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Patterns and levels of gene flow in *Rhododendron metternichii* var. *hondoense* revealed by microsatellite analysis

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Abstract

Parentage analysis was conducted to elucidate the patterns and levels of gene flow in *Rhododendron metternichii* Sieb. et Zucc. var. *hondoense* Nakai in a 150 × 70 m quadrat in Hiroshima Prefecture, western Japan. The population of *R. metternichii* occurred as three subpopulations at the study site. Seventy seedlings were randomly collected from each of three 10 × 10 m plots (S1, S2, and S3) on the forest floor of each subpopulation (A1, A2, and A3). Almost all parents (93.8%) of the 70 seedlings were unambiguously identified by using 12 pairs of microsatellite markers. Within the quadrat, adult trees less than 5 m from the centre of the seedling bank (plots S1, S2, and S3) produced large numbers of seedlings. The effects of tree height and distance from the seedling bank on the relative fertilities of adult trees were highly variable among subpopulations because of the differences in population structure near the seedling bank: neither distance nor tree height had any significant effect in subpopulation A1; distance from the seedling bank had a significant effect in subpopulation A2; and tree height had a significant effect in subpopulation A3. Although gene flow within each subpopulation was highly restricted to less than 25 m and gene flow among the three subpopulations was extremely small (0–2%), long-distance gene flow from outside the quadrat reached 50%. This long-distance gene flow may be caused by a combination of topographical and vegetational heterogeneity, differences in flowering phenology, and genetic substructuring within subpopulations.

Keywords: gene flow, microsatellite genetic marker, parentage analysis, pollen flow, population structure, seedling bank

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Introduction

The mating system of a plant population is the process by which parental genes are reassociated in the next generation. In seed plants, mating patterns within and among populations are decided by gene dispersal (seed and pollen movement) and establishment. Populations of long-lived woody species normally consist of a range of individuals of various sizes (or ages) and stages of maturity with overlapping generations, such as the combinations of mature adult trees and seedlings established on special microhabitats known as 'safe sites' (Harmon & Franklin 1989; Shibata & Nakashizuka 1995; Kameyama *et al*. 1999). Spatial and temporal dynamics in plant populations result in the development of complex genetic structures with overlapping generations (Alvarez-Buylla *et al*. 1996; Epperson & Alvarez-Buylla 1997; Kitamura *et al*. 1997). Patterns and levels of gene flow into seedling banks are, therefore, critical components of the ecological and evolutionary genetics of plant populations.

The conventional approach to quantifying gene flow has been to transform measures of population structure (Wright 1969) into indirect estimates of the average number of migrants exchanged per generation among a set of populations (Neigel 1997), most commonly using an island model. Because this model assumes that all populations are equal sources of migrants and yields estimates that are stable, it does not reflect variations in gene exchange among populations or changes in dispersal processes (Sork *et al*. 1999).

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Direct estimation of contemporary gene flow is possible by comparing genotypes of potential parents with genotypes of seeds collected from maternal trees (paternity analysis: Godt & Hamrick 1993; Broyles *et al*. 1994; Schnabel & Hamrick 1995) or of naturally established seedlings or juveniles (parentage analysis: Meagher & Thompson 1987; Schnabel & Hamrick 1995). Such exclusion analysis has shown great progress with the emergence of microsatellite genetic markers characterized by their high polymorphism, codominant alleles at a single locus, and the use of polymerase chain reaction (PCR) amplification of DNA (Bruford & Wayne 1993; Queller *et al*. 1993; Ashley & Dow 1994). High polymorphism and codominant alleles of microsatellites permit exclusion in most cases of potential pollen donors (paternity analysis: Dow & Ashley 1998; Streiff *et al*. 1999; Kameyama *et al*. 2000; Miyazaki & Isagi 2000) or candidate parents (parentage analysis: Dow & Ashley 1996; Isagi *et al*. 2000), but only by the use of several loci. However, there are still very few studies using microsatellite genetic markers for the analysis of parentage in natural plant populations.

Rhododendron metternichii Sieb. et Zucc. var. *hondoense* Nakai is an evergreen shrub distributed mainly in the mountainous regions of western Japan. Pink flowers bloom in the middle of May and are pollinated mainly by bumblebees such as *Bombus diversus diversus* Smith and *B. ardens ardens* Smith. The population of *R. metternichii* is often composed of subpopulations along the northern slopes of steep ridges, owing to its special demands for habitat.

The aim of this study was to elucidate patterns and levels of gene flow within and among three subpopulations of *R. metternichii*. We used 12 pairs of microsatellite markers for parentage analysis of seedlings randomly sampled from seedling banks on the forest floor of the subpopulations. By identifying the parents of each seedling, we elucidated: (i) the amount of gene exchange among the subpopulations; (ii) the effect of tree height and distance on relative fertilities of adult trees; and (iii) the patterns of pollen flow within the subpopulations.

Materials and methods

Field investigations

The study site was located at Mt. Kamakuraji, Hiroshima Prefecture, Japan (lat. 34°32′ N, long. 132°41′ E, 613 m a.s.l.). The mean annual precipitation and air temperature from 1988 to 1997 in Higashi–Hiroshima City (13 km south of Mt. Kamakuraji, elevation 224 m a.s.l.) were 1512.2 mm and 12.1 °C. We set a 150 × 70 m quadrat near the top of the mountain. It contained three stripe-shaped subpopulations of *Rhododendron metternichii* (A1, A2, and A3 from west to east) along the northern slopes of branch ridges off the main ridge (Fig. 1). Each subpopulation continued intermittently along the branch ridges beyond the northern boundary of the quadrat, and another subpopulation grew 20 m east of the quadrat. The southern and western sides of the quadrat were isolated

