

# Mating patterns and pollen dispersal in a heterodichogamous tree, *Juglans mandshurica* (Juglandaceae)

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## Summary

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- Mating patterns in heterodichogamous species are generally considered to be disassortative between flowering morphs, but this hypothesis has hitherto not been vigorously tested. Here, mating patterns and pollen dispersal were studied in *Juglans mandshurica*, a heterodichogamous wind-pollinated species that is widely distributed in northern and north-eastern China.
- Paternity analyses carried out on 11 microsatellite loci were used to estimate morph-specific rates of outcrossing and disassortative mating. Pollen dispersal and genetic structure were also investigated in the population under study.
- The mating pattern of *J. mandshurica* was highly outcrossing and disassortative. Pairwise values of intramorph relatedness were much higher than those of intermorph relatedness, and a low level of biparental inbreeding was detected. There was no significant difference in outcrossing and disassortative mating rates between the two morphs. The effective pollen dispersal distribution showed an excess of near-neighbor matings, and most offspring of individual trees were sired by one or two nearby trees.
- These results corroborate the previous suggestion that mating in heterodichogamous plant species is mainly disassortative between morphs, which not only prevents selfing but also effectively reduces intramorph inbreeding.

**Key words:** heterodichogamy, *Juglans mandshurica*, mating patterns, microsatellites, paternity analysis, pollen dispersal, protandry, protogyny.

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## Introduction

Populations of flowering plants typically contain a single sexual phenotype that is capable of mating with all other conspecific individuals. Although reproductive traits commonly vary in these sexually monomorphic populations, the variation is largely quantitative and probably insufficient to cause consistent nonrandom mating. In contrast, populations of sexually polymorphic species are reproductively subdivided into either separate sexes (e.g. dioecy) or distinct mating groups that differ in morphology (e.g. heterostyly) or phenology (e.g. heterodichogamy). Theory suggests that disassortative pollen transfer in polymorphic species, that is, greater transfer

between individuals of different morphs ('legitimate' transfer) than between individuals of the same morph ('illegitimate' transfer; see Barrett, 1989), evolves to promote efficient pollen exchange between individuals (Lloyd & Webb, 1992). Additionally, physiological barriers to self- and intramorph fertilization may have developed in heterostylous species as a means to prevent selfing and the deleterious effects of inbreeding (Richards, 1986; Barrett, 1988). Both mechanisms lead to an equal morph ratio at equilibrium (isoplethy; Finney, 1952) as a result of negative frequency-dependent selection (Clark *et al.*, 1988). However, morph ratios can also be influenced by morph-specific differences in mating patterns, and higher levels of assortative mating within either morph would

lead to fixation of that morph (Baker *et al.*, 2000). An extensive literature concerning morph-specific differences in outcrossing rates exists for heterostylous plants (e.g. Ganders, 1975; Charlesworth, 1979; Barrett, 1987, 1989; Eckert & Barrett, 1994; Baker *et al.*, 2000; Hodgins & Barrett, 2006), but an exact estimate of assortative vs disassortative mating between morphs has been available for only very few plant species (Eckert & Barrett, 1994; Ishihama *et al.*, 2006).

As a temporal analog to heterostyly, heterodichogamy is a polymorphic sexual system in phenology, which involves two genetic morphs that occur generally at a 1 : 1 ratio within populations (Renner, 2001). Genetically determined temporal dimorphism comes in two forms. In the simplest case, both morphs are either protandrous or protogynous, but their flowers open on different days or at different times during the day. In the second form, flowers of both morphs open simultaneously, but one morph is protandrous and the other is protogynous. Heterodichogamy is phylogenetically widespread (Renner, 2001), occurring in nine orders, 12 families, and 18 genera of flowering plants such as *Corylus* (Müller, 1875), *Juglans* (Knuth, 1906; Stout, 1928; Wood, 1934; Gleeson, 1982; Kimura *et al.*, 2003; Bai *et al.*, 2006), *Carya* (Thompson & Romberg, 1985; McCarthy & Quinn, 1990), *Acer* (Gabriel, 1968; Asai, 2000; Sato, 2002), *Grayia* (Pendleton *et al.*, 1988, 2000), *Thymelaea* (Domme *et al.*, 1990, 1995), *Alpinia* (Li *et al.*, 2001) and *Hernandia* (Endress & Lorence, 2004). In these species, the flowering phases of the two mating types are synchronous and reciprocal, so their mating patterns have been characterized as disassortative (Gleeson, 1982; Pendleton *et al.*, 1988; Domme *et al.*, 1990; Kimura *et al.*, 2003; Bai *et al.*, 2006). However, the hypothesis that flowering phenology dimorphism in heterodichogamous plants promotes proficient cross-pollination between morphs has not been examined in any detail; to our knowledge, the only work carried out in this area is that of Rink *et al.* (1994) and Busov *et al.* (2003), who estimated outcrossing rates as population averages in *Juglans nigra*. Compared with heterostylous plants, heterodichogamous plants have received little attention with regard to self- or intramorph compatibility levels and morph-specific mating patterns. A recent review by Renner (2001) suggests that about half of the heterodichogamous taxa are self-incompatible, but Juglandaceae are entirely self-compatible (Gleeson, 1982; Thompson & Romberg, 1985).

