Analysis of Mating Patterns and Spatial Genetic Structure in *Acer mono* Using Microsatellite Genetic Markers in Conserved and Fragmented Forests

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Introduction

Acer mono Maxim. (Aceraceae) is a deciduous canopy tree widely distributed in eastern Asia, and one of the major components in deciduous cool-temperate forests of Japan. The genus *Acer* exhibits great variability in its reproductive systems, including monoecy, androdioecy, and dioecy, and *A. mono* shows heterodichogamy, as do most *Acer* species (de Jong 1994). Heterodichogamy, by definition, refers to a breeding system that involves two types of bisexual individuals (protandrous and protogynous individuals) in a population and has been reported from 11 families and 17 genera of flowering plants, including *Grayia* (Chenopodiaceae), *Juglans* (Juglandaceae), and *Acer* (Aceraceae). In heterodichogamous taxa in genus Acer, male individuals occasionally occur at a low frequency (heterodichogamous androdioecy, Gleiser & Verdú 2005).

Several previous studies addressing sex expression in these taxa have revealed the reciprocal and synchronous nature of two flowering types, and heterodichogamy is considered an effective mechanism to avoid selfing and to ensure outcrossing by promoting between-type mating (Gleeson 1982, Pendleton *et al*. 1988, Asai 2002, Sato 2002, Kimura *et al*. 2003, Bai *et al*. 2006). However, no empirical studies have explored the exact mating patterns of these taxa. Understanding mating systems and patterns in these taxa will provide important insight into the ecological role of this mating system.

Here we characterize microsatellite genetic markers for parentage analysis and examine mating patterns of *A. mono* within a natural forest stand. We address the following questions: (1) is flowering of the two mating types, protandry and protogyny, in *A. mono* reciprocal and synchronous? (2) Does heterodichogamy in *A. mono* effectively avoid selfing (3) and promote between-type mating? (4) What other factors affect mating patterns?

Another issue in this study is the effects of forest fragmentation on the mating system and gene flow in *A. mono*. However, we could not perform direct paternity testing on seeds in fragmented populations to compare with the above data from continuous populations because of our experimental restrictions. Here we employ indirect methods to estimate the levels of gene flow in conserved and fragmented populations based on the genetic structure of adult trees, and predict the possible effects of forest fragmentation on gene flow and genetic diversity.

Materials and Methods

The species

Acer mono Maxim. var. *marmoratum* (Nichols.) Hara f. *dissectum* (Wesmael) Rehder (hereafter *A. mono*) is a deciduous tree species that reaches a height of about 20 m and is one of the major components of cool-temperate forests in Japan. Sex expression in *A. mono* was first described in detail by Mitigami *et al*. (1989); males and two types of hermaphroditic (protandrous and protogynous) individuals were found in a population. This maple is known to be visited by various types of insects such as flies, hover flies, and small solitary bees of the Halictidae and Andrenidae (Matsui 1991) and is considered to have generalist pollination systems.

The field study and sampling

The field study was conducted in a 6-ha plot in Ogawa Forest Reserve (36˚56' N, 140˚35' E, 610-660 m above sea level) and in a neighboring fragmented forest site in Ibaraki and Fukushima Prefecture (Fig. 1). All trees with diameter at breast height (DBH) >5 cm in the 6-ha plot had been tagged and mapped, and DBH of each tree had been measured every four years.

All tagged individuals of *A. mono* in the 6-ha plot were checked for anthesis and flowering type (male, protandry, or protogyny) in 2003. In 2005, flowering phenology and sex expression of all flowering trees in the plot were examined; the sex of flowers in bloom was recorded for each flowering tree in the plot every 3-5 days from 5 to 20 May by collecting three to five inflorescences. Sex phases of flowering individuals at a given time were classified into three categories including female, male, and cosexual stages. The flowering rate of a single plant was estimated by the proportion of flowering shoots in its crown. Potential mating probability $P(i, j)$, which refers to the degree of temporal overlap between the male stages of individuals with flowering type i and the female stages of those with flowering type j, was calculated following the methods of Sato (2002).

Fig. 1. Map of the study sites. The shaded area indicates natural forests in the conserved and the fragmented sites. Dots show the locations of sampled individuals of *A. mono*.

