

Reciprocal herkogamy promotes disassortative mating in a distylous species with intramorph compatibility

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Summary

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- Mating patterns in heterostylous species with intramorph compatibility have the potential to deviate from symmetrical disassortative mating owing to ecological and reproductive factors influencing pollen dispersal. Here, we investigate potential and realized patterns of mating in distylous *Luculia pinceana* (Rubiaceae), a species with intramorph compatibility. Our analysis provides an opportunity to test Darwin's hypothesis that reciprocal herkogamy promotes disassortative pollen transfer.
- We combined measurements of sex-organ reciprocity and pollen production to predict potential pollen transfer and mating patterns in a population from SW China. Marker-based paternity analysis was then used to estimate realized patterns of disassortative and assortative mating at the individual and floral morph levels.
- Both potential and realized mating patterns indicated a significant component of disassortative mating, satisfying theoretical conditions for the maintenance of floral dimorphism. Levels of assortative mating (37.7%) were significantly lower than disassortative mating (62.3%), but numerous offspring resulting from intramorph mating were detected in the majority of maternal seed families in both floral morphs.
- Our results provide empirical support for Darwin's cross-promotion hypothesis on the function of reciprocal herkogamy, but indicate that in most heterostylous species strong diallelic incompatibility may be a general requirement for complete disassortative mating.

Introduction

The reproductive systems of animal-pollinated angiosperms typically involve nonrandom mating because plant immobility and local pollen dispersal results in spatially structured mating between individuals in close proximity (Levin & Kerster, 1974; Heywood, 1991; García *et al.*, 2007; Hodgins & Barrett, 2008). Nonrandom mating is also promoted in plants with sexual polymorphisms because of the sub-division of populations into different mating groups (or sexual morphs) that differ in morphological and physiological traits influencing pollination and fertilization (Barrett & Hodgins, 2006; Shang *et al.*, 2012). Understanding the diverse factors influencing nonrandom mating in plant populations represents a key challenge for evolutionary biologists, as the pattern of mating is an important determinant of population genetic structure and the maintenance of genetic polymorphism.

Heterostyly is a conspicuous floral polymorphism in which nonrandom mating is usually enforced by a strong diallelic self-incompatibility system that ensures intermorph (disassortative) mating by preventing self and intramorph (assortative) mating. Populations of heterostylous species contain either two (distyly)

or three (tristyly) floral morphs that differ reciprocally in the relative positions of stigmas and anthers, a condition known as reciprocal herkogamy (Ganders, 1979; Barrett, 1992; Lloyd & Webb, 1992a). Darwin (1877) proposed that heterostyly is a floral mechanism that promotes pollinator-mediated cross-pollination between floral morphs. Experimental studies of pollen loads on stigmas in natural populations of heterostylous plants have provided empirical support for Darwin's hypothesis of the function of heterostyly (reviewed in Lloyd & Webb, 1992b; Barrett & Shore, 2008; and see Cesaro & Thompson, 2004; Baena-Díaz *et al.*, 2012). Studies of pollen dispersal also commonly report substantial amounts of intramorph pollen on stigmas of heterostylous plants (Ganders, 1974; Barrett & Glover, 1985). In species with strong diallelic incompatibility, assortative pollination has no influence on the mating patterns of maternal parents. However, not all heterostylous species possess diallelic incompatibility and in such cases mating patterns may potentially deviate from strict disassortative mating, including some degree of assortative mating.

Investigations of the types of self-incompatibility systems in heterostylous species have revealed a wide range of conditions, in addition to classical diallelic incompatibility (reviewed in Barrett

& Cruzan, 1994). For example, heterostylous plants may be self-compatible (Ganders, 1975; Eckert & Barrett, 1994), have different morph-specific strengths of self-incompatibility (Barrett & Anderson, 1985) and intramorph compatibility (Ornduff, 1988), or possess entirely different self-recognition systems unrelated to diallelic incompatibility that permit intramorph mating despite strong self-incompatibility (Schou & Philipp, 1984; Sage *et al.*, 1999). In these cases, mating patterns may deviate from symmetrical disassortative mating and result in biased morph ratios in heterostylous populations (Schou & Philipp, 1984; Barrett *et al.*, 2004; Weber *et al.*, 2013).