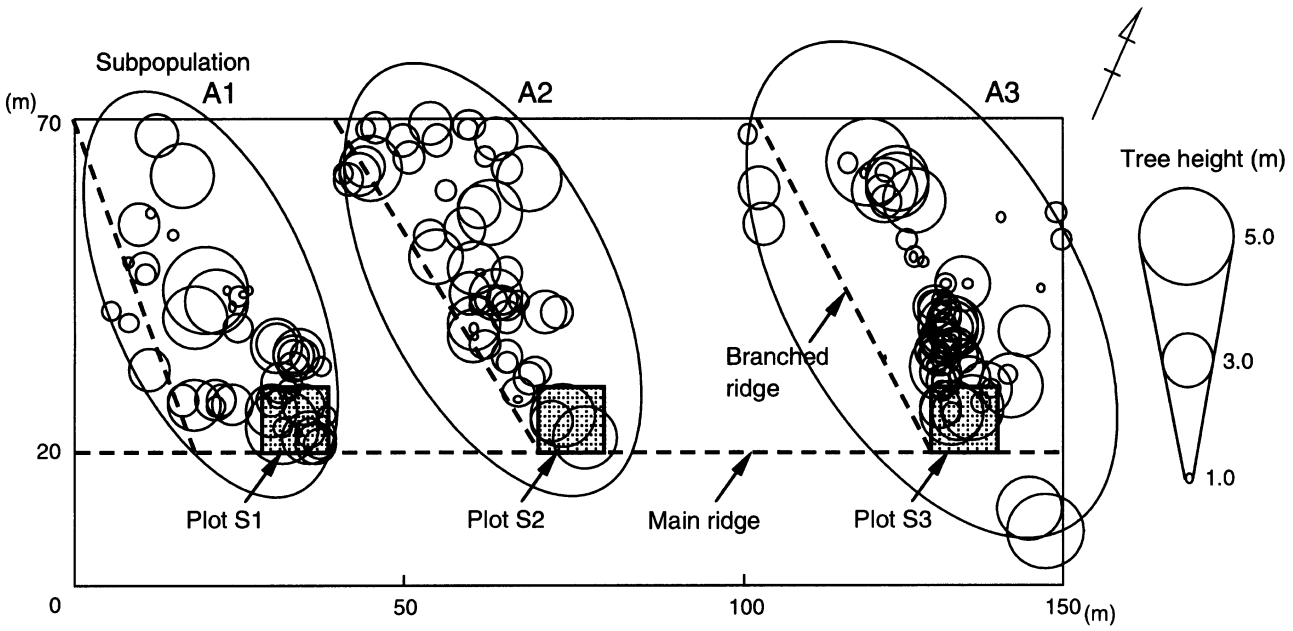

Fig. 1 Map of study site. Three stripe-shaped subpopulations of *Rhododendron metternichii* (A1, A2, A3) grew along the northern slopes of branch ridges off the main ridge. Seventy seedlings were randomly collected from small plots (S1, S2, S3) within each subpopulation for parentage analysis. Each subpopulation continued intermittently along the branch ridges beyond the northern boundary of the quadrat. The southern and western sides of the quadrat were isolated by several hundred meters from other *R. metternichii* populations.

Table 1 Numbers of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), estimated exclusion probabilities, and null allele frequencies at 12 microsatellite loci in 174 adult trees of *Rhododendron metternichii* var. *hondoense*

Locus	Number of alleles	H_O	H_E	Exclusion probability		Null allele frequency
				first parents	second parents	
RM1D1	10	0.759	0.781	0.402	0.581	0.0128
RM1D5	16	0.839	0.859	0.555	0.716	0.0116
RM1D9	9	0.452	0.740	0.333	0.509	0.2447
RM1D12	33	0.942	0.932	0.753	0.859	−0.0076
RM2D2	10	0.810	0.840	0.508	0.677	0.0168
RM2D6	16	0.750	0.894	0.646	0.785	0.0831
RM3D1	11	0.614	0.767	0.385	0.566	0.1097
RM3D2	21	0.648	0.899	0.654	0.791	0.1605
RM3D4	7	0.713	0.741	0.344	0.524	0.0171
RM7D9	13	0.775	0.848	0.530	0.695	0.0460
RM9D1	12	0.770	0.775	0.401	0.580	0.0010
RM9D6	6	0.776	0.784	0.389	0.568	0.0021

Exclusion probabilities and null allele frequencies were calculated with CERVUS 1.0 (Marshall *et al*. 1998).

by several hundred meters from other *R. metternichii* populations.

The sizes and positions of all adult trees within the quadrat were measured in 1998. We found 174 adult trees within the quadrat: 49 in subpopulation A1, 50 in subpopulation A2, and 75 in subpopulation A3. Adult trees of *R. metternichii* were defined as individuals if they were ≥50 cm in height, based on our previous study of the flowering history of 148 individuals (Kameyama *et al*. 1999). Flowering history was judged by presence of inflorescences that remain on trees several years after flowering. In our previous study, 36 of the 37 plants >150 cm in height had flowered, 19 of the 33 plants 50–150 cm in height had flowered, and 78 plants <50 cm in height had never flowered. Based on these results, we defined 'seedlings' as individuals <50 cm in height and 'adults' as individuals ≥50 cm in height. Although there are no data on the age of trees, several tens of years may be required to reach flowering size under natural conditions.

Three 10 × 10 m quadrats (plots S1, S2, and S3 from west to east) were set on the forest floor of subpopulations A1, A2, and A3 (Fig. 1). Seventy seedlings <50 cm in height were randomly collected from each plot for parentage analysis.

DNA extraction and electrophoresis

We extracted DNA from the leaves of 174 adult trees and 210 seedlings (70 seedlings from each of S1, S2, and S3). Genotypes of each DNA sample were scored by using 12 pairs of microsatellite PCR primers. Six of the 12 were developed by Naito *et al*. (1998): RM2D2, RM3D1, RM3D2, RM3D4, RM9D1, and RM9D6. We designed six additional primers — RM1D1, RM1D5, RM1D9, RM1D12, RM2D6, and RM7D9 — for the clones containing microsatellite loci isolated by K. Naito, N. Iiyama and Y. Isagi (unpublished data). PCR amplifications were performed with a thermal cycler (GeneAmp PCR System 9600, ABI). The size of PCR products was determined by automated fluorescent scanning detection with an ABI377 autosequencer and GENESCAN analysis software (ABI).