In a separate field study, flowering patterns of *Juglans mandshurica*, a heterodichogamous and wind-pollinated tree, were reported (Bai *et al.*, 2006). In this species, the flowering periods of the two mating types were reciprocal and synchronous, but female flowering peaks occurred earlier than male flowering peaks for the two flowering periods within a reproductive season, especially for the first period (Bai *et al.*, 2006). From flowering phenology, the protogynous morph is expected to exhibit a higher level of assortative mating than the protandrous morph. However, the pollen dispersal pattern and spatial genetic structure determine the frequency distri-

bution of genetic relatedness of pollen receipt by female gametes (Herlihy & Eckert, 2004).

Darwin's hypothesis of heterodichogamy as an outcrossing mechanism has been widely accepted since its first formulation (Darwin, 1877), because dichogamy, that is, the temporal separation in male and female flowering within an individual, is sufficient to reduce selfing, but may be insufficient to avoid inbreeding through sib-matings. Therefore, it is important to determine whether the genetic dimorphism of heterodichogamy could effectively reduce inbreeding. In this paper, the focus is on the use of paternity analysis based on microsatellite markers for determining the mating patterns and pollen dispersal of *J. mandshurica*. Specifically, four questions are addressed. What are the exact rates of outcrossing and disassortative mating at morph level? Are there any morph-specific differences in the rates of outcrossing and disassortative mating? What is the distribution of pollen dispersal in this wind-pollinated heterodichogamous species? Do genetic structure and inbreeding exist among adult trees in the study population?

## Materials and Methods

### Material

*Juglans mandshurica* Maxim. is a monoecious, wind-pollinated, deciduous tree, mainly distributed in northern and north-eastern China, and often located along a brook. Trees are c. 10–20 m tall and commence flowering in early spring (April/May). Staminate inflorescences (catkins) are pendulous and 9–20 cm long; pistillate inflorescences (spikelets) are erect and 3–5 cm long, and typically consist of 10–18 flowers arranged spirally on the floral axis. Flowers mature acropetally. The gynoecium comprises a single ovule, a sessile style and two feathery stigmas. The fruits of *J. mandshurica* (walnuts) are big (c. 3.5–7.5 cm in diameter), single-seeded drupes. Fruits mature in early autumn and are eaten by squirrels, suggesting that long-distance, animal-mediated seed dispersal is possible. If not eaten, the fruits typically fall in the vicinity of parental plants, facilitating seed collection. Protogynous and protandrous trees are randomly distributed within a population. It has been suggested that Juglandaceae are entirely self-compatible (Gleeson, 1982; Thompson & Romberg, 1985; Renner, 2001).

### Study site and sampling design

The study site was in the Dongling Mountain, 100 km west of Beijing, China (N39°58', E115°26'; 1120 m above sea level). This region is dominated by mountain brown earth, with a temperate continental monsoon climate. The mean annual temperature is 4.8°C (January, –10.1°C and July, 18.3°C). Average annual precipitation is 612 mm yr<sup>-1</sup>, 78% of which occurs in June–August (Li & Chen, 1999). *Juglans mandshurica* is a common species in this region. Our study population is located in a long, narrow valley and is surrounded by *Pinus*

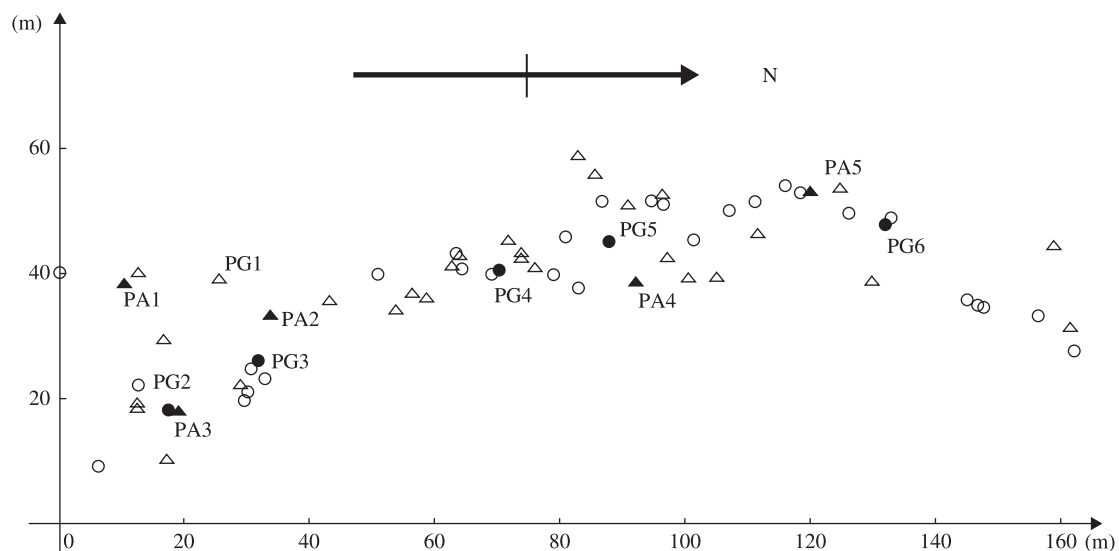
*tabulaeformis*. The adult trees are distributed on the valley floor and surrounding hill slopes. Protogynous and protandrous mating types were found to occur in roughly equal proportions. A total of 35 protogynous and 38 protandrous flowering trees were mapped in 2004, and were randomly dispersed both at the study site and with respect to each other (Fig. 1). In early May 2004, young leaves were collected from all 73 adult trees at the study site for genotyping. All the individuals in the population were thus included in the study as potential pollen donors. A total of 459 walnuts (238 from five protandrous trees and 221 from six protogynous trees) were collected from five protandrous (PA) trees (PA1, PA2, PA3, PA4 and PA5) and six protogynous (PG) trees (PG1, PG2, PG3, PG4, PG5 and PG6) in late August 2004 (Fig. 1).