Heterostylous species with intramorph compatibility provide an opportunity to evaluate Darwin's hypothesis on the function of heterostyly. Without the confounding influence of diallelic incompatibility, the extent to which reciprocal herkogamy acting alone promotes disassortative mating can be evaluated using genetic markers. This approach has been used to examine whether tristylous promotes disassortative mating in self-compatible *Eichhornia paniculata* (Barrett *et al.*, 1987; Kohn & Barrett, 1992), but has not been investigated in a distylous species with intramorph compatibility. One of the main goals of this study is therefore to evaluate the extent to which reciprocal herkogamy promotes disassortative mating in *Luculia pinceana* (Rubiaceae), a distylous species.

Studies of pollen-tube growth and seed set following controlled pollinations in diverse taxa of Rubiaceae have revealed considerable variation in the expression of the physiological components of heterostyly. This variation includes distylous species that exhibit self-compatibility (Richards & Koptur, 1993; Riveros *et al.*, 1995), cryptic self-incompatibility (Wu *et al.*, 2010), morph-specific differences in the sites of pollen tube arrest (Bawa & Beach, 1983) and strength of self-incompatibility (Pailler & Thompson, 1997), as well as species with conventional diallelic self-incompatibility (Sobrevila & Arroyo, 1982; Murray, 1990), that may often vary in overall strength (Sobrevila *et al.*, 1983). In addition to variation in incompatibility expression, members of Rubiaceae commonly exhibit weak reciprocity of stigma and anther heights (Richards & Koptur, 1993; Lau & Bosque, 2003) with consequences for disassortative pollen transfer and patterns of mating.

Luculia pinceana possesses a self-incompatibility system unlike that reported elsewhere in the family. A controlled crossing programme by Ma *et al.* (2009) revealed that intramorph crosses produced amounts of fruit set similar to those resulting from intermorph crosses; however, the long-styled morph (hereafter L-morph) set abundant seed following self-pollination, whereas the short-styled morph (hereafter S-morph) was self-incompatible. A geographical survey of morph ratios in 25 populations of *L. pinceana* in SW China revealed that most populations were isoplethical (1:1 morph ratio), but some populations were L-morph biased and others lacked the S-morph entirely (see fig. 1 in Zhou *et al.*, 2012). These authors suggested that intramorph compatibility and its influence on mating may contribute to the observed variation in morph frequencies in populations.

Here, we investigate mating patterns in a natural population of *L. pinceana* using microsatellite markers and paternity analysis to

determine if reciprocal herkogamy promotes disassortative mating. We first measured variation in sex-organ reciprocity and pollen production in the floral morphs to evaluate the extent to which these traits may promote disassortative pollen transfer. We then assayed open-pollinated progeny from mapped individuals of the floral morphs to address the following specific questions: are realized patterns of mating similar to those predicted from the influence of floral traits on pollen transfer? Is the level of disassortative mating sufficient to maintain floral dimorphism and how frequent is assortative mating? To what extent is the spatial location of individuals important for influencing patterns of disassortative and assortative mating? Following the presentation of our results, we discuss the relevance of our findings to Darwin's hypothesis for the function of heterostyly.

Materials and Methods

Study species

Luculia pinceana Hook. (Rubiaceae) is a perennial distylous shrub distributed from the southeast margin of the Tibetan Plateau in SW China to adjacent countries at altitudes between 350 and 1800 m a.s.l. (Luo *et al.*, 1999). The species flowers from August to December and produces compact inflorescences composed of showy pink or white tubular flowers (mean flowers per inflorescence = 7.8, SD = 2.4, $n = 48$) that last for up to 8 d. Pollinators of *L. pinceana* are mainly long-tongued nectivorous insects (bumblebees, moths and butterflies) and pollen-collecting bees (*Apis florae*).