Parentage analysis

Parentage was assigned by comparing genotypes of candidate parents and seedlings (Dow & Ashley 1996; Isagi *et al*. 2000). Adults that had no matching haplotypes with seedlings were excluded. If the combination of only two haplotypes of adult trees could explain the genotype of a given seedling (exact matches), these were presumed to be the parents. In cases where more than two combinations of adult trees could explain the genotypes of a given seedling (multiple matches), unambiguous parent pairs could not be identified.

It is common for microsatellites to have nonamplifying alleles (null alleles). This often occurs because of mutations in one or both primer binding sites, sufficient to prevent effective amplification of the microsatellite allele (Pemberton *et al*. 1995). In parentage analysis, the possible existence of null alleles must be taken into account because it could cause mismatches of genotypes among the true parent–offspring pairs. We estimated null allele frequencies with the computer program CERVUS 1.0 (Marshall *et al*. 1998), which is based on the estimation algorithm of Summers & Amos (1997). High levels of null allele frequencies were observed in several microsatellite loci (Table 1). To prevent the exclusion of true candidate

parents, individuals that appeared to be homozygous at seven loci (RM1D9, RM2D2, RM2D6, RM3D1, RM3D2, RM3D4, and RM7D9), which had null allele frequencies of >0.015, were assumed to be heterozygous for the null allele. This method may overestimate the number of matches within the stand but would not exclude any true parent (Dow & Ashley 1996). The average null allele frequency of the remaining five loci (RM1D1, RM1D5, RM1D12, RM9D1, and RM9D6) was 0.00398, which might be negligible. Although mutations in microsatellite sequences could cause true parent–offspring mismatches, the mutation rate for microsatellites has been estimated to be between 10^{-4} and 5×10^{-6} per locus per generation (e.g. Dallas 1992; Edwards *et al*. 1992); this indicates the negligible effect of microsatellite mutations for parentage analysis in ecological applications.

To estimate the amount of gene flow, it is important to evaluate the amount of cryptic gene flow — the probability that an adult tree within the quadrat matched an unrelated seedling. Exclusion probabilities of first and second parents calculated with CERVUS are shown in Table 1. The total exclusion probabilities calculated from the exclusion probabilities of each locus were 0.999817 for the first parent and 0.999999 for the second parent. Therefore, the probability of correctly excluding all unrelated adults (174 trees) within the quadrat was $0.999817^{174} = 0.968598$ for the first parent, and $0.999999^{174} = 0.999762$ for the second parent. If we analysed 100 seedlings, the amount of cryptic gene flow could be estimated as $100 \times (1-0.968598) = 3.140$ for the first parent, and $100 \times (1-0.999762) = 0.024$ for the second parent.

Results

Gene flow among subpopulations

The 12 microsatellite markers are highly variable, having 6–33 alleles per locus (Table 1), with an average of 13.7. Observed heterozygosities ranged from 0.452 to 0.942. These highly variable microsatellites made it possible to exclude almost all unrelated candidate parents by exclusion analysis. Of the 70 seedlings that were randomly collected from each plot, multiple matches were observed for six seedlings in plot S1, three in S2, and four in S3. These 13 seedlings, whose parents could not be identified, were excluded from later analysis. Thus, the numbers of analysed seedlings were 64 in plot S1, 67 in S2, and 66 in S3.

Of the 64 seedlings in plot S1, 15 had both first and second parents within the quadrat, 34 had only one parent within the quadrat, and 15 had both parents outside the quadrat (Table 2a). Although these results indicate that 64 of the 128 haplotypes (50.0%) within plot S1 were derived from outside the quadrat, actual gene flow from outside the quadrat was $[(34.02 + 17.02 + 17.02)/128] \times 100 = 53.17\%$ because of cryptic gene flow. Similarly, while gene flow from other subpopulations within the quadrat seems to be $(1/128) \times 100 = 0.78\%$ from subpopulation A2 and $(2/128) \times 100 = 1.56\%$ from subpopulation A3, actual gene flow was $\{[31.21 \times (1/34)]/128\} \times 100 = 0.72\%$ from subpopulation A2 and $\{[31.21 \times (2/34)]/128\} \times 100 = 1.43\%$ from subpopulation A3. Although cryptic gene flow should be considered to accurately estimate the rate of gene flow, these calculations and explanations are quite complicated for comparing the difference in gene flow at the individual level. Moreover, the actual rate of cryptic gene flow was relatively low in this study. Thus, we did not consider the effect of cryptic gene flow in the later comparison.

Of the 67 seedlings in plot S2, 31 had both parents within the quadrat, 28 had only one parent within the quadrat, and eight had both parents outside the quadrat (Table 2b). Gene flow from outside the quadrat was $(44/134) \times 100 = 32.8\%$. The gene flows from subpopulations A1 and A3 were 1.49% and 0.75%, respectively.

Of the 66 seedlings collected from plot S3, 37 had both parents within the quadrat, 22 had only one parent within the quadrat, and seven had both parents outside the quadrat (Table 2c). Gene flow from outside the quadrat was $(36/132) \times 100 = 27.3\%$. There was no gene flow from subpopulations A1 and A2. The numbers of seedlings produced by self-pollination were two (3.1%) in plot S1, four (6.0%) in plot S2, and four (6.1%) in plot S3, with an average of 5.1%.

Gene flow within subpopulations: the effect of distance and tree height

Because gene flow among subpopulations was quite small (Table 2), the effects of ecological factors — distance from seedling bank and tree height of adults — on gene flow were analysed in detail within each subpopulation. The numbers of parents analysed were 15 pairs of adults in subpopulation A1 (30 parents in total), 29 pairs of adults in subpopulation A2 (58 parents in total), and 37 pairs of adults in subpopulation A3 (74 parents in total).