Leaf samples for genetic analysis were collected from all 46 flowering trees and eight non-flowering trees from the 6-ha plot, and from 130 flowering individuals from the fragmented forest site. Seeds for paternity analysis were collected in October 2003 from four maternal trees in the 6-ha plot, including one protandrous (tree number *PP6587*) and three protogynous (*PP4762*, *PP5249*, *PP6487*) individuals. To detect pollen flow from nearby areas outside the plot, additional leaf samples were collected from 33 flowering trees in the surrounding area with a width of 50 m outward from each side of the 6-ha plot.
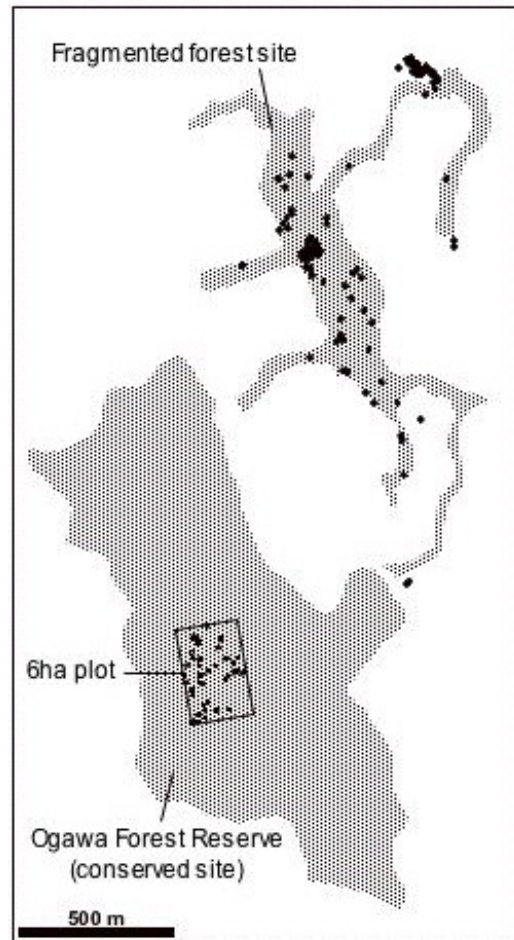
Development of microsatellite markers in Acer mono

We characterized nuclear microsatellite markers in *A. mono* as described in Kikuchi & Shibata (in press). Microsatellites were developed from a genomic library enriched for (CT) dinucleotide repeats in *A. mono* Maxim. var. *marmoratum* (Nichols.) Hara and screened for PCR amplification and polymorphism using 34 individuals from the 6-ha plot. Thirteen polymorphic loci were characterized, with an average of 13.8 alleles per locus (Table 1).

Genetic analysis

Six (Am116, Am118, Am607, Am742, Am775, and Am909) of the developed microsatellite markers were employed in further analysis according to their robustness of PCR amplification, their reliability of allele scoring, and the low frequency or absence of null alleles. Moreover, we used two microsatellite loci, MAP09 (Pandey *et al.* 2004) and Aca24 (Terui *et al.* 2006), which have been reported in other *Acer* species (*A. pseudoplatanus* and *A. capillipes*, respectively).

DNA samples of adult individuals were extracted from leaf tissues using the cetyltrimethylammonium bromide (CTAB) method described by Murray & Thompson (1980) and modified by Mukai & Yamamoto (1997). DNA from seeds was extracted using a DNeasy Plant Mini Kit (Qiagen). PCR amplifications were performed in a 6-μL reaction mixture consisting of approximately 1 ng of template DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.25 μM of each primer, and 0.25 U *Taq* polymerase. The PCR conditions included an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at an annealing temperature of 50 to 60°C (for details see Table 1), and 30 s at 72°C, followed by a final extension step of 5 min at 72°C. Alleles were scored on an ABI 3100 automated sequencer, using the software GeneScan 3.7.1 and Genotyper 2.5 (Applied Biosystems).

Genetic data analysis

Estimators of microsatellite genetic diversity, measured as the number of alleles per locus (N_A) and expected heterozygosities (H_E), were computed for two groups of adult trees, one from the 6-ha plot and the other from the fragmented forest, using the program GenAlex (Peakall & Smouse 2006). The inbreeding coefficient F_{IS} and the fixation index F_{ST} were calculated according to Weir & Cockerham (1984) using FSTAT software (Gouldet 2001).

Genotype data of adult individuals from the 6-ha plot and the fragmented forest were also used to examine spatial genetic structure. We employed a spatial autocorrelation analysis between individuals based on multilocus genotypes. A relationship coefficient *r* (Wang 2002) was computed using SPAGeDi 1.2 software (Hardy & Vekemans 2002). Ten distance classes up to 303.7 m and 15 up to 1277.4 m were automatically set up at irregular distance intervals using SPAGeDi to allocate a relatively equal number of individual pairs to each distance class.