Study site and sampling design

The present study was conducted at Xiaolu mountain, Lancang County, Yunnan Province, SW China (22°45' N, 99°43' E, 1750 m a.s.l.). This region is dominated by yellow-brown earth soils and experiences a subtropical monsoon climate. The mean average annual temperature is 18.6°C and the average annual precipitation is 1400–1800 mm yr⁻¹, of which 85% occurs during the rainy season from June to October. The study population is located on a valley floor in an area of *c.* 270 × 250 m, with a steep cliff on the southern edge and forests surrounding the remaining boundary of the population. Rock outcrops are abundant and *L. pinceana* and *Bidens* sp. (Asteraceae) are the predominant species at the site. The nearest *L. pinceana* population to our study population was located 2.5 km away and was coded XLS in our previous phylogeographic study of the species (see fig. 1 in Zhou *et al.*, 2012).

In mid-September 2011, we identified and tagged the floral morphs of all flowering individuals in the population. A total of 62 L-morph and 58 S-morph plants were recorded; the morph ratio was not significantly different from 1:1 ($G = 0.13$; $P = 0.715$). Most individuals occurred in patches with a few individuals that were more isolated. We mapped the location of all flowering individuals in the population (Fig. 1). We sampled leaf tissue from all individuals and this was dried in silica gel for genotyping. To investigate floral traits of importance to pollen

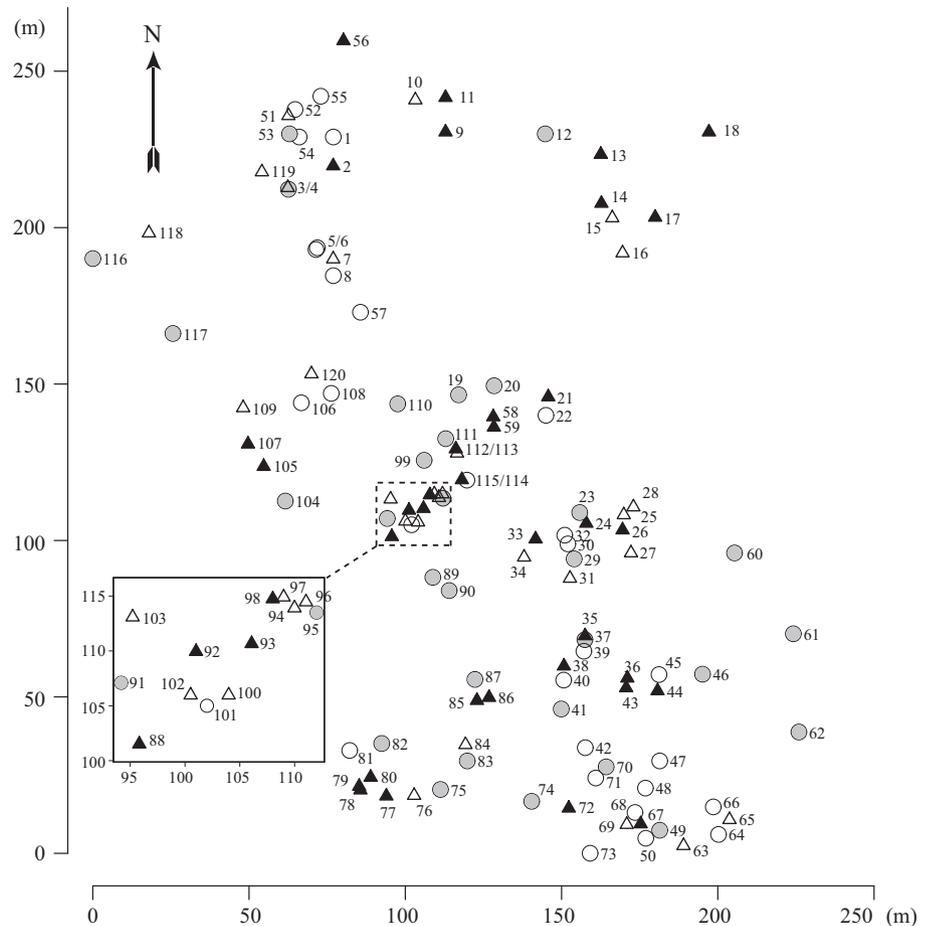


Fig. 1 Spatial location of the 120 *Luculia pinceana* adult individuals in the population studied. Triangles, L-morph ($n = 62$); circles, S-morph ($n = 58$); closed triangles and circles, maternal parents used for paternity analysis. The inserted box shows an area of high density.