### Molecular typing

Total genomic DNA was extracted from the dried leaf tissue using a modified Cetyltrimethylammonium bromide (CTAB) method. Approximately 20 mg of material was homogenized for 30 s in 700  $\mu$ l of 2  $\times$  CTAB solution using a FastPrep<sup>TM</sup> FP120 machine (Thermo Electron Corporation, Waltham, MA, USA), and incubated at 65°C for 1 h. DNA was isolated using chloroform-isoamylalcohol (24 : 1) extraction, precipitated in isopropanol, and washed in 75% ethanol twice. Extracted DNA was dissolved in 100  $\mu$ l of Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer. Total DNA was extracted from the walnut cotyledon using a plant genomic DNA Kit (Tiangen, Beijing, China). The genotypes of the DNA samples were scored using 11 pairs of the microsatellite polymerase chain reaction (PCR) primers that were developed in *J. nigra* (Woeste *et al.*, 2002; Dangl *et al.*, 2005; Table 1). To ensure that our PCR products were indeed microsatellite fragments, the

products of one or two individuals for each of the 11 loci were sequenced using the ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA) (Table 1).

PCR amplification of primer pairs was performed with a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA, USA) using 20- $\mu$ l reactions. The PCR reaction mixture contained 50 mM Tris-HCl (pH 8.0), 500 mg ml<sup>-1</sup> KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, 0.4  $\mu$ M of (each) primer (the upper primers had been labelled with fluorescent dye; 6-FAM, TAMRA or HEX (Applied Biosystems)), 20 ng of DNA template, and 0.6 U *Taq* polymerase (TaKaRa Company, Tokyo, Japan). PCR amplifications were performed as follows: an initial denaturation step at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 1 min at an annealing temperature and 1 min at 72°C, and a final extension step at 72°C for 10 min. The annealing temperature was 54°C for *WGA27*, *WGA32*, *WGA72*, and *WGA79*; 52°C for *WGA4*; and 50°C for *WGA7*, *WGA009*, *WGA089*, *WGA118*, *WGA202*, and *WGA276*. PCR products were separated on an ABI 3100 automated sequencer using a 50-cm capillary, polymerPOP-6<sup>TM</sup> (Applied Biosystems), and fragment sizes were assessed using GENEMAPPER software and GeneScan-500 ROX (Applied Biosystems) as a size standard. Allele size determinations were performed twice manually to reduce scoring error. Moreover, the error rate was calculated by repeating the marker amplification for 73 adult trees and counting the number of inconsistent genotypes between the first and second attempts. The error rate was expressed as the number of incorrect alleles divided by the total number of alleles, which was estimated to be 1.14% for *J. mandshurica*. Sources of error included adjacent allele heterozygote scoring error (0.38%), the dropout of a single allele during PCR amplification (0.48%), and faint alleles (0.16%).



**Fig. 1** Map of the study stand. Circles, protogynous trees ( $n = 35$ ); triangles, protandrous trees ( $n = 38$ ). The *Juglans mandshurica* trees used for paternity analysis were protandrous trees PA1–PA5 and protogynous trees PG1–PG6.

