Differences in spatial autocorrelation between four sub-populations of *Alnus trabeculosa* Hand.-Mazz. (Betulaceae)

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To investigate the spatial structure of *Alnus trabeculosa* Hand.-Mazz., we compared three sub-populations at Imaichi in Tochigi Prefecture and one sub-population at Juo in Ibaraki Prefecture, Japan. A total of 269 trees, covering 0.71 ha in total, were mapped and genetically analyzed using nine enzyme systems encoding 13 isozyme loci. There were no significant differences between the four sub-populations in terms of N_a , N_e , H_o , H_e and F_{IS} . However, according to spatial autocorrelation analysis, the Juo sub-population, which is younger than the others, showed an aggregation of multi-locus genotypes, especially within 25 m radii. In contrast with the Juo sub-population, those in Imaichi showed no clear

spatial structuring. In the three Imaichi sub-populations, gametic phase disequilibrium is attributable to heterogeneity of genotype frequencies, but in the Juo sub-population it seems to be due to other factors, connected with the composition of the trees in the sub-population and/or its founder population, gene flow and asexual propagation. To conserve as much as possible of the genes or genotypes in restricted areas, conservation of populations that do not show clear family structures, such as those in Imaichi, would be most effective.

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Introduction

Alnus trabeculosa Hand.-Mazz. is a deciduous broad-leaved tree species which grows only in open swampy places. The species is sparsely distributed in the warm- and intermediate-temperate zones of Honshu, with northernmost limits in Yuda in Iwate Prefecture, and Kyushu (Miyazaki Prefecture) in Japan. It is also found in the southeastern part of continental China (Kurata, 1971). Propagation is accomplished not only by sexual reproduction via seeds but also by asexual reproduction via sprouts (Handa *et al.*, 1996). Because its habitat is dwindling and human activities are having an adverse influence, populations of the species are declining, and it is now classed as 'near threatened' (Environment Agency of Japan, 2000).

Genetic variation and especially family structure can result from several evolutionary processes including, amongst others, 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 in various studies using the technique (Hamrick *et al.*, 1993; Epperson and Alvarez-Buylla, 1997; Parker *et al.*, 2001). The cited authors found genetic structure in the younger classes which disappeared in older classes as the populations were thinned. It also revealed striking differences in genetic structure between populations with differing histories of anthropogenic disturbance (Knowles *et al.*, 1992; Takahashi *et al.*, 2000). Strong structuring has also been detected using spatial autocorrelation in a population in which vegetative reproduction and limited dispersal of pollen and seeds were observed (Shapcott, 1995).

It is true that assessing subtle differences among evolutionary processes by spatial autocorrelation is unrealistic. Nevertheless, investigating family structure can help us 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 sampling populations for breeding programs. Family structure should also be taken into account in order to maximize diversity, and to avoid making erroneous estimates of the diversity of populations (Epperson, 1989).

Miyamoto *et al.* (2000) detected the existence of family structure in the Juo population of *A. trabeculosa* using spatial autocorrelation. They 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.

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They also suggested that the species' mode of seed dispersal and asexual propagation may be involved in the formation of family structure. However, there is no information about family structures in other populations of *A. trabeculosa*, so it is not clear whether family structure is a common phenomenon in this species, or whether any other factors, such as tree size and historical factors, influence such structure.

In this study, we investigated the genetic variation and family structure of three sub-populations in Imaichi, Tochigi Prefecture, Japan, using spatial autocorrelation, and compared them with similar data from the sub-population in Juo, Ibaraki prefecture, to obtain fundamental information for conserving populations of this species.

Materials and methods

Study site

The *A. trabeculosa* population investigated at Imaichi, Tochigi Prefecture (Figure 1, Table 1) (175 m.a.s.l.; 36°37'N, 139°44'E) is composed of 123 individuals. The height and diameter at breast height (d.b.h.) of all these individuals were measured and mapped. The individuals are situated in two areas (about 200 m apart), separated by paddy fields, roads and watercourses. The sub-population in the northern area 'sub-population A' was designated AI, and the subpopulation in the southern area 'sub-population B' was designated BI. Within AI, trees in

Figure 1 Geographical distribution of trees in AI (LAI and SAI), BI and JUO. Grid with dashed lines indicates 10 m intervals.