Table 1. Characteristics of 13 microsatellite loci in *Acer mono*. These include the locusname, DDBJ accession number, primer sequence, and annealing temperature (T_a). Repeat motif and expected product size are derived from the sequenced clone. Number of alleles (A) and observed (H_O) and expected (H_E) heterozygosity were based on 34 samples (cited from Kikuchi & Shibata in press).

Locus	Accession No.	Primer sequences (5'-3')	Ta	Repeat motif	Size	A	HO	HE
Am096	AB303348	F: HEX-TAAGCTTCATACGCCATCAACCT R: GGCATCACCAAATCCAGACAC	58	$(CT)_{22}$	180	16	0.647	0.906
Am106	AB303349	F: TCCACCACGGTCCCACCTA R: NED-GAGATTGGCACTCGACGACAAG	58	$(CT)_5CA(CT)_{10}$	128	17	0.824	0.871
Am116	AB303350	F: AACGCTACCGACTTCGCCAACT R: 6-FAM-TGGAGGTCAAGTGCTGGAAACAA	58	$(CT)_{20}$	258	18	0.882	0.887
Am118	AB303351	F: GAGGGAGGAGGCTGAGAAGA R: HEX-TATCAAAGAAGCCAAGGAAGGTG	58	$(CT)_{16}$	171	15	0.971	0.897
Am258	AB303352	F: CCGGTGCATCTATCTCCAT R: HEX-CATCCATAAAGTAAAAATTGAGGG	58	$(CT)_{17}$	181	13	0.794	0.876
Am340	AB303353	F: CGGAGCCAACTTGAGAGTAGAG R: NED-ATTGAAGGTCCTTAATCCACGTC	58	$(AG)_{22}$	189	23	0.824	0.939
Am412	AB303354	F: NED-AAATTGTGACTTGTAGCGAACTC R: AACGAACCAACCAAACCTT	58	$(AG)_{23}$	128	14	0.706	0.777
Am607	AB303355	F: 6-FAM-CACACATGGGCTTCTCTATGAGT R: CATCCGCCAGTTGGTGAAT	58	$(AG)_{15}$	139	10	0.676	0.828
Am668	AB303356	F: NED-AACAACTCGGGCACTTCTC R: TGTTATTTTTACTCCCAAAGGTCT	60	$(AG)_3AA(AG)_{18}GG(AG)_4$	214	22	0.912	0.945
Am742	AB303357	F: HEX-AGAACAGGCGGAGAGTTTCGAGTC R: CCCGACGACAACCACCCAT	58	$(AG)_{17}$	163	9	0.853	0.822
Am748	AB303358	F: 6-FAM-CCCTTGAACCCGACTATTT R: GGATTGGTAAGAGGGTACATACTA	58	$(AG)_{15}$	295	3	0.147	0.140
Am775	AB303359	F: NED-AATCCACAACCACAGCCGCATCAG R: GGTGGCGACGGCAGCTAGGGTTAG	58	$(CT)_{19}$	151	12	0.824	0.860
Am909	AB303360	F: GACACAAGTATGGACGGTGATTTC R: HEX-GGCCAACTTTGAGATAAGC	58	$(AG)_{18}A(AG)_4$	258	7	0.618	0.663

Paternity of seeds collected from four trees in the 6-ha plot was determined using the program Cervus version 3.0 (Marshall *et al*. 1998, Kalinowski *et al*. 2007). We used likelihood methods implemented in Cervus to find the most likely pollen parents. Cervus calculates likelihood ratios (the likelihood that the candidate parent is the true parent divided by the likelihood that the candidate parent is not the true parent) and LOD scores (the natural log of the product of the likelihood ratios at each locus). Paternity was assigned to an individual with the highest LOD scores and Delta values (difference in LOD scores between the most likely parent and the second most likely parent) exceeding the 90% confidence level. If none of the candidates in the 6-ha plot plus its surrounding area (a total of 12 ha) had positive LOD scores, seeds were scored as being sired by an individual outside the population. In any other case, paternity was identified as "not determined."

