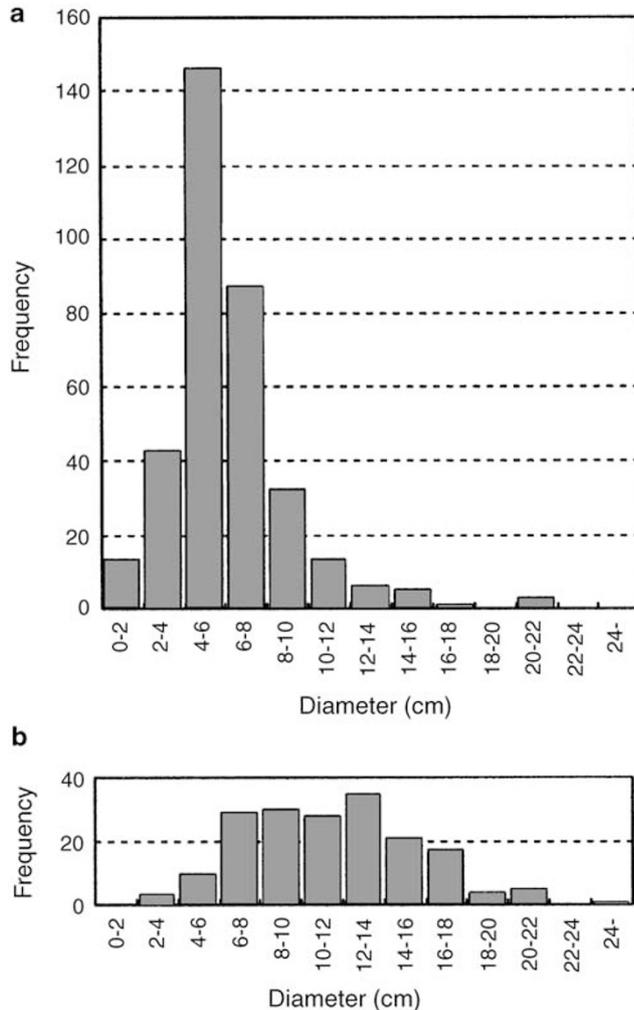Effects of cutting in an *Alnus trabeculosa* Hand.-Mazz.
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Figure 2 Distribution of DBH of ramets taller than 1 m in portions A and B in the Yuda population of *A. trabeculosa*.

Figure 3 Distribution of the spread of type individuals in portions A and B of a *A. trabeculosa* population.

The spread and type of individuals

The proportion of type 2 individuals was very similar in the two portions; 51% in portion A and 48% in portion B (Table 1). However, the average number of ramets per type 2 individual was higher in portion A (283/68 or 4.2) than in portion B (136/44 or 3.1), and there was a significant difference between these numbers according to *t*-tests ($p < 0.05$). The largest individual was 10.8 m wide in portion A. The most frequently observed spread class was 1.0–2.0 m, and the average spread of these individuals ($\pm$SE) was 2.1 ± 0.18 m (2.3 ± 0.27 and 1.6 ± 0.20 m in portions A and B, respectively; Figure 3). There was a significant difference between these values according to *t*-tests ($p < 0.05$).