the northeastern part (height, 16.1 m $\pm$ 2.4; d.b.h., 25.1 cm $\pm$ 10.0) appear to differ substantially in age compared with those in the southwestern part (height, 10.0 m $\pm$ 1.7; d.b.h., 11.1 cm $\pm$ 3.6). In 1972, the southwestern part of AI was reclaimed, and at that time many seeds fell onto this site from the trees that were cut down (Natsuka, 1996). The two parts of AI were separated; the northeastern part with large trees was called 'sub-population LA' (or LAI), and the southwestern part with small trees was called 'sub-population SA' (or SAI). BI was composed solely of trees of about the same size as those in LAI (height, 17.6 m $\pm$ 1.6; d.b.h., 24.5 cm $\pm$ 6.1).

Miyamoto *et al* (2000) have already described the family structure of the sub-population of *A. trabeculosa* at Juo in Ibaraki Prefecture (Figure 1, Table 1) (50 m.a.s.l.; 36°42'N, 140°42'E; designated JUO), which is composed of 146 individuals (height, 8.9 m $\pm$ 2.6; d.b.h., 11.0 cm $\pm$ 4.7). This sub-population is located along the course of a small river, and no sub-populations of the species are located upstream. This sub-population was with the three sub-populations at Imaichi (LAI, SAI and BI).

Isozyme analysis

Experimental samples (winter buds) were collected from all the mapped individuals in Imaichi in the winter of 1999, and 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*, nine enzyme systems, encoding the 13 putative isozyme loci used in this analysis have been reported (Miyamoto *et al*, 2000, 2001); *Aap* (alanine aminopeptidase; AAP, EC 3.4.11.1), *Aco* (aconitase; ACO, EC 4.2.1.3), *Amy-2* (amylase; AMY, EC 3.2.1), *Dia-1*, *Dia-2*, *Dia-3* (diaphorase; DIA, EC 1.6.4.3), *G6p* (glucose-6-phosphate dehydrogenase; G6PD, EC 1.1.1.49), *Got-1*, *Got-2* (glutamate oxaloacetate transaminase; GOT, EC 2.6.1.1), *Lap* (leucine aminopeptidase; LAP, EC 3.4.11.1), *Pgi-1*, *Pgi-2* (phosphoglucose isomerase; PGI, EC 5.3.1.9) and *Shd* (shikimate dehydrogenase; ShdDH, EC 1.1.1.25).

Genetic variation

Genetic variation was calculated for all the trees in LAI, SAI, BI and JUO percentage of polymorphic loci (PI), mean number of alleles per polymorphic locus (Na), effective number of alleles per polymorphic locus (Ne), observed heterozygosity (H_o) and expected heterozygosity (H_e) (Hamrick and Godt, 1989). The Ne values were derived from $1/\sum p_i^2$ (Kimura and Crow, 1964). The H_e values were derived by averaging h_e over 13 loci, where $h_e = 2n(1-\sum p_i^2)/(2n-1)$ (Nei, 1987), p_i is the frequency of the i th allele at a given locus, and n = sample size.

Inbreeding coefficients and gametic phase disequilibrium

The inbreeding coefficient (F_{IS}) and heterogeneity in genotype frequencies at each locus were calculated to assess whether the observed genotype frequencies deviated from the frequencies expected under Hardy-Weinberg equilibrium. F_{IS} was derived by averaging f_{is} over polymorphic loci, where $f_{is} = 1 - h_o/h_e$, h_e is expected heterozygosity and h_o is observed heterozygosity. We also calculated the gametic phase disequilibrium between all pairs of detected loci. Heterogeneity in genotype frequencies at each of the polymorphic loci and gametic phase disequilibrium between all pairs of 13 loci were examined using the chi-squared test of the GDA program version 1.0 (Lewis and Zaykin, 2001).

Table 1 Environmental characteristics for the four *Alnus trabeculosa* sub-populations

Population	Altitude (m)	Latitude	Longitude	No. of trees	Area (m ²)	Density (/ha)	Height (m)	d.b.h. (cm)
LAI	175	36°41'N	140°42'E	34	1400	243	16.1 ± 2.4	25.1 ± 10.0
SAI	–	–	–	37	500	740	10.0 ± 1.7	11.1 ± 3.6
BI	–	–	–	52	1700	306	17.6 ± 1.6	24.5 ± 6.1
JUO	50	36°37'N	139°44'E	146	3500	417	8.9 ± 2.6	11.0 ± 4.7

d.b.h.: diameter at breast height.