The numbers of adult trees of each distance class from the centre of the seedling bank and the numbers of seedlings produced by adults of each distance class were compared within each subpopulation (Fig. 2). Adult trees adjacent to the seedling bank (<5 m from it) produced very large numbers of seedlings. A distance class histogram of numbers of adults and numbers of seedlings produced showed significant differences (Mann–Whitney *U*-test: $P < 0.0001$ in subpopulations A1, A2, and A3) (Fig. 2). Moreover, the numbers of seedlings produced by adults of each distance class were

Table 2a Gene flow into plot S1

		Source of gene flow				
	first/second parents	A1	A2	A3	outside the quadrat	Total
Seedlings having parent pairs within the quadrat	first	15 (2)	0	0	—	15 (13.77)
	second	15 (2)	0	0	—	15 (14.97)
Seedlings having first parents within the quadrat	first	31	1	2	—	34 (31.21)
	second	—	—	—	34	34 (34.02)
Seedlings having no parents within the quadrat	first	—	—	—	15	15 (17.02)
	second	—	—	—	15	15 (17.02)
Total		61	1	2	64	128

Table 2b Gene flow into plot S2

		Source of gene flow				
	first/second parents	A1	A2	A3	outside the quadrat	Total
Seedlings having parent pairs within the quadrat	first	0	31 (4)	0	—	31 (28.79)
	second	1	29 (4)	1	—	31 (30.97)
Seedlings having first parents within the quadrat	first	1	27	0	—	28 (26.00)
	second	—	—	—	28	28 (28.02)
Seedlings having no parents within the quadrat	first	—	—	—	8	8 (10.11)
	second	—	—	—	8	8 (10.11)
Total		2	87	1	44	134

Table 2c Gene flow into plot S3

		Source of gene flow				
	first/second parents	A1	A2	A3	outside the quadrat	Total
Seedlings having parent pairs within the quadrat	first	0	0	37 (4)	—	37 (34.40)
	second	0	0	37 (4)	—	37 (36.97)
Seedlings having first parents within the quadrat	first	0	0	22	—	22 (20.45)
	second	—	—	—	22	22 (22.02)
Seedlings having no parents within the quadrat	first	—	—	—	7	7 (9.08)
	second	—	—	—	7	7 (9.08)
Total		0	0	96	36	132

The number in parentheses under 'Source of gene flow' is the number of seedlings that could be explained by the genome of 1 parent (self-pollination). The number in parentheses under 'Total gene flow' is the number of seedlings corrected for the value of cryptic gene flow. Because the numbers of seedlings whose parents were unambiguously identified were 64 in plot S1, 67 in plot S2, and 66 in plot S3, the totals sum up to 128 in plot S1, 134 in plot S2, and 132 in plot S3.

significantly different among subpopulations (one-way factorial ANOVA; $F_{2,159} = 8.876$, $P = 0.0002$) because of a relatively long-distance gene flow in subpopulation A2 compared with the other two subpopulations (Scheffé's test: A1 vs. A2, $P = 0.0055$; A2 vs. A3, $P = 0.0010$; A1 vs. A3, $P = 0.9512$).

The effect of tree height on the number of offspring produced was different in each subpopulation (Fig. 3). In

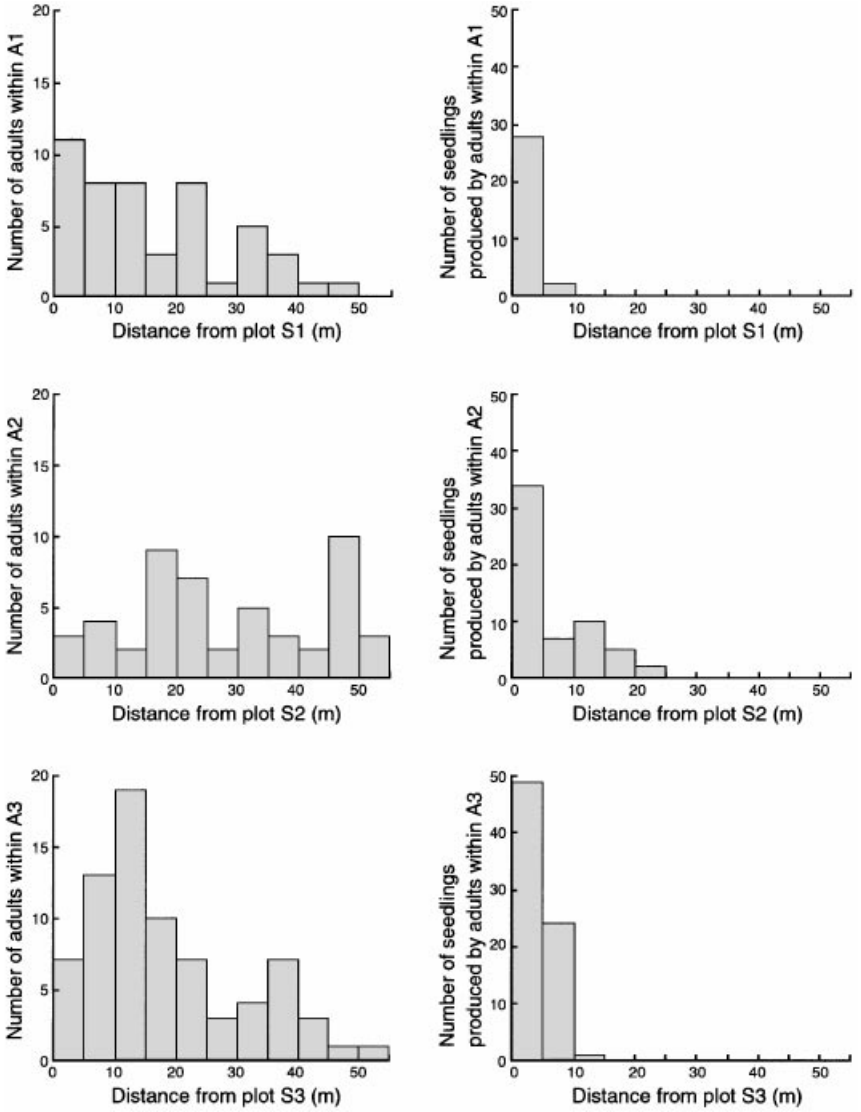

Fig. 2 Numbers of adult trees of each distance class from the centre of the seedling bank (left) and numbers of seedlings produced by adults of each distance class (right) in subpopulations A1 (upper), A2 (middle), and A3 (lower).