**Table 1** Microsatellite loci analysed, polymerase chain reaction (PCR) conditions, motif and allele size ranges in *Juglans mandshurica*

Locus	Motif	Allele range (bp)	T <sub>a</sub> (°C)	Label <sup>a</sup>	Reference
WGA4	(GA) <sub>14</sub>	231–57	52	6-FAM	Woeste <i>et al.</i> (2002)
WGA7	(GA) <sub>9</sub>	201–21	50	TAMRA	Woeste <i>et al.</i> (2002)
WGA27	(GA) <sub>10</sub>	206–16	54	HEX	Woeste <i>et al.</i> (2002)
WGA32	(CT) <sub>38</sub>	183–41	54	HEX	Woeste <i>et al.</i> (2002)
WGA72	(GT) <sub>11</sub>	140–58	54	HEX	Woeste <i>et al.</i> (2002)
WGA79	(GA) <sub>12</sub>	207–49	54	TAMRA	Woeste <i>et al.</i> (2002)
WGA009	(CT) <sub>12</sub>	227–65	50	6-FAM	Dangl <i>et al.</i> (2005)
WGA089	(AG) <sub>18</sub> (TG) <sub>12</sub>	184–238	50	HEX	Dangl <i>et al.</i> (2005)
WGA118	(CT) <sub>17</sub> (CA) <sub>7</sub>	200–24	50	TAMRA	Dangl <i>et al.</i> (2005)
WGA202	(CT) <sub>12</sub>	241–59	50	6-FAM	Dangl <i>et al.</i> (2005)
WGA276	(CT) <sub>14</sub>	143–215	50	TAMRA	Dangl <i>et al.</i> (2005)

<sup>a</sup>Forward primers were modified at the 5' end with a fluorescent label: HEX (green), 6-FAM (blue), or TAMRA (yellow) (see the Materials and Methods, PCR amplification).

T<sub>a</sub>, annealing temperature; WGA, *Juglans regia* microsatellite locus name at NCBI.

### Data analysis

The number of alleles, observed and expected heterozygosity, and the inbreeding coefficient for all 11 loci were analysed for 73 adult trees and 459 seeds, using the software FSTAT version 2.9.3 (Goudet, 2001). The presence of null alleles was tested using MICROCHECKER (Van Oosterhout *et al.*, 2004).

### Paternity analysis

Paternity analyses (the assignment of a putative father to a genetically known mother–offspring pair) were performed using CERVUS version 3.0 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007), a software program based on maximum-likelihood methods (Meagher, 1986). The simulation parameters were as follows: 10 000 cycles, 104 candidate parents (the approximate size of the male gene pool for our study population), 0.70 as the proportion of candidate parents sampled, 0.997 as the proportion of loci typed, 0.0114 as the rate of typing error (calculated from repeat genotyping), 0.95 for the strict confidence level and 0.80 for the relaxed confidence level. Note that the number of candidate male parents specified in the simulation was the approximate size of the male gene pool for the study population, which should include both sampled and unsampled male parents. The total number of male parents and the proportion of male parents sampled were adjusted, always keeping the product of these two numbers equal to 73. A simulation with 104 male parents (70% of the sampled individuals) was found to yield the best match between observed and expected assignments.

### Mating pattern

The proportions of walnuts sired by the same morph or selfed were determined directly by paternity assignment. Differences in outcrossing rate, disassortative mating rate, and pollen flow rate

between the two morphs were assessed by pairwise comparisons of bootstrap estimates following the method of Eckert & Barrett (1994). With this approach, morphs were considered to differ significantly if 100(1 –  $\alpha_{PC}/2$ ) per cent of the differences between paired bootstrap values (where  $\alpha_{PC}$  is the type I error rate per contrast) were either greater than zero or less than zero.

The relatedness among all 73 adult trees was calculated using the RELATEDNESS software, version 5.0.5 (Goodnight, 1999). The potential relatedness values under completely intermorph mating, random mating, completely intraprotandrous mating, and completely intraprotogynous mating were calculated and compared by independent *t*-test. The significance of the difference between expected relatedness, obtained using the calculated disassortative mating rate, and observed relatedness was assessed by randomization tests. These processes were iterated 10 000 times. A deviation from expectation caused by an excess of high relatedness (*R*) values could result from nonrandom mating among related individuals. The statistical test for the correlation between the genetic relatedness and spatial distance parameters was performed using TFGA version 1.3 software (Miller, 1997). The 5000 times randomization in the Mantel test was adopted. Inbreeding depression ( $\delta = 1 - (\text{fitness of selfed progeny}/\text{fitness of outcrossed progeny})$ ) was also measured for survival from seed to reproductive maturity from the outcrossing rate (*t*) and parental inbreeding (*F*) using the equilibrium estimator of Ritland (1990):

$$\delta = 1 - \left[ \frac{2tF}{(1-t)(1-F)} \right]$$

### Pollen dispersal

Measurements consisted of the distance between the mother and the putative father when the most likely father was assigned. In addition, the distances between each mother tree and all other candidate fathers were also measured to determine

whether the observed intermate distance was influenced by the spatial arrangement of the adult trees. A comparison was made between the frequency distributions of observed and potential intermate distances using the Wilcoxon matched pairs test (Zar, 1999). Data were pooled over all five protandrous and six protogynous mother trees.