Data analysis

Spatial genetic structure within sub-populations was assessed with estimates of coancestry (Cockerham, 1969) between all possible pairs of individuals at different interplant distances. 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 comprised 2, 1, or 0 copies of the respective alleles (Heywood, 1991). Estimates of coancestry (ρ_{ij}) were then calculated as:

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

where p_i and p_j are the frequencies of homologous 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 in the equation 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 homologous alleles at a given locus (ρ_{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 sub-population. Tests of significance for estimated values of ρ_{ij} were performed using randomization procedures to generate populations under the null hypothesis of no spatial structure. In each sub-population, intact multilocus genotypes were randomly drawn, with replacement, from the sampled data and assigned to points occupied by plants. New ρ_{ij} values were then calculated. This randomization procedure was repeated 399 times for each sub-population, giving (together with the originally sampled data) 400 ρ_{ij} values, from which 95% and 99% intervals were constructed. The procedure tests the significance of the correlation in multilocus genotypes at each distance interval (Slatkin and Arter, 1991). The software developed by Nason (1997, 1998) for the autocorrelation analyses of coancestry was used.

Results

Isozyme analysis

Thirty alleles were detected, but for three loci (*Aco*, *Dia-1* and *Pgi-2*) no variation was in any of the sub-populations (Table 2). All other loci had at least two alleles (the maximum being four alleles at *Shd*). Four out of 10 polymorphic loci were fixed in some of the sub-populations, but the other six (*Aap*, *Amy-2*, *Dia-3*, *G6p*, *Lap* and *Pgi-1*) showed segregation in every population at the 95% polymorphic level. Some alleles were observed in only one sub-population (*Shd^c* and *Shd^e* in SAI; *Dia-2^a* and *Lap^c* in JUO).

Comparison of the growth of individuals between the populations

The LAI and BI populations were almost the same in terms of height and d.b.h. (Figure 2; Table 1). However, these two populations differed in size from the SAI and JUO sub-populations, which were also composed of similarly sized (and thus similarly aged) individuals.

Genetic variation

There were no significant differences in genetic variation parameters amongst the four sub-populations (Table 3).

Inbreeding coefficients, heterogeneity and gametic phase disequilibrium

The inbreeding coefficients (F_{IS}) were –0.112 in LAI, 0.001 in SAI, –0.085 in BI and –0.018 in JUO, there were no significant differences in these parameters between the four sub-populations. Heterogeneity of genotype frequencies was detected at *Pgi-1* in LAI, and at *Aap* in SAI and BI. But when these loci were excluded, no gametic phase disequilibrium between the pairs of loci was observed in LAI, SAI or between any pairs except one (*G6p/Got-2*) in BI. In contrast to the three sub-populations in Imaichi, we did not observe heterogeneity of genotype frequencies in JUO, but we did detect gametic phase disequilibrium between 12 pairs of loci (*Aap/Dia-2*, *Aap/Dia-3*, *Aap/Got-2*, *Aap/Pgi-1*, *Aap/G6p*, *Aco/G6p*, *Amy-2/Lap*, *Dia-1/G6p*, *Dia-2/Dia-3*, *Dia-2/Got-2*, *Dia-2/G6p*, *Got-2/G6p*) in this sub-population.

Fine-scale genetic structure

In the LAI and SAI sub-populations, coancestry values were within the range of 99% confidence limits in all of the distance classes (Figure 3). In the BI sub-population too, the coancestry values were not significantly different from zero, with the exception of significant positive and negative correlations at the closest distance class and 45