A1, there were no significant differences between the height class distribution of adults and numbers of seedlings produced by adults of each height class (Mann–Whitney *U*-test; $P = 0.1345$). In A2 and A3, taller adults produced more seedlings than smaller ones (Mann–Whitney *U*-test; $P < 0.0001$). The numbers of seedlings produced by adults of each height class were significantly different among subpopulations (one-way factorial ANOVA; $F_{2,159} = 23.966$, $P < 0.0001$), but the difference between A1 and A2 was relatively small (Scheffé's test: A1 vs. A2, $P = 0.0644$; A2 vs. A3, $P < 0.0001$; A3 vs. A1, $P < 0.0001$).

The fertilities of adult trees, defined as the numbers of seedlings produced by adults, were compared within and among subpopulations in relation to distance from the seedling bank and tree height (Fig. 4). In subpopulation A1, nine trees were parents of the seedlings in plot S1 (Fig. 4a). These nine trees were all adjacent to the seedling bank, <10 m distant, and the fertilities of the contributing adults were similar. In subpopulation A2, adult trees relatively far from the seedling bank contributed as parents (Fig. 4b). However, the fertilities of the adult trees were extremely high for adjacent parents. In subpopulation A3, 13 trees <10.6 m from the seedling bank contributed as parents of seedlings (Fig. 4c). Of these 13 trees, only four, about 3 m or more in height, had extremely high fertilities, producing 10 or more seedlings.

Multiple regression analyses were conducted to reveal the effects of distance from the seedling bank and tree height on the fertilities of parents (Table 3). The dependent variable was the relative fertilities of adult trees that

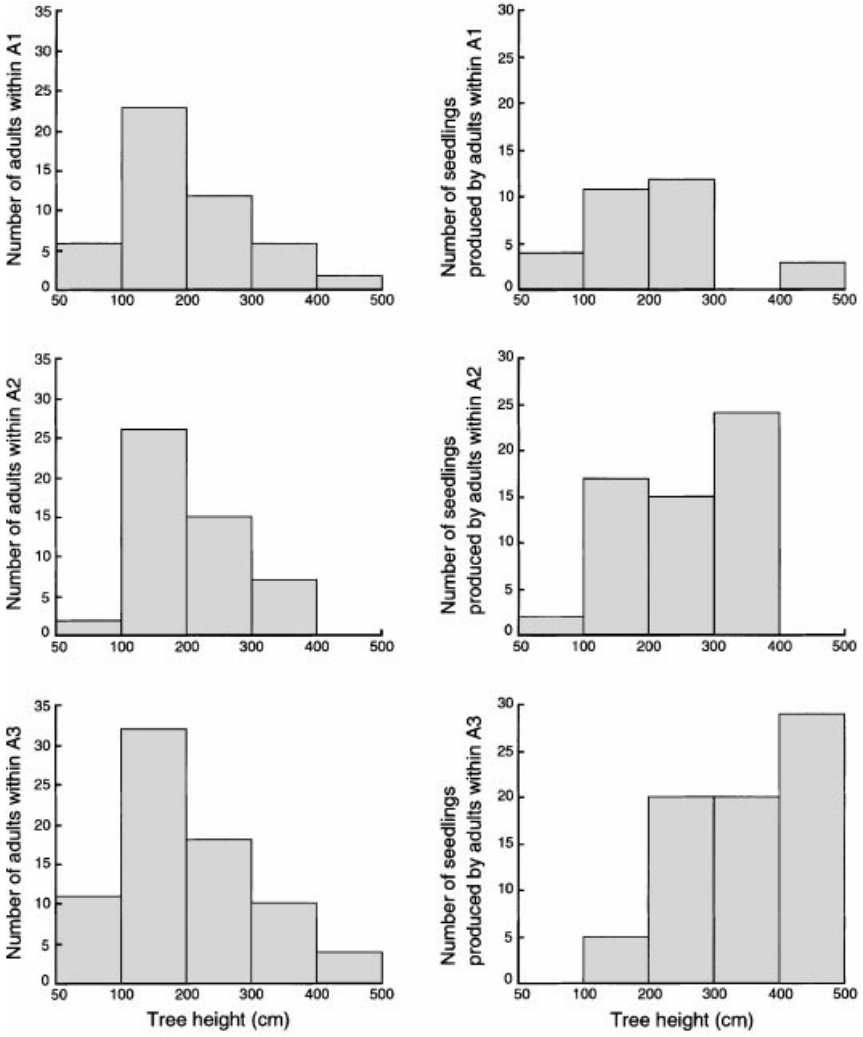

Fig. 3 Height class distribution (left) and numbers of seedlings produced by adults of each height class (right) in subpopulations A1 (upper), A2 (middle), and A3 (lower).

contributed as parents of the seedlings, and the independent variables were tree height and the natural logarithm of the distance from the seedling bank: ln(distance from seedling bank). The effects of these ecological factors were highly different in each subpopulation. The relative fertility of adults within subpopulation A1 was not associated with either tree height or ln(distance from seedling bank). In A2, a negative association was found between ln(distance from seedling bank) and the relative fertility of adults ($P = 0.0021$). It played a major role in predicting adult fertility ($R^2 = 0.709$). On the other hand, the relative fertility of the adults in A3 was positively associated with tree height ($P = 0.0015$), and a relatively low negative association was found for ln(distance from seedling bank) ($P = 0.0902$), in which 72.6% of the total variation in adult fertility was explained. For the three subpopulations in total, tree height was positively associated ($P = 0.0040$) and ln(distance from seedling bank) was negatively associated ($P = 0.0039$) with the relative fertility of adult trees, explaining 40.6% of total variance.