Physical analysis by root excavation

Physical connections between ramets of two individuals were consistent with the identification based on the MLG analysis. Two individuals showed that ramets were connected within the range of 2.0 ± 0.20 m. This was almost the same as the mean value derived from the MGC analysis (2.1 ± 0.18 m).
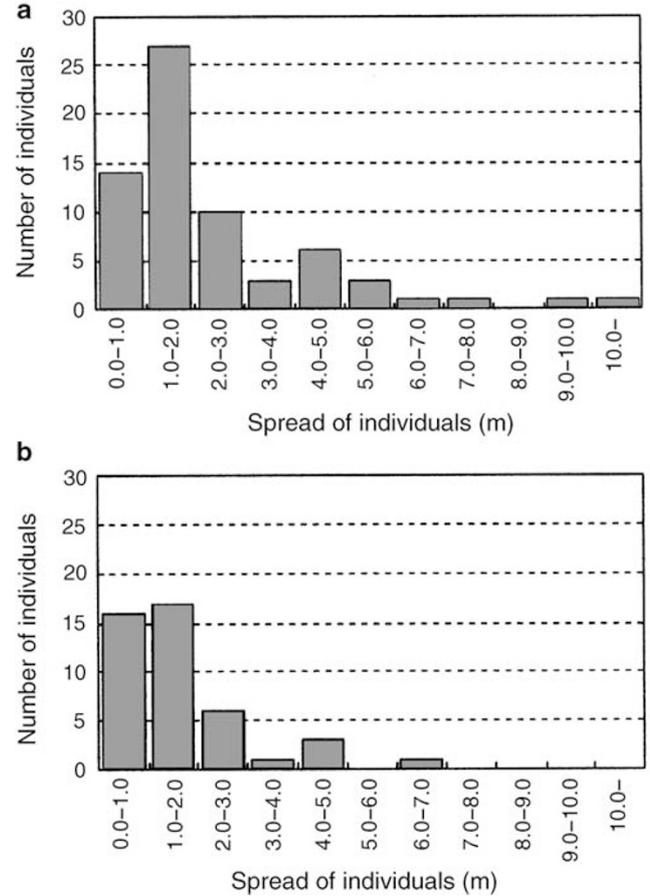
Genetic variation

No significant differences were found between the two portions in genetic diversity parameters (Table 2).

Gene distribution analysis in each portion

In portion B, coancestry values were within the range of 99% confidence limits in all of the distance classes, that is, coancestry values were not significantly different from zero. In contrast, the coancestry coefficients in portion A were higher than the expected range in the first two distance classes, but fell to within the expected interval in the third distance class (upper limit, 15 m). These data indicate that individuals within 10 m of each other tend to be genetically similar in portion A (Figure 4).

Discussion

Influence of cutting on sprouting

A. trabeculosa has previously been reported to produce sprouts from its roots (Handa *et al*, 1996). The distribution of trunks we found with the same MLGs strongly corroborates this report. We estimated the influence of cutting in terms of the spread of area spanned by individuals and the number of ramets belonging to each individual. The maximum spread of an individual clone is about 10 m, and the average range of individuals including more than two ramets is about 2.1 m. This

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Table 2 Genetic diversity parameters (*Pl*, N_a, N_e, H_o and H_e) in portions A and B, and the total population of *A. trabeculosa* at Yuda

	Pl	N_a	N_e	H_o	H_e
Portion A	41.7	1.6 (0.229)	1.22 (0.120)	0.127 (0.059)	0.125 (0.056)
Portion B	41.7	1.6 (0.193)	1.24 (0.119)	0.144 (0.067)	0.133 (0.060)
Difference between the two portions (*t*-test)		NS	NS	NS	NS
Total	41.7	1.7 (0.225)	1.23 (0.118)	0.134 (0.060)	0.129 (0.056)

Averages and standard errors are shown in parentheses. *Pl*=percentage of polymorphic loci (0.99 criterion); N_a=mean number of alleles per locus; N_e=effective number of alleles per locus; H_o=observed heterozygosity; H_e=expected heterozygosity.
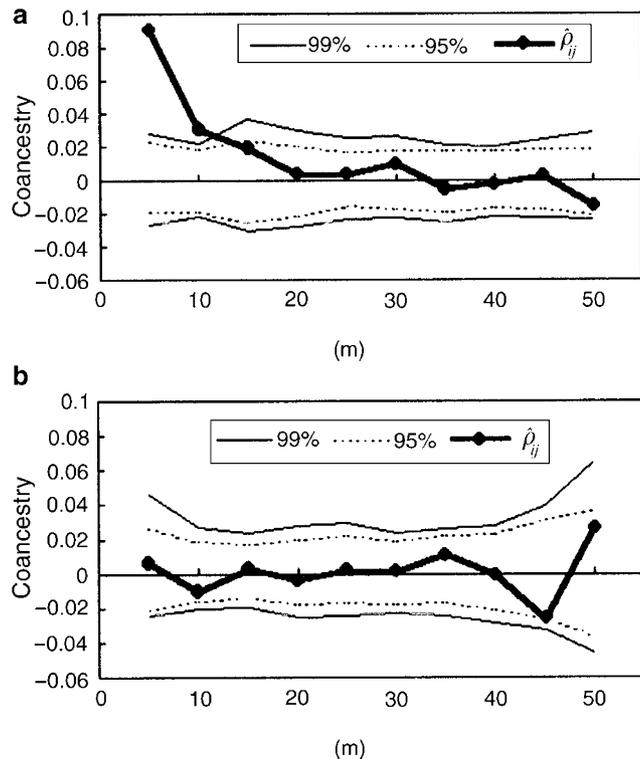