## Results

### Paternity analysis

The cross-species amplification of microsatellite loci has become an efficient way of screening markers for closely related species. Eleven *J. nigra* (black walnut)-derived microsatellite markers successfully amplified polymorphic loci in *J. mandshurica* (Table 1). There are differences in the times of repeat motif and allele range between these two species. Diversity parameters for the 11 microsatellite loci and the exclusion probability (EP) for the parental population are shown in Table 2. For the 11 microsatellite loci analysed, the expected heterozygosity ( $H_E$ ) varied from 0.361 to 0.942; observed heterozygosities ( $H_O$ ) varied from 0.361 to 0.937. Reproducing individuals had inbreeding coefficients very close to zero, with a multilocus estimate  $F_1 = 0.006$ . Null alleles were not detected at 11 loci by MICROCHECKER. Diversity parameters for the 11 microsatellite loci for walnuts were as follows: the multilocus expected heterozygosity ( $H_E$ ) and observed heterozygosity ( $H_O$ ) were 0.698 and 0.672, respectively, and the inbreeding coefficient for walnuts was 0.052, higher than that of reproducing adults (0.006) (Table 3).

Using CERVUS, paternity assignments gave the following results: a single most likely father was assigned to 328 (71.5%) (PA: 186; PG: 142) offspring with 95 and 80% confidence

**Table 2** Number of observed alleles ( $A_o$ ), observed and expected heterozygosities ( $H_O$  and  $H_E$ ), paternity exclusion probability (EP), and inbreeding coefficient ( $F_1$ ) at each locus, for 73 adult individuals of *Juglans mandshurica*

Locus	$A_o$	$H_O$	$H_E$	EP	$F_1^a$
WGA4	8	0.699	0.723	0.510	0.028
WGA7	6	0.575	0.595	0.366	0.027
WGA27	4	0.411	0.361	0.190	-0.147
WGA32	25	0.959	0.937	0.861	-0.030
WGA72	5	0.630	0.570	0.329	-0.113
WGA79	15	0.849	0.844	0.685	-0.013
WGA009	10	0.466	0.546	0.331	0.059
WGA089	14	0.918	0.850	0.697	-0.087
WGA118	12	0.836	0.837	0.673	-0.006
WGA202	9	0.634	0.721	0.530	0.064
WGA276	25	0.861	0.906	0.807	0.043
Multilocus	12.1	0.713	0.719	0.997	0.006

<sup>a</sup>Significance levels were determined after 11 000 randomizations; all were nonsignificant ( $P > 0.05$ ).

WGA, *Juglans regia* microsatellite locus name at NCBI.

**Table 3** Number of observed alleles ( $A_o$ ), observed and expected heterozygosities ( $H_O$  and  $H_E$ ), and inbreeding coefficient ( $F_1$ ) at each locus, using the genotypes of 459 walnuts (*Juglans mandshurica*)

Locus	$A_o$	$H_O$	$H_E$	$F_1$
WGA4	10	0.625	0.676	0.075*
WGA7	8	0.583	0.612	0.046 <sup>ns</sup>
WGA27	8	0.569	0.577	0.012 <sup>ns</sup>
WGA32	16	0.800	0.791	-0.011 <sup>ns</sup>
WGA72	5	0.288	0.326	0.114*
WGA79	34	0.921	0.896	-0.016 <sup>ns</sup>
WGA009	13	0.517	0.566	0.080*
WGA089	17	0.825	0.831	0.014 <sup>ns</sup>
WGA118	13	0.779	0.817	0.050 <sup>ns</sup>
WGA202	12	0.691	0.704	0.066*
WGA276	33	0.795	0.882	0.174*
Multilocus	15	0.672	0.698	0.052*

Significance levels were determined after 11 000 randomizations. ns, nonsignificant ( $P > 0.05$ ); \* $P < 0.05$ .

WGA, *Juglans regia* microsatellite locus name at NCBI.

levels among a total of 459 offspring; for 131 (28.5%) (PA: 51; PG: 80) offspring, the most likely father assigned was rejected at the 0.80 confidence level, indicating that their pollen parents were likely to have come from outside the research site.