Results

Flowering phenology in A. mono

Forty-six out of 173 tagged trees of *A. mono* (DBH >5 cm in 2001) in the 6-ha area were found bearing flowers during the study period. Most (95.2%) of the trees with DBH >20 cm produced flowers, whereas none of the trees with DBH <15 cm flowered (Fig. 2; data based on Forestry and Forest Products Research Institute 2003). Sex expression of all flowering individuals in the plot was investigated in 2005, and 25 trees

were found to be protandrous and 21 were protogynous. Male individuals were not found in the plot in 2005. Female and male flowering in the protogynous and the protandrous individuals, respectively, were at their peak in early May; female flowers of 17 (81%) protogynous individuals and male flowers of 20 (80%) protandrous individuals were in bloom from 5 to 12-13 May. The peak in male flowering of the protogynous individuals occurred from 16 to 17 May and was synchronized with the female-flowering peak of protandrous individuals (Fig. 3); during this period, 19 protogynous individuals had male flowers in bloom, of which two were in the cosexual stage, and 20 protandrous individuals were in the female stage (Fig. 3). The second male stage was observed in 12 protandrous individuals in mid to late May, after the female phase.
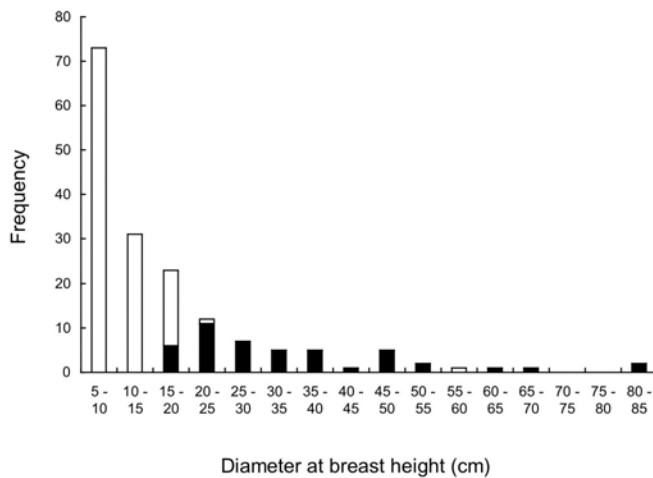

Fig. 2. Size-class distribution of *A. mono* trees with DBH >5 cm in the 6-ha plot, based on data collected in 2001 (Forestry and Forest Products Research Institute 2003). Filled and open bars indicate trees found bearing and not bearing flowers, respectively.
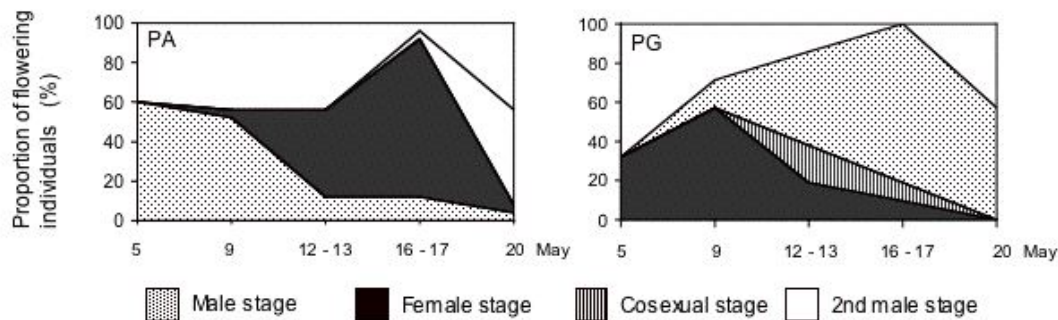

Fig. 3. The proportion of flowering individuals in each flowering phase at a given time of observation for protandrous (PA, above) and protogynous (PG, below) individuals.

The potential mating probability was 0.71 for between-type mating, 0.40 for P (PG, PA) and 0.31 for P (PA, PG), where PA and PG denote protandrous and protogynous individuals, respectively. The potential probability for within-type mating was 0.29, 0.10 for P (PA, PA) and 0.19 for P (PG, PG).

Genetic diversity and structure

Using eight microsatellites, 128 alleles were detected in 211 samples. An average of 13.25 alleles per locus were found in 81 samples from the 6-ha plot plus its surrounding area in the conserved site, whereas N_A was 14.25 in 130 samples from the fragmented site (Fig. 1). H_E was 0.801 and 0.781 in samples from the conserved and the fragmented sites, respectively. F_{IS} values were not significantly different from the null hypothesis in the former (F_{IS} = -0.007), but were significantly positive (F_{IS} = 0.031, p <0.05) in the latter. F_{ST} was 0.029 (95% confidence interval of 0.013-0.047), suggesting low but significant genetic divergence between the two sites.