Table 2 Allele frequencies in the four *Alnus trabeculosa* sub-populations

Enzyme	Locus	Allele	LAI	SAI	BI	JUO
AAP	<i>Aap</i>	<i>a</i>	0.367	0.157	0.365	0.837
		<i>b</i>	0.017	0.029	0.048	0.059
		<i>c</i>	0.550	0.643	0.423	0.104
		<i>d</i>	0.067	0.171	0.163	–
AMY	<i>Amy-2</i>	<i>a</i>	0.191	0.203	0.183	0.190
		<i>b</i>	0.809	0.797	0.817	0.810
DIA	<i>Dia-2</i>	<i>a</i>	–	–	–	0.117
		<i>b</i>	1.000	1.000	1.000	0.883
	<i>Dia-3</i>	<i>a</i>	0.235	0.214	0.212	0.048
		<i>b</i>	0.647	0.729	0.615	0.767
		<i>c</i>	0.118	0.057	0.173	0.185
G6PD	<i>G6p</i>	<i>a</i>	0.647	0.784	0.596	0.308
		<i>b</i>	0.353	0.216	0.404	0.692
GOT	<i>Got-1</i>	<i>a</i>	0.941	1.000	1.000	0.993
		<i>b</i>	0.059	–	–	0.007
	<i>Got-2</i>	<i>a</i>	0.044	–	0.127	0.141
		<i>b</i>	0.956	1.000	0.873	0.859
LAP	<i>Lap</i>	<i>a</i>	0.941	0.973	0.673	0.962
		<i>b</i>	0.059	0.027	0.327	0.028
		<i>c</i>	–	–	–	0.010
PGI	<i>Pgi-1</i>	<i>a</i>	0.676	0.797	0.510	0.886
		<i>b</i>	0.029	–	0.096	0.114
		<i>c</i>	0.294	0.203	0.394	–
ShDH	<i>Shd</i>	<i>a</i>	–	0.014	0.106	0.090
		<i>b</i>	1.000	0.932	0.894	0.910
		<i>c</i>	–	0.014	–	–
		<i>e</i>	–	0.041	–	–

m, respectively. In contrast, the coancestry coefficients in the JUO sub-population were higher than the expected range in the first five distance classes, falling into the expected interval only at the sixth distance class (upper limit, 30 m). These data indicate that individuals within 25 m of each other may tend to be genetically similar in the JUO sub-population.

Discussion

Based on the data of Miyamoto *et al* (2001), the mean parameters of genetic variation within seven previously investigated populations of *A. trabeculosa* in Japan $PI = 64.3$, $Na = 2.76$, $Ne = 1.54$ and $H_e = 0.199$ are roughly intermediate in comparison with the values obtained for the four sub-populations in this study. According to the ANOVA for the values of genetic variation, there were no significant differences between the four sub-populations, four sub-populations have almost the same level of within-population genetic variation.

However, the spatial autocorrelation revealed striking differences among these sub-populations. The JUO sub-population showed an aggregation of multilocus genotypes, which was not seen in the other sub-populations. This indicates the possible existence of family structure in the JUO sub-population, which is consistent with observations reported by Miyamoto *et al* (2000), but not

in the others. The data suggest that the spatial range of the autocorrelation for individuals of the JUO sub-population is about 25 m.

JUO and SAI are composed of trees with very similar sizes and these two sub-populations have very similar levels of genetic variation. However, the former showed a remarkable tendency for individuals displaying autocorrelation to be located close to each other, while the latter did not. In several species, the genetic structure in younger populations is reported to disappear in older populations as they are thinned (Hamrick *et al*, 1993; Epperson and Alvarez-Buylla, 1997; Parker *et al*, 2001). In the same way, the only sub-population that showed genetic structure was JUO; which is relatively young. However, the SAI sub-population, which is of similar age to JUO, did not show obvious genetic structure. Hence, we can speculate that in each population, spatial structures have been affected by various factors such as the composition of the mother population, micro-geographic features and climatic factors.

Heterogeneity of genotype frequencies in the three Imaichi sub-populations and gametic phase disequilibrium in all four sub-populations were observed. The gametic phase disequilibria of combined genotype frequencies between the *Pgi-1* locus and other loci at LAI, the *Aap* locus and other loci at SAI, and the *Aap* locus and other loci at BI are attributable to the heterogeneity

Figure 2 Distributions of diameter at breast height (d.b.h.) in the four *Alnus trabeculosa* sub-populations.

of genotype frequencies at these loci. However, in the JUO sub-population gametic phase disequilibria in 12 pairs of combinations were detected, despite the absence of heterogeneity of genotype frequencies. Gametic phase disequilibrium may be due to various causes, including the admixture of two or more populations that differ in genetic composition, residual disequilibrium persisting from gametic phase disequilibrium in the founder popu-

Figure 3 Spatial correlograms of coancestry coefficients (ρ_{ij}) for the four *Alnus trabeculosa* sub-populations. Dashed lines (95%) and solid lines (99%) represent upper and lower confidence limits for zero relationships.