### Intermorph mating pattern

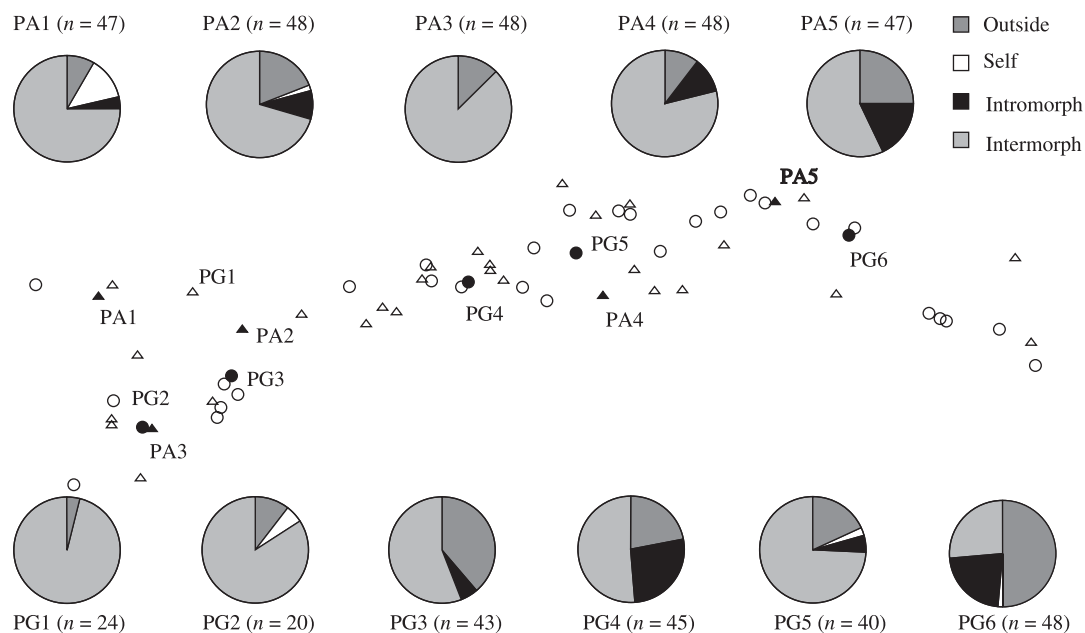
Among 328 offspring that had one male parent within the stand, the proportions of seeds sired by the opposite morph (disassortative mating) and outcrossed were 91.9 and 96.2%, respectively, in protandrous mothers, compared with 91.5 and 98.6% in protogynous mothers. Disassortative mating rates varied greatly among individual trees, ranging from 82.6% (PA5) to 100% (PA2) in protandrous mothers and from 57.9% (PG6) to 100% (PG1, PG2, and PG3) in protogynous mothers (Table 4). Outcrossing rates ranged from 88.4% (PA1) to 100% (PA2 and PA4) in protandrous mothers and from 96.3 (PA4) to 100% (PA1, PA2, PA3, and PA6) in protogynous mothers (Fig. 2). There were no significant differences in the disassortative mating rates and outcrossing rates between the two morphs.

A negative and significant correlation between genetic distance and spatial distance was detected, but the  $r$ -value was low ( $r = -0.106$ ,  $P < 0.01$ ; Mantel test). The pairwise value of relatedness under completely intermorph mating ( $-0.018 \pm 0.046$ ; mean  $\pm$  standard error) was significantly lower than that under random mating ( $0.060 \pm 0.162$ ) or under completely intraprotandrous ( $0.107 \pm 0.188$ ) or completely intraprotogynous ( $0.118 \pm 0.215$ ) mating (independent sample  $t$ -test;  $P < 0.05$ ). This indicated that biparental inbreeding might occur more frequently intramorph than intermorph, and that sexual dimorphism of heterodichogamy could effectively reduce intramorph inbreeding. However, the empirical values of

Maternal tree	Number of pollen donors	Main pollen donor			Median distance (m)
		Tree (%)	Distance (m)	Relatedness	
PA1	7	PG7 (67.4)	10.6	0.495	10.6
PA2	9	PG12 (25.0)	12.7	0.038	31.4
PA3	10	PG7 (27.2)	29.1	0.060	11.5
PA4	13	PG5 (43.2)	7.7	0.152	13.9
PA5	13	–	39.8		
PG1	8	PA6 (59.1)	16.0	0.314	16.0
PG2	8	PA15 (37.5)	5.0	0.106	5.0
PG3	11	PA11 (24.0)	4.8	0.063	18.8
PG4	13	PA23 (40.7)	5.7	0.176	37.2
PG5	16	–	12.4		
PG6	13	–	33.7		

**Table 4** Mating parameters for *Juglans mandshurica* inferred using the maximum likelihood method (CERVUS)

PA, protandrous tree; PG, protogynous tree.