The relationship coefficient *r* was significantly positive for the four shortest distance classes (<101.7 m) and negative at six of the eight longest distance classes (>334.4 m) for the fragmented forest site at the 95% significance level. It was positive and negative only in the shortest (<44.7 m) and the longest distance classes (213.8-303.7 m) in the 6-ha plot (Fig. 4).
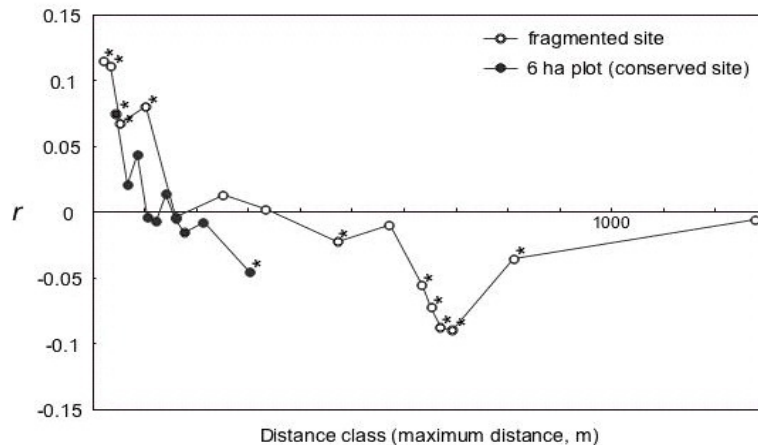

Fig. 4. The results of spatial autocorrelation analysis within the 6-ha plot in the conserved site (filled circles) and in the fragmented site (open circles). Asterisks indicate significant values for relationship coefficients *r* (p <0.05) based on 1000 permutations.
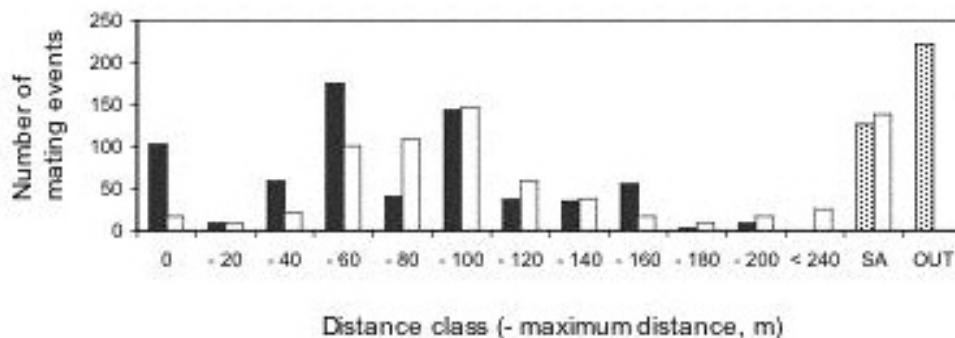

Fig. 5. Distribution patterns of mating distance inferred from parentage analysis. Filled and shaded bars indicate the number of mating events in each distance class. SA and OUT denote the frequency of seeds sired by individuals from the surrounding area and the frequency of seeds in which paternity was not decided, respectively. Open bars indicate expected values based on the number of individual pairs at a given distance class.
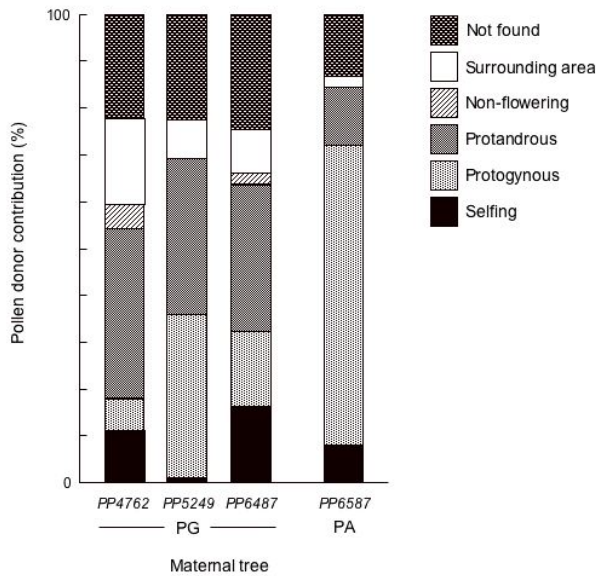
Fig. 6. The proportion of selfed seeds and outcrossed seeds sired by each type of pollen donor (protogynous and protandrous individuals, those not found bearing flowers in the plot [shown as "Non-flowering"], and those in the surrounding area). The proportion of seeds without a pollen parent among the sampled adults is indicated as "Not found."
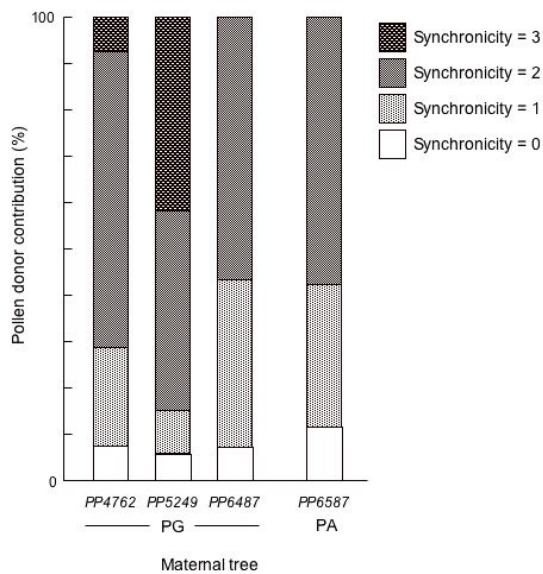