lation, or selection favoring individuals with specific pairs of genotypes. Sometimes it is also caused by chance in small populations. Furthermore, gametic phase disequilibrium tends to be maintained through generations. If the cause of this kind of gametic phase disequilibrium is removed, it will decrease slowly, but persist for a long

Table 3 Genetic variation parameters (Pl , Na , Ne , H_o , H_e and F_{IS}) for the four *Alnus trabeculosa* sub-populations (means with standard errors in parentheses)

	Pl	Na	Ne	H_o	H_e	F_{IS}
LAI	61.5	2.5 (0.3)	1.60 (0.16)	0.225 (0.070)	0.203 (0.063)	-0.112 (0.034)
SAI	53.8	2.7 (0.4)	1.50 (0.14)	0.165 (0.054)	0.165 (0.055)	0.001 (0.049)
BI	61.5	2.5 (0.3)	1.89 (0.21)	0.287 (0.075)	0.265 (0.071)	-0.085 (0.037)
JUO	76.9	2.3 (0.2)	1.33 (0.07)	0.181 (0.044)	0.177 (0.042)	-0.018 (0.027)

Pl = percentage of polymorphic loci; Na = mean number of alleles per polymorphic locus; Ne = effective number of alleles per polymorphic locus; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = inbreeding coefficient.

time. On the other hand, heterogeneity of genotype frequencies may be caused by non-random mating or selection, but it will be cancelled by one random mating in these cases (Falconer, 1989; Hartl and Clark, 1997). Takahashi *et al* (2000) investigated the gametic phase disequilibrium in two populations of Japanese beech (*Fagus crenata*) that have different histories in terms of cutting. A population cut in the past showed gametic phase disequilibrium, whereas the other, uncut population showed no such disequilibrium. The cited authors point out that if the natural composition of the gene set is assumed to be the most highly adapted to a given environment, gametic phase disequilibrium implies reductions in the adaptability of populations in succeeding generations, which could be detrimental to conservation of important genetic resources. The heterogeneity and gametic phase disequilibrium data gathered for the JUO sub-population indicate that random mating may have occurred in its mother population, but there are distortions in the composition of the trees in the sub-population and/or its founder population. Moreover, asexual propagation may occur in JUO (Miyamoto *et al*, 2000), which may also contribute to the gametic phase disequilibrium.

SAI occupies a site that was reclaimed in 1972 (Natsuka, 1996), when the mother trees of the trees now forming this sub-population were probably cut down. Indeed, in SAI we detected two alleles (Shd^c , Shd^e) that were not found in LAI or BI, despite the proximity of these sub-populations to SAI, indicating that the mother trees of the SAI sub-population do not exist now or are located outside of AI and BI.

As we noted above, differences between the spatial structure and the probable cause of gametic phase disequilibrium were detected among the sub-populations in Imaichi and Juo. Another possible reason for these differences is that there are a lot more small sub-populations of the species around the sites we studied at Imaichi (Natsuka, 1996) than there are at JUO. So, we can speculate that fewer trees may have contributed genetically to the members of the JUO sub-population. In contrast with the case of the JUO sub-population, dispersal of pollen by the wind, or migration of seeds by wind and/or water from other sub-populations might homogenize the sub-populations genetically in Imaichi. Differences in the size of gene sources may be one of the reasons for the differences detected between the Imaichi and Juo sub-populations.

From the conservation perspective, all the populations of this species cannot be conserved. So, to conserve as much as possible of the species' genes and genotypes, information about their distribution within populations

is very helpful. In this study, the three sub-populations at Imaichi did not show any clear indications of family structure. These populations have fewer individuals and cover smaller areas than the JUO population. However, it seems highly likely that the populations at Imaichi are composed of genetically varied individuals. For gene and genotype conservation, conserving such populations should be valuable and effective. For the JUO sub-population, however, the existence of family structure indicates that conserving just a part of it will not be sufficient for maintaining its genetic variation. Doing so would risk losing its genetic variation through selection and genetic drift that will occur in the future. Moreover, some of the individuals in the SAI and JUO sub-populations are considered to host rare alleles. Conserving such individuals via *in situ* and/or *ex situ* conservation & would be important. To obtain further information concerning *A. trabeculosa* for establishing effective conservation measures, researching the spatial structure in other populations, and evaluation of the gene flow and asexual reproduction ability of this species would be needed.

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