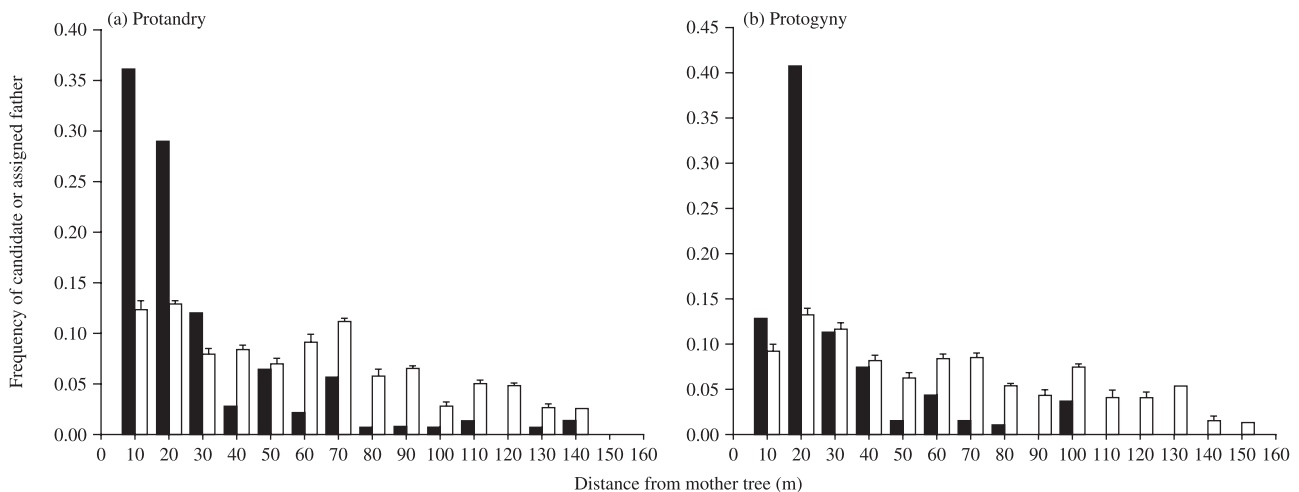
**Fig. 2** Composition of fathers in individual trees of the protandrous morph (PA, upper) and the protogynous morph (PG, lower) in *Juglans mandshurica*. *n*, number of seeds analysed in each tree.

relatedness in protandry (0.122) and protogyny (0.055) were more than those expected (protandry:  $-0.032 \pm 0.01$ ; protogyny:  $0.015 \pm 0.01$ ). The distribution of observed pairwise values of relatedness provided evidence that the *J. mandshurica* population is somewhat inbred, especially in protandrous mothers. The inbreeding coefficient of walnuts (0.052) was higher than that of the parents (0.006), indicating that few inbred offspring survive to reproductive maturity because of strong inbreeding depression ( $\delta = 0.548$ ).

#### Pollen dispersal distance

The median observed intermate distances were 14.9 m (range 0–134.1 m) and 11.5 m (range 0–99.2 m) for protandrous and protogynous trees, respectively, much lower than the

median potential distances of 45.2 and 50.9 m. The difference in frequency distribution between observed and potential intermate distances was significant for both morphs (Wilcoxon matched pairs test;  $P < 0.05$ ) (Fig. 3), with the shorter distance class having a much higher observed frequency. About 70% of the effective pollen was exchanged between trees located within 30 m of each other, and only 3.8% (protogyny) and 4.3% (protandry) was exchanged between individuals more than 100 m apart. Pollen pool heterogeneity was relatively low, with the averages for pollen donors being 10.4 for protandry and 11.5 for protogyny; moreover, most offspring of individual trees were pollinated by one or two pollen parents near to them in our study site, which would produce more full-sib offspring. For tree PA1, 67.4% of offspring were pollinated by PG7 (distance: 10.6 m; relatedness: 0.495); for



**Fig. 3** Comparison of the potential (open bars) and pollinating (closed bars) male parent distributions as a function of the distance to the female parent. (a) *Juglans mandshurica* protandrous trees and (b) protogynous trees both showed a significant difference between potential and effective distributions at the 5% significance level in a Wilcoxon matched pairs test.

tree PG1, 59.0% of offspring were pollinated by PA6 (distance: 16.0 m; relatedness: 0.314). When pollen flow from outside the research site was considered, the median observed intermate distances increased. Although pollen flow for the first flowering period (36.2%) was greater than that for the second (21.4%) in our study site, there was no significant difference in pollen flow rate between the two flowering periods.

## Discussion

### Outcrossing rates

Only *Juglans* (Knuth, 1906; Stout, 1928; Wood, 1934; Gleeson, 1982; Kimura *et al.*, 2003; Bai *et al.*, 2006) and *Carya* (Thompson & Romberg, 1985; McCarthy & Quinn, 1990) have been reported to be heterodichogamous in the Juglandaceae to date. Although the Juglandaceae are entirely self-compatible (Thompson & Romberg, 1985; Renner, 2001), our data from paternity analysis showed that selfing events appear to be rare in the study population and the mating system of *J. mandshurica* can be characterized as completely outcrossing, consistent with observations on flowering phenology within individual trees (Bai *et al.*, 2006). As previously found for several heterodichogamous plant species (e.g. Gleeson, 1982; Rink *et al.*, 1994; Busov *et al.*, 2003; Kimura *et al.*, 2003), the present study reconfirms that heterodichogamy represents an effective way of reducing self-pollination or geitonogamy and facilitates outcrossing.