Pollen flow within subpopulations

The distance of seed dispersal could not be estimated because parentage analysis gave no information about which of the parent pair was the seed parent or the pollen donor. The distance of pollen flow, however, could be estimated from the positions of parent pairs. Seedlings whose parent pairs were unambiguously identified were used for the estimation of pollen flow within subpopulations: 15 seedlings in subpopulation A1; 29 in A2; and 37 in A3 (Table 2).

The distance class distribution of pollen flow estimated by parentage analysis was compared with both nearest

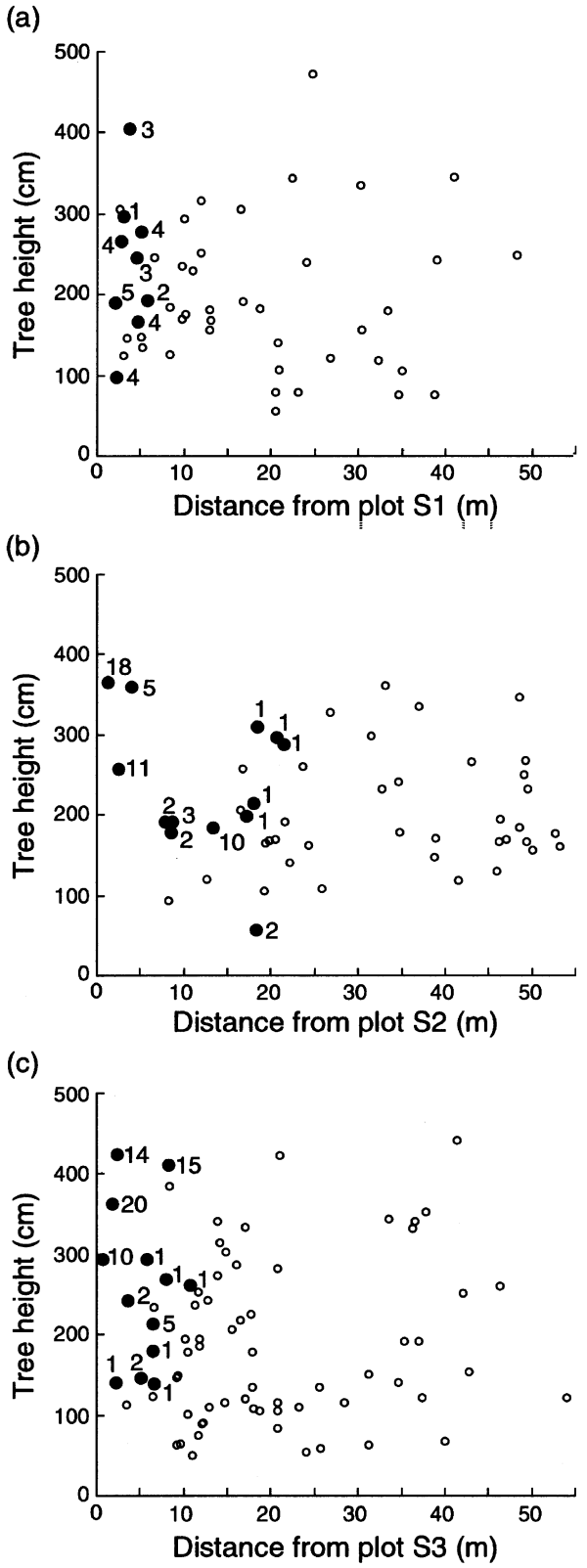

Fig. 4 Fertilities of adult trees in relation to distance from seedling bank and tree height: (a) within A1, (b) A2, (c) A3. Adult trees that produced seedlings are shown by closed circles. Figures beside the circles show the numbers of seedlings produced by each adult.

Table 3 Results of multiple regression analysis, in which the dependent variable is the relative fertility of the adult trees (%) that contributed as parents of the seedlings, and the independent variables are adult tree height and the natural logarithm of the distance from the centre of the seedling bank

Dependent variables	R^2 (adjusted)	F	P	Independent variables	Standardized coefficient	T	P
Relative fertility of adults within A1	0.213	0.814	0.4867	Constant	18.053	3.207	0.0184
				Tree height	−0.286	−0.767	0.4721
				ln(distance from S1)	−0.301	−0.809	0.4495
Relative fertility of adults within A2	0.709	12.173	0.0021	Constant	26.614	3.389	0.0069
				Tree height	−0.020	−0.103	0.9201
				ln(distance from S2)	−0.851	−4.446	0.0012
Relative fertility of adults within A3	0.726	13.223	0.0016	Constant	−5.278	−0.966	0.3570
				Tree height	0.732	4.333	0.0015
				ln(distance from S3)	−0.317	−1.875	0.0902
Relative fertility of adults within A1, A2, and A3	0.406	12.617	<0.0001	Constant	1.608	0.975	0.3368
				Tree height	0.422	3.100	0.0040
				ln(distance from seedling bank)	−0.423	−3.113	0.0039

F, *F* statistic = (mean square of regression)/(mean square of residual); *P*, *P* value (or significant level); *T*, *T* statistic = (standardized coefficient)/(standard error).