Fig. 7. The relative contribution of each type of pollen donor among the outcrossed seeds sired by flowering adults within the plot, in relation to flowering synchronicity between the male phase of pollen donors and the female phase of maternal trees.

Parentage analysis

A total of 1036 seeds collected from four mother trees were genotyped for paternity tests. Paternity was assigned to individuals within the plot in 60.6% of the seeds and to individuals in the surrounding area in 12.1% of seeds, and 21.4% were assumed to be sired by outside individuals. Paternity was not determined for only 0.29% of the seeds. The distribution of mating distance was illustrated in Fig. 5. Selfing rates

ranged from 0.01% for the maternal tree *PP5249* to 15.6% for *PP6487* (Fig. 6). Of 554 outcrossed progenies sired by flowering trees in the plot, 396 were sired by trees of the reciprocal morph, significantly exceeding the number sired by the same heterodichogamous morph (p <0.0001, Chi-square test). However, between-type mating was largely superior to within-type mating for three of the maternal trees, *PP4762*, *PP6587* (p <0.001), and *PP6487* (p <0.01), but not for *PP5249*.

Next, we examined the effects of reciprocal flowering synchronicity between pollen donors and mother plants on mating patterns. The degree of reciprocal synchronicity between a maternal tree and each pollen donor was numerically scored from 0 to 3 based on the number of observations at which the female flowering of the maternal tree coincided with the male flowering of the pollen donor. Contribution of pollen donors with flowering synchronicity ≥1 (i.e., with male flowering period overlapping female flowering of the maternal tree for at least one observation) was disproportionately higher for all the maternal trees (p <0.001, chi-square test; Fig. 7).

To address the factors determining mating patterns in *A. mono*, the relative contribution of pollen donors was regressed with their flowering synchronicity to a maternal tree, mating distance, and their flower production. The relative flower production of each individual was roughly estimated from its crown size as determined from DBH and the proportion of flowering shoots in the crown. Here we applied a simple nonlinear power function model relating crown width (*CW*) and DBH of the congener *A. rubrum* (Bragg 2001):

$$CW = 1.64 + 0.249 \, (DBH)^{0.876}.$$

The estimation of relative flower production was therefore obtained by multiplying (*CW*)*2π/4 and the proportion of flowering shoots. Pollen donor contribution was correlated with relative flower production (r = 0.550, p <0.001) and flowering synchronicity (r = 0.349, p <0.001), but not with mating distance (Fig. 8).
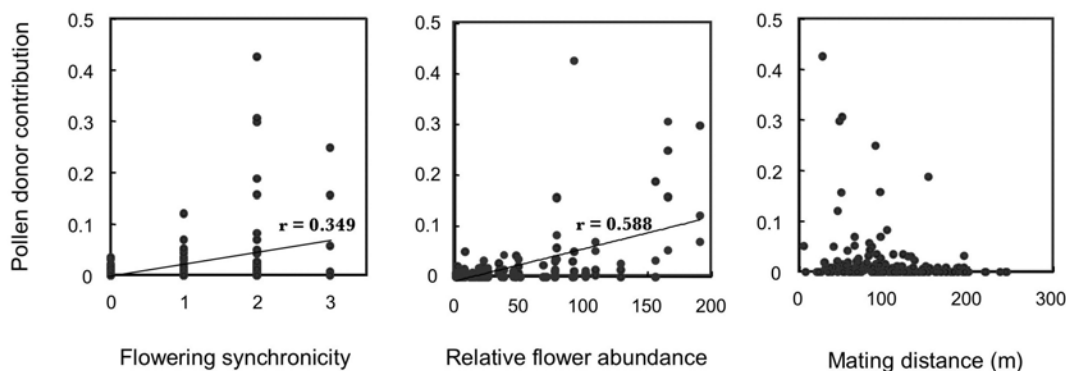