dispersal and mating success (e.g. stigma and anther position, pollen production) we obtained a random sample of flowers from both morphs and preserved these in FAA (10% formalin, glacial acetic acid, and 70% ethanol; 5/5/90 v/v) for subsequent measurement. Our sample included a single flower from 46 and 41 plants of the L- and S-morphs, respectively.

In late February 2012 we sampled mature fruits from 68 plants in the population of both morphs. Later, *c.* 20 seeds from each maternal plant were separately sown in pots in a glasshouse at the Kunming Institute of Botany (Chinese Academy of Science). Approximately 1 month later offspring from all maternal plants had established, but three families of the S-morph were excluded from further use because they contained less than five seedlings. In total we collected leaf tissue for parentage analysis from 1051 offspring (range 7–18 per family, mean = 16) from 35 L-morph parents ($n = 566$ offspring) and 30 S-morph parents ($n = 485$); the parental individuals are identified in Fig. 1.

DNA extraction and microsatellite genotyping

We extracted total genomic DNA from the dried (adult) or fresh (offspring) leaf tissue using a modified cetyl trimethyl ammonium (CTAB) protocol (Doyle, 1991). Quantification of DNA was carried out with a SmartSpec™ Plus Spectrophotometer (Bio-Rad). Working stocks of DNA were then prepared based on

these estimates and stored in $0.1 \times$ TE buffer. We used pairs of microsatellite PCR primers: LP54, LP198, LP45, LP162, LP4, LP107, LG11, LG26 and LG33 to score genotypes at nine loci in parents and offspring (Supporting Information Table S1), (Zhou *et al.*, 2010, 2011). The chromosome number for *L. pinceana* is reported as $2n = 44$ and the species listed as tetraploid (Mehra & Bawa, 1969). However, the scoring of genotypes at microsatellite loci was straightforward with no evidence of multiple alleles at individual loci, as in our earlier studies of this species (Zhou *et al.*, 2011, 2012).

We performed PCR amplification using the following protocol: 25- μ l reaction volume containing 20 ng of genomic DNA template, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1 U Taq polymerase (Fermentas, Shenzhen, China), 0.1 μ M of each primer (the 5' side of the forward primers were labelled with fluorescent dye: HEX, TAM or 6-FAM) and $10 \times$ PCR buffer. We conducted PCR amplifications on a thermocycler (Perkin-Elmer, Foster City, CA, USA) under the following conditions: 95°C for 3 min followed by 30 cycles at 95°C for 30 s for denaturation, 1 min for annealing, 72°C for 1.5 min for extension, and a final extension step at 72°C for 10 min. The annealing temperature was 58°C for LP54, LG33, LP45, LP4 and LP107; 55°C for LG11, LP198 and LP162; and 52°C for LG26. We checked PCR products on 1% agarose gels stained with ethidium bromide. All PCR products were separated and

visualized using an ABI 3730 XL automated sequencer (Applied Biosystems, Foster City, CA, USA). We first determined allele sizes using GENE Mapper 3.7 software with GeneScan-500 ROX as an internal-lane size standard (Applied Biosystems) and then rechecked the data manually to reduce scoring errors. Five randomly selected PCR products for each locus were purified and sequenced in both directions to examine the character of the repeat motif. Sequencing primers were identical with those used in the PCR, and were conducted in forward and reverse reactions individually.