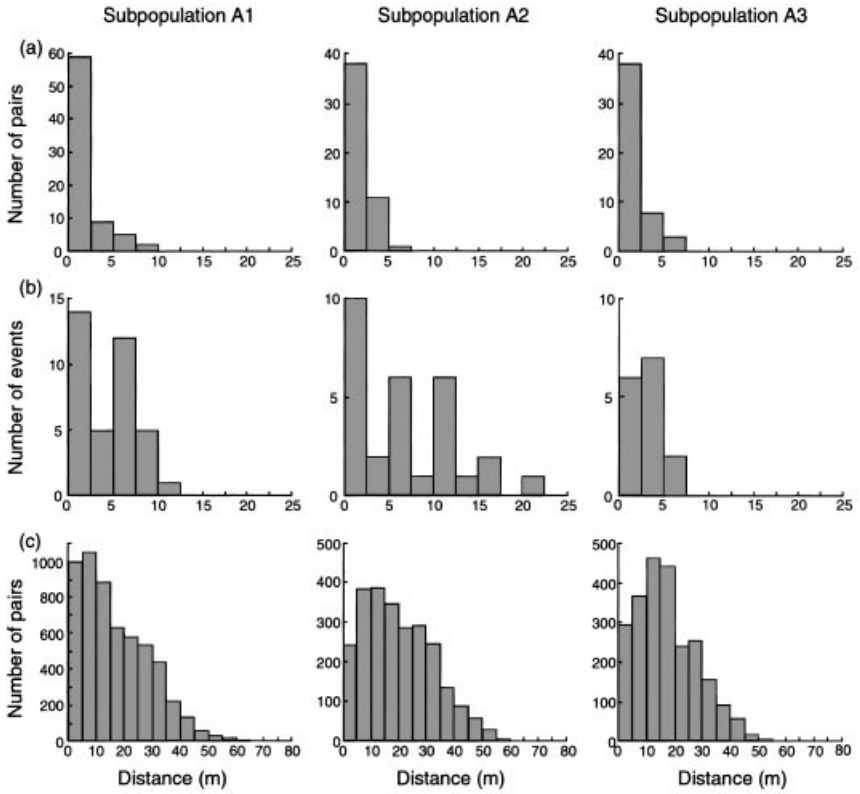

Fig. 5 (a) Distance class distributions within each subpopulation of nearest neighbours for each adult, (b) pollen movement inferred from parentage analysis, and (c) random pairs of adult trees.

neighbours for each adult and random pairs of adult trees within each subpopulation (Fig. 5). In all subpopulations, the distance of pollen flow was significantly longer than the distance to the nearest neighbours of adult trees (Mann–Whitney *U*-test: $P = 0.0416$ in subpopulation A1; $P < 0.0001$ in A2 and A3), and significantly smaller than that between random pairs of adult trees (Mann–Whitney *U*-test: $P < 0.0001$ in A1, A2, and A3). Distance class distributions of pollen flow were also significantly different among subpopulations (one-way factorial ANOVA; $F_{2,78} = 5.697$, $P = 0.0049$) because of relatively long-distance pollen flow in subpopulation A2 (Scheffé's test: A1 vs. A2, $P = 0.0105$; A2 vs. A3, $P = 0.0447$; A1 vs. A3, $P = 0.5083$).

Discussion

Parentage analysis of seedlings by the use of microsatellite markers enables a wide range of analyses of population structure. Most studies of gene flow in natural plant populations have been limited to the analysis of pollen movement (paternity analysis: Godt & Hamrick 1993; Broyles *et al*. 1994; Schnabel & Hamrick 1995; Dow & Ashley 1998; Streiff *et al*. 1999; Kameyama *et al*. 2000; Miyazaki & Isagi 2000). Gene flow, however, is accomplished by both pollen and seed dispersal, with the establishment of genes after dispersal (e.g. Endler 1977). Thus, parentage analysis of established seedlings or juveniles would provide the realized reproductive success of adult trees or gene flow better than standard paternity analysis, because most of the seedlings and juveniles have already successfully established (parentage analysis: Meagher & Thompson 1987; Schnabel & Hamrick 1995; Dow & Ashley 1996; Isagi *et al*. 2000).

Gene flow within subpopulations

In this study, we primarily analysed gene flow within subpopulations. Distance from the seedling bank was the primary force deciding the patterns and levels of gene flow within subpopulations. In all three subpopulations, adult trees <5 m from the centre of the seedling bank produced extremely large numbers of seedlings, and no adults >25 m from it contributed as parents of the seedlings (Fig. 2). Distance class distributions of seedling numbers produced by adult trees, however, were significantly different among subpopulations; longer-distance gene flow was found in subpopulation A2 than in the other two subpopulations.

More seedlings were produced by taller than shorter adults within subpopulations A2 and A3 but not within A1 (Fig. 3). The numbers of seedlings produced by adults of each height class were significantly different among

subpopulations; extremely large numbers of seedlings were produced by taller adults in subpopulation A3.

A few comparable studies analysed the effects of plant size and distance on adult fertility based on parentage analysis of naturally established seedlings (plant size: Meagher & Thompson 1987; plant size and distance: Schnabel & Hamrick 1995). Meagher & Thompson (1987) studied genealogical relationships of the dioecious plant species *Chamaelirium luteum* using 11 enzyme markers to estimate genetic likelihoods. They found a negative correlation between reproductive success and rosette and inflorescence size. This result, however, does not deny the general finding in plant populations that size is a reflection of overall vigor and that bigger plants produce more and probably better seed (Harper & White 1974; Solbrig 1981; Cook & Lyons 1983). This is because the observed correlation in *C. luteum* involved only plants that flowered; larger plants in the overall population (Meagher & Antonovics 1982). Schnabel & Hamrick (1995) studied pollen gene flow of the subdioecious tree species *Gleditsia triacanthos* (Leguminosae) in two populations in eastern Kansas, USA. Within the populations, a multiple regression model showed that the maximum-likelihood estimate of fertility for a given male on a given maternal tree was negatively associated with distance between mates and positively associated with male size. However, the multiple regression model explained <16% of the total variation in male fertility.

The results of our study indicate that the effects of distance and plant size on adult fertility would vary with differences in plant population structure. Both distance and tree height had no significant effect on adult fertility if the height of trees within 10 m was relatively small — <~300 cm in *Rhododendron metternichii* (Fig. 4a, Table 3). If the number of candidate parents within 5–10 m is limited, relatively distant individuals could produce seedlings, their relative fertilities decreasing exponentially with distance (Fig. 4b, Table 3). Plant size had a significant positive effect on adult fertility if there were various sizes of adult trees (100–450 cm in height) within 10 m from the seedling bank (Fig. 4c, Table 3).