Fig. 8. Relationships between the contribution of pollen donors and their flowering synchronicity with the maternal tree, their flower abundance, and mating distance.

Discussion

Previous studies on flowering systems of heterodichogamous taxa have revealed reciprocal and synchronous flowering of protandrous and protogynous mating types within a population (Gleeson 1982, Pendleton *et al*. 1988, Asai 2002, Sato 2002, Kimura *et al*. 2003, Bai *et al*. 2006), and heterodichogamy has been considered an effective breeding system to avoid selfing and to ensure outcrossing by promoting between-type mating.

This study investigated exact mating patterns in *A. mono* and serves as an important empirical case addressing the ecological role of heterodichogamy in this maple.

In this study, flowering phases of the two mating types in *A. mono* were synchronous and reciprocal (Fig. 3). The potential mating probability, which was 2.4 times higher between the mating types than within the same type, is comparable with that of *A. japonicum* (Sato 2002). Temporal separation of male and female flowering within individual trees was apparent, but not perfect, with a cosexual stage in a few protogynous trees, as has been reported in previous studies (Pendleton *et al*. 1988, Sato 2002 and Kimura *et al*. 2003).

Paternity analysis using highly polymorphic microsatellites demonstrated high levels of outcrossing in *A. mono*. However, a certain amount of self-fertilization occurred in this natural population, suggesting no obligate self-incompatibility systems in *A. mono*. Self-compatibility is considered common in heterodichogamous taxa, including *Juglans* and *Acer* (reviewed by Renner 2001). Gabriel (1966) reported self-compatibility without a gametophytic or sporophytic incompatibility system in the heterodichogamous maple *A. saccharum* by experimental pollination, but also demonstrated lower seed set, probably resulting from post-zygotic abortion. Thus, the selfing rate found in this study may be an underestimate of self-fertilization in the natural population.

Self-fertilization in a natural population requires overlap of the male (or pollen remaining viable) and the female stages (or stigmas remaining receptive) within individuals. Therefore, it is considered that the degree of temporal overlap between male and female functions within individuals may be an important factor contributing to the variable selfing rates among maternal trees (0.01-16.2%). Specifically, the maternal tree *PP4762* with 9.6% of seeds self-fertilized showed a cosexual stage, in which female flowers remained in bloom in the upper layer of the tree while male flowers occurred in the lower layer. Although our observations in this study detected cosexual stages only in a few protogynous individuals, Sato (2002) described cosexual stages in protandrous individuals of *A. japonicum* between the male phase and the second male phase, suggesting a probability of self-fertilization in protandrous individuals. We conclude that the apparent but imperfect segregation of male and female flowering periods ensures high outcrossing in *A. mono*.

The hypothesis of between-type mating was not supported for all of the maternal trees (Fig. 6). Otherwise, a higher contribution of pollen donors just requires synchronicity between male flowering of pollen donors and female flowering of maternal trees (Fig. 7). Specifically, in the maternal tree PP5249, the female flowering phase lasted until the male flowering periods of many of the protogynous individuals in the plot, resulting in high pollen contribution from the same flowering type. Subsequently, the ratio of between-type mating to within-type mating was 2.94, which exceeded the expected value of potential mating probability (2.44).

Other than flowering synchronicity, flower production of pollen donors was considered a factor that increases their pollen contribution (Fig. 8). Interaction of these factors may well explain mating patterns of *A. mono* within this plot. We did not detect a significant negative relationship between distance and effective pollen dispersal. Effective pollination occurred over distances greater than 150 m (Fig. 8). Moreover, although the number of mating events dropped at distances greater than 180 m, more than 30% of the seeds

were still sired by individuals not found within the plot (Fig. 5). Extending the spatial scale of paternity testing will be required to capture the negative effect of distance on pollen dispersal.