However, we found that a low level of biparental inbreeding existed in the study population of *J. mandshurica*, which was also found for *J. nigra* (Busov *et al.*, 2003), possibly as a result of local pollen and seed dispersal. The spatial genetic structure found in our population showed that trees located near each other were often full-sibs or half-sibs, which was caused by walnut seed being mainly dispersed by gravity. Also, because

pairwise values of intramorph relatedness were much higher than those of intermorph relatedness, intramorph inbreeding would be more frequent than intermorph inbreeding in our population. Compared with dichogamy, heterodichogamy effectively reduces inbreeding intramorph, although it may not avoid inbreeding intermorph. The inbreeding coefficient of walnuts (0.052) was higher than that of the parents (0.006), indicating that few inbred offspring survive to reproductive maturity because of strong inbreeding depression, as demonstrated in a few other *Juglans* species (Beineke, 1989; Rink *et al.*, 1994).

### Disassortative mating rates

The exact disassortative mating rates of 91.9% (protandry) and 91.5% (protogyny) determined by paternity analysis provide the first direct evidence of predominant intermorph mating in a heterodichogamous species. This finding is also in accordance with field observations of flowering phenology in other heterodichogamous species (Knuth, 1906; Gleeson, 1982; Pendleton *et al.*, 1988; Sato, 2002; Kimura *et al.*, 2003; Bai *et al.*, 2006). All available evidence suggests that the sexual functions of the two morphs in heterodichogamous plant species are synchronous and reciprocal within a population.

Theoretical studies on the maintenance of sexual polymorphism, such as distyly, indicate that dimorphism will always be maintained when levels of disassortative mating are greater than those of assortative mating, and that morph-specific differences in these mating parameters give rise to populations with biased morph ratios (Baker *et al.*, 2000). Our results for morph ratios and disassortative mating rates between morphs were consistent with the model predictions. The dates of female flowering peaks were earlier than those of male flowering peaks for the two flowering periods, especially for

the first flowering period (Bai *et al.*, 2006). This observation would lead us to expect a higher level of assortative mating in protogyny, which was not supported by microsatellite data. The discrepancy between paternity analysis and flowering phenology could result from intramorph incompatibility in the sense that intramorph crosses have lower fertility than intermorph crosses. It is noteworthy that pairwise values of intramorph relatedness were much higher than those of intermorph relatedness. It was inferred that both flowering phenology and intramorph incompatibility promote more proficient pollen transfer between, rather than within, morphs in heterodichogamous plants. Further study is needed on the mating pattern and fitness of the progeny of intramorph matings to clarify the mechanisms underlying heterodichogamy in *J. mandshurica*.

### Pollen dispersal pattern

Pollen dispersal was studied, for the first time, using paternity analysis in a heterodichogamous plant population. Overall, *J. mandshurica* showed a pattern of predominant short-distance pollen transfer in both pollination episodes within a reproductive season (Fig. 3). Similar results were obtained for other wind-pollinated tree species, including *Quercus robur* (Streiff *et al.*, 1999), *Pinus densiflora* (Chunlan *et al.*, 2001), and *Picea abies* (Burczyk & Chybicki, 2004). However, the median pollen dispersal distances (14.9 and 11.5 m) in our study were much shorter than those of other wind-pollinated tree species in continuous forests; for example, 140 m in *Pinus flexilis* (Schuster & Mitton, 2000), 68 m in *P. densiflora* (Chunlan *et al.*, 2001) and 43.6 m in *Q. robur* (Streiff *et al.*, 1999). Two reasons might account for this difference. First, our study population is spatially limited, with the maximum distance between trees being only 152.0 m. Furthermore, the density of adult trees is relatively high, with a median distance between trees of 50.9 m for protogyny and 45.2 m for protandry (Fig. 1). Secondly, most offspring of individual trees were pollinated by one or two pollen parents near to them, which would lead to relatively low pollen pool heterogeneity and a relatively high frequency of full-sib offspring. The pairwise relatedness between trees was relatively high, and genetic similarity may cause their flowering phenology to be more synchronous (Table 4). For example, 60.5% of offspring of tree PA1 were pollinated by PG7 (distance, 10.6 m; relatedness, 0.495); for tree PG1, 50.0% of offspring were pollinated by PA6 (distance, 16.0 m; relatedness, 0.314).

A review of recent paternity studies in tree species showed that, for the experimental designs generally used, individuals outside the stand under study account for as much as 30% of pollination in wind-pollinated species, and 15% in insect-pollinated species, even when the study populations are spatially isolated (Dick, 2001; Slavov *et al.*, 2002). These levels of gene flow should be high enough to override the diversifying effects of genetic drift and/or inbreeding. Our

estimates of 36.2 and 21.4% pollen flow for the first and the second flowering periods in *J. mandshurica* were consistent with those of previous studies in wind-pollinated species. For example, Dow & Ashley (1996) showed that more than half of the saplings in a bur oak (*Quercus macrocarpa*) population had paternal parents outside the stand. Although there was no significant difference in pollen flow rate between the two flowering periods, the higher pollen flow rate for the first flowering period may have resulted from the fact that the female flowering phase of protogynous individuals was less well synchronized with the male flowering phase of protandrous individuals (Bai *et al.*, 2006). The proximity to other populations in the study area may be one reason for the high level of gene flow in our population.

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