Variation in reciprocal herkogamy and pollen production

In order to evaluate the extent to which pollen transfer between and within the floral morphs may be influenced by the degree of reciprocal herkogamy, we used a modification of the method proposed by Lau & Bosque (2003) in their study of pollen dispersal in distylous *Palicourea fendleri* (Rubiaceae). This method provides insight into potential patterns of pollen transfer and mating in a population based on floral traits, but ignores post-pollination mechanisms that may alter the frequency of fertilizations resulting from different pollen sources. Using our sample of flowers of the L- and S-morph we measured using digital calipers the length of the style, stigma, filament and anther; style and filament length were measured from the base of the ovary. The frequency distribution of stigma and anther height in each floral morph was determined using an interval of 0.4 mm. We used normal family functions to fit the distributions and parameters were estimated using the maximum-likelihood (ML) method in R v2.51 (R Development Core Team, 2011). We estimated the ratio of overlap between the distributions of stigma and anther heights between and within the floral morphs.

In order to estimate pollen size (L-morph – 10 flowers, 117 pollen grains; S-morph – 10 flowers, 94 pollen grains) and production ($n=26$ flowers per morph), we measured the polar and equatorial axis of pollen grains under a scanning electron microscopy (SEM, S-4800, Hitachi, Tokyo, Japan) and counted pollen grains using a hemacytometer. We used two sample t -tests to determine whether there were significant differences between the floral morphs in pollen size and production.

Genetic diversity and paternity analysis

We estimated the number of alleles per locus (A), observed and expected heterozygosity (H_O , H_E), and the inbreeding coefficient (F_{IS}) from the 120 parents and 1051 offspring using nine loci and the software FSTAT v2.9.3 (Goudet, 1995). We used GenePop v3.4 (Raymond & Rousset, 1995) to test for departures from Hardy–Weinberg equilibrium (HWE) for each locus. We estimated the probability of excluding a randomly chosen nonfather based on allele frequency (total paternity exclusion probability or PEP) and the presence of null alleles using the program CERVUS v3.0 (Kalinowski *et al.*, 2007).

We performed paternity analysis on each seedling using CERVUS v3.0 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007), a computer

program that uses a ML method to assign parentage to offspring (Meagher, 1986). We performed a random mating simulation of 10 000 offspring using allele frequencies observed in the parental genotypic data, with 120 (the total flowering population size) as probable candidate parents, 1.0 as the sampled candidate male parent proportion because all flowering individuals in the study population were screened, and 0.0143 as the error rate, which was estimated from the number of mother-offspring mismatches over all nine loci. Because in *L. pinceana* the L-morph is self-compatible (SC) but the S-morph is self-incompatible (SI) (Ma *et al.*, 2009), both SC and SI models were conducted separately using the same parameter settings in each simulation. Both strict (95%) and relaxed (80%) confidence levels of LOD (natural logarithm of the likelihood ratio) scores were estimated using the same values for the likelihood error rate and genotyping error rate.

Mating patterns and pollen dispersal

We determined the mating patterns of the floral morphs of *L. pinceana* by identifying seedling to which a single male parent could be assigned. We used goodness-of-fit tests to examine significance differences between mating patterns under two models. In the first, we compared the observed four possible mating combinations (L-morph sired by S-morph: q_{SL} , S-morph sired by L-morph: q_{LS} , L-morph sired by L-morph: q_{LL} , and S-morph sired by S-morph: q_{SS}) with those expected from random mating. We estimated random mating assuming that there were the same opportunities for intermorph and intramorph mating. In the second, we determined the levels of disassortative and assortative mating for each floral morph and compared these values to those predicted based on our measurements of overlap in sex-organ positions and differences in pollen production of the morphs.

In order to determine whether the observed mating distance based on our paternity assignment was influenced by the spatial arrangement and floral morph of adults, a comparison was made between the frequency distributions of observed and potential mating distance using a Mann–Whitney U -test. Potential mating distance was calculated by determining how many mating pairs were present at various distance classes from each maternal parent. Because in *L. pinceana* intramorph pollen transfer is compatible (Ma *et al.*, 2009), this involved all flowering plants in the population. Data were analysed separately for disassortative and assortative mating components.