Analysis of spatial genetic structure provides an indirect estimate of the levels of gene flow via pollen and seeds. Spatial autocorrelation analysis in this study clarified significant genetic structure both within the 6-ha plot in the conserved site and within the fragmented site. The x-axis intercepts, where the relationship coefficient r first crossed the x-axis, suggest that gene flow of *A. mono* becomes restricted at the geographical scale of about 100-300 m. In the fragmented site, high r-values at distance classes up to 100 m represent spatial clustering of genetically related individuals in two younger forest stands with high density of this maple (Fig. 1). Moreover, the inbreeding coefficient F_{IS} was significantly positive in the fragmented site, whereas it did not depart from the null hypothesis in the conserved site. This evidence suggests reduced gene flow of *A. mono* throughout the fragmented forest.

Effective pollination should occur over hundreds of meters; however, isolation of reproductive individuals of *A. mono* by forest fragmentation may reduce the availability of suitable pollen donors with reciprocal flowering synchronicity and flower abundance. This study suggests two possible scenarios: increased selfing, if female and male functions temporarily overlap within individuals, and reduced seed production, if such overlap does not occur. Further analysis of the exact mating patterns within fragmented sites should be conducted.

References

Asai T (2000) Dichogamy in fullmoon maple (*Acer japonicum* THUNB.). *Bulletin of the Hokkaido Forestry Research Institute* **37**: 27-40 (in Japanese).

Bai W, Zeng Y, Liao W, Zhang D (2006) Flowering phenology and wind-pollination efficacy of heterodichogamous *Juglans mandshurica* (Juglandaceae). *Annals of Botany* **98**: 397-402.

Bragg DC (2001) A local basal area adjustment for crown width prediction. *Northern Journal of Applied Forestry* **18**: 22-28.

de Jong PC (1994) Taxonomy and reproductive biology of maples. In: *Maples of the World* (eds. Van Gelderen DM, de Jong PC, Oterdoom HJ), pp. 69-103. Timber Press, Portland, OR, USA.

Forestry and Forest Products Research Institute (2003) Forest Dynamics Database. http://fddb.ffpri-108.affrc.go.jp/ Viewed 15 August 2007.

Gabriel WJ (1966) Reproductive behavior in sugar maple: self-incompatibility, cross-compatibility, agamospermy, and agamocarpy. *Silvae Genetica* **16**: 165-168.

Gleeson SK (1982) Heterodichogamy in walnuts: inheritance and stable ratios. *Evolution* **36**: 892-902.

Gleiser G, Verdú M (2005) Repeated evolution of dioecy from androdioecy in *Acer*. *New Phytologist* **165**: 633-640.

Gouldet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). http://www.unil.ch/izea/softwares/fstat.html.

Hardy OJ, Vekemans X (2002). SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* **2**: 618-620.

Kalinowski ST, Taper ML & Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* **16**: 1099-1006.

Kikuchi S, Shibata M (2007) Development of polymorphic microsatellite markers in *Acer mono* Maxim. *Molecular Ecology Notes* (in press).

Kimura M, Seiwa K, Suyama Y, Ueno N (2003) Flowering system of heterodichogamous *Juglans ailanthifolia*. *Plant Species Biology* **18**: 75-84.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7**: 639-655.

Mitigami Y, Mizui N, Kikuzawa K (1989) Flowering patterns and fruit characteristics of *Acer mono*. *Transactions of the Meeting in Hokkaido Branch of the Japanese Forestry Society* *37: 30-32 (in Japanese)*.

Mukai Y, Yamamoto N (1997) DNA and RNA isolation method of woody plants. In: *PCR Experimental Protocols*

for Plants (eds. Shimamoto K, Sasaki T), pp. 57-62. Shujunsha, Tokyo (in Japanese).

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**: 4321-4325.

Pandey M, Gailing O, Fischer D, Hattemer HH, Finkeldey R (2004) Characterization of microsatellite markers in sycamore (*Acer pseudoplatanus* L.). *Molecular Ecology Notes* **4**: 253-255.

Peakall, R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288-295.

Renner SS (2001) How common is heterodichogamy? *Trends in Ecology and Evolution* **16**: 595-597.

Sato T (2002) Phenology of sex expression and gender variation in a heterodichogamous maple, *Acer japonicum*. Ecology **83**: 1226-1238.

Terui H, Lian CL, Saito Y, Ide Y (2006) Development of microsatellite markers in *Acer capillipes*. *Molecular Ecology Notes* **6**: 77-79.

Wang J. 2002. An estimator for pairwise relatedness using molecular markers. *Genetics* **160**: 1203-1215.

Weir BS, Cockerham CC 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**: 1358-1370.