Figure 4 Spatial correlograms of coancestry coefficients ($\hat{\rho}_{ij}$) for portions A and B of the Yuda population of *A. trabeculosa*. Dashed lines (95%) and solid lines (99%) represent upper and lower confidence limits for no spatial structure.

value is consistent with the range (2.0 m) obtained by physically tracing connections through root excavation. Previously reported ranges of individuals or clumps of ramets include 2–4 m in *Fagus japonica* (Kitamura *et al*, 1992), 4 m in *Quercus chrysolepis* (Montalvo *et al*, 1997), 8 m in *Fagus grandifolia* (Jones and Raynal, 1987), and areas of 50 m² in *Thujopsis dolabrata* (Hashimoto *et al*, 1999) and up to 43 ha in *Populus tremuloides* (Kemperman and Barnes, 1976). Thus, the *A. trabeculosa* range found in this study was similar to those of *F. japonica* and *Q. chrysolepis*.

Comparison of the height and DBH of trees in the two portions showed that ramets in portion B, which had not been cut, were about twice as large as those in portion A. which has been cut. The proportion of type 2 individuals, that is, those with two or more ramets, was similar in portions A and B, but the average number of ramets per type 2 individual was significantly higher in portion A. Two factors may have contributed to this difference. Firstly, type 2 individuals may become type 1 indivi-

duals, as some ramets die. Alternatively, since portion A is considered to be younger and immature, the stand in portion A may be in a transitional state towards that of portion B. Secondly, tree species are generally thought to increase by asexual propagation when the trunks die due to damage caused by fungi, or because they have fallen down or been blown down by the wind (Hallé *et al*, 1978; Watanabe, 1994). Portion A was cut down approximately 30 years ago; so it is presumed that the cutting might have induced sprouting in this species.

Influence of cutting on the genetic variation and gene distribution

No significant differences were found between the two portions in terms of genetic diversity parameters. However, the coancestry analyses revealed striking differences between the allele and genotype distributions in the two portions. Portion A showed higher degrees of clustering of individuals with identical genes than portion B, and the range of the clusters with similar genetic components appears to be up to 10 m. Since portion A was partly cut down in the past, it is now thought to be composed of large remnant trees together with small seedlings or sprouts from the trees that were cut down. There are many potential candidates for the mother trees of these seedlings, but it is highly likely that they were trees that were cut and/or remnant trees. In contrast, in portion B, the mother trees may have been located in the other portion, and dispersed seeds by wind to portion B, or they may have dropped seeds that floated to the present portion B, which was a safe site for *A. trabeculosa*. So, we can speculate that the gene and genotype concentrations seen in portion A reflect the locations and limited pool of mother trees before and just after the cutting, while in portion B, clustering of genes and genotypes occurs to a much lower degree because the seeds came from more widely dispersed trees.

Miyamoto *et al* (2000) suggested that the mode of seed dispersal and asexual propagation in the Juo population of the species may be related to the formation of family structure. In addition, this study suggests that the cutting may have caused clustering of individuals with identical genes and thus created the family structure. This indicates that the uncut portion may be more suitable for conservation, because of its more random distribution of genes, than the portion that was cut in the past.

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Mr M Ueta, Mr N Miura, Mr T Hasebe and Ms T Wakai for their help in collecting samples and Ms K Tanaka and Ms Y Miyata for their assistance in the laboratory.

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