There are different views of the patterns and levels of gene flow (reviewed in Ellstrand 1992). At one extreme, gene flow is viewed as highly restricted (Levin 1981); at the other, extensive (Muona 1990). A third view is that gene flow in plants is idiosyncratic, ranging from very low to very high, and varying among species, populations, individual plants, and even over a season (Ellstrand & Marshall 1985; Hamrick 1987; Slatkin 1987). Although gene flow within each of our subpopulations of *R. metternichii* was highly restricted <25 m from the seedling bank, it is notable that the patterns of gene flow vary among subpopulations because of differences in population structure near the seedling bank.

Pollen flow within subpopulations

Pollen flow within subpopulations was highly restricted. The distribution of pollen flow distance within a subpopulation was much smaller than that of random pairs of adult trees, but larger than that of nearest neighbours of adult trees (Fig. 5). This restricted pollen movement was observed in other studies (Levin & Kerster 1974; Willson 1983; Hamrick 1987). Relatively long-distance pollen flow, however, was found in subpopulation A2, where there were fewer adult trees within 5–10 m from the seedling bank than in the other two subpopulations (Fig. 4b). It is reported that gene flow rate is greater in years of lower flower production than in most years, because dense flowers reduce pollinator foraging distance and increase nearby matings or self-pollination (Handel 1983; Fenster 1991; Godt & Hamrick 1993; Schnabel & Hamrick 1995). Our result is consistent with those conclusions: the presence of a small number of adult trees induced long-distance pollen flow.

Long-distance gene flow

Two important questions should be answered to explain the patterns and levels of gene flow in *R. metternichii* populations. This first is why gene flow from outside the quadrat reached 50% while most gene flow within subpopulations was highly restricted. The second is why gene flow among subpopulations was extremely small (0–2%) in spite of long-distance gene flow from outside the quadrat.

To answer the first question, long-distance gene flow could occur by pollen movement. Recent studies employing microsatellite markers for paternity analysis reported extensive pollen movement in wind-pollinated trees — *Quercus* spp. (Dow & Ashley 1998; Streiff *et al*. 1999) — and insect-pollinated trees — *Pithecellobium elegans* (Chase *et al*. 1996) and *Magnolia obovata*. (Isagi *et al*. 2000). Dow & Ashley (1998) suggested the existence of some mechanism in *Q. macrocarpa* that allows female flowers to 'choose' pollen from distant sources over more dense pollen produced by nearby trees. Streiff *et al*. (1999) concluded that effective pollen dispersal of *Q. petraea* (Matt.) Liebl and *Q. robur* L. could be a combination of two processes: (i) local dispersion; and (ii) long-distance transport. In a study of insect-pollinated tropical trees (*Tachigali versicolor* and *Platypodium elegans*), Hamrick & Murawski (1990) observed extensive (>20%) pollen movement of >750 m. They concluded that the breeding structure of these tropical species appeared to be a mixture of near-neighbour (30–50%) and long-distance (10–25%) pollen movement. Our previous study of *R. metternichii* also showed 20–30% of pollen flow from outside the quadrat, >40 m from maternal trees, although much pollen movement occurred between adjacent trees (Kameyama *et al*. 2000). The

combination of near-neighbour and long-distance pollen flow could be attributed to variations in phenology or in flowering behaviour between adult trees. In the case of episodic flowering without synchronization in a population, only limited combinations of adult trees can produce offspring. In *R. metternichii*, directional pollen flow from late-blooming flowers to early ones could also occur because of dichogamy, in which anthers mature earlier than pistils (Kameyama *et al*. 2000). Cumulative pollen flow from the tail of the leptokurtic curve of pollen dispersal and inbreeding depression may be important, too. Leptokurtic dispersal curves are characterized by tails that contain more long-distance dispersals than the tails of an equivalent normal distribution (Hamrick 1987). This indicates that the cumulative contribution of long-distance pollen flow could become significant. If populations were genetically structured and inbreeding depression occurred, some kind of natural selection could decrease the contributions of neighbouring trees (Waser 1993; Dow & Ashley 1998).

Because we could not distinguish between pollen donors and seed parents by the parentage analysis of seedlings, it is impossible to estimate the relative contributions of pollen and seed dispersal to long-distance gene flow from outside the quadrat. If most long-distance gene flow in *R. metternichii* could be attributed to pollen flow, the second parents of seedlings with only first parents within the quadrat might be pollen donors: 34 seedlings in subpopulation A1, 28 in A2, and 22 in A3 (Table 2). However, the second parents of seedlings with no parents within the quadrat would be maternal trees. This indicates that the minimum estimate of seed dispersal from outside the quadrat is $(15/128) \times 100 = 11.7\%$ in subpopulation A1, $(8/134) \times 100 = 6.0\%$ in A2, and $(7/132) \times 100 = 5.3\%$ in A3 (Table 2). The seed size of *R. metternichii* is 1.0–2.0 mm in length. These seeds could be dispersed several tens of meters by wind.

Each subpopulation of *R. metternichii* continued intermittently along the branch ridges beyond the northern boundary of the quadrat (Fig. 1). To answer our second question, the contradictions between extensive gene flow from outside the quadrat and restricted gene flow among subpopulations imply directional gene flow along each subpopulation because of some kind of barrier between them. Steep ridges covered by sparse vegetation lie to the west of each subpopulation, and developed vegetation lies to the east. Because the vegetation cover over each subpopulation was relatively thin, pollinators may move more frequently along each subpopulation than between subpopulations. The steep ridge to the west of each subpopulation may prevent seed dispersal between subpopulations. Physical and biological differences between subpopulations should be considered to reveal patterns and levels of gene flow at the larger scale.

Gene flow within each subpopulation of *R. metternichii* was highly restricted and showed an idiosyncratic pattern. This idiosyncratic gene flow should be due to differences in population structure near the seedling bank. The patterns and levels of long-distance gene flow, however, may be determined by combinations of topographic and vegetational heterogeneity, differences in flowering phenology, and genetic substructuring within subpopulations.

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