Mating patterns and maintenance of dimorphism

In order to investigate whether the observed mating patterns in the *L. pinceana* population would maintain a stable dimorphism we used the model of Lloyd & Webb (1992b) for the maintenance of stilar dimorphism in a pollen-limited population (condition 1) and for a nonpollen-limited population (condition 2):

$$q_{LS} + q_{SL} > 2q_{LL} \text{ and } q_{LS} + q_{SL} > 2q_{SS} \quad \text{Condition 1}$$

$$q_{SL} > q_{LL} \text{ and } q_{LS} > q_{SS} \quad \text{Condition 2}$$

In these equations, q_{LS} , q_{SL} , q_{LL} and q_{SS} are the rates of the four mating types with the former and the latter letter indicating the floral morph of the paternal and maternal parent.

Results

Pollen size and production

In common with most heterostylous species, there was a significant difference in the mean pollen size of the floral morphs of *L. pinceana*. Pollen grains of the S-morph were *c.* 1.2 times larger than the L-morph with respect to the polar axis (S-morph = 25.224 μm , L-morph = 21.154 μm , $t = 29.547$, $df = 180.624$, $P < 0.001$) and the equatorial axis (S-morph = 16.229 μm , L-morph = 12.789 μm , $t = 27.703$, $df = 183.338$, $P < 0.001$). The number of pollen grains produced per flower was also significantly different between the L- and S-morph ($t = -2.674$, $df = 6$, $P = 0.037$). The L-morph averaged 15.65×10^4 pollen grains per flower whereas the S-morph averaged 9.95×10^4 pollen grains per flower.

Reciprocity and potential mating patterns

Variation in stigma and anther height in the floral morphs of *L. pinceana* was normally distributed (L-morph: $\mu = 30.0$, $\sigma = 3.577$ for stigmas, $\mu = 21.0$, $\sigma = 3.577$ for anthers; S-morph: $\mu = 21.4$, $\sigma = 4.963$ for stigmas, $\mu = 30.4$, $\sigma = 4.386$ for anthers). As expected for a distylous species, there was much higher spatial matching between corresponding stigma and anther heights of the floral morphs (Fig. 2). Thus, 89.58% of the distribution of stigma height in the L-morph overlapped with anthers of the S-morph (Fig. 2a). This is more than four times as much as the

overlap in distribution between the stigmas and anthers of the L-morph (20.84%; Fig. 2a,b). Similarly, there was considerable overlap (83.39%; Fig. 2c) between the height of S-morph stigmas and anthers of the L-morph. This was about twice as high as the overlap between the stigmas and anthers of the S-morph (33.47%; Fig. 2c,d). The normalized overlap ratios were therefore: $o_{LS} : o_{SL} : o_{LL} : o_{SS} = 0.36 : 0.39 : 0.09 : 0.14$. Taking into account sex-organ overlap and differences in pollen production between the L- and S-morphs (L-morph/S-morph = 1.57), we can calculate the expected rates of pollen transfer based on floral traits by multiplying o_{LS} and o_{LL} by 1.57, resulting in predicted values of: $e_{LS} : e_{SL} : e_{LL} : e_{SS} = 0.46 : 0.31 : 0.12 : 0.11$ (Fig. 3). All else being equal, reciprocal herkogamy should promote significantly higher levels of disassortative than assortative pollen transfer.

Genetic diversity and paternity assignment

The nuclear microsatellite loci we investigated in *L. pinceana* were highly polymorphic, with an average of 6.22 alleles per locus. Population genetic parameters and the paternity exclusion probability (PEP) based on nine loci for the parental population are presented in Table 1. Observed and expected heterozygosity varied from 0.075 to 0.817 (H_O) and 0.080 to 0.698 (H_E). Table 2 presents the data for progeny, and values of observed and expected heterozygosity were 0.397 (H_O) and 0.388 (H_E). Inbreeding coefficients (F_{IS}) for both adults (-0.090) and offspring (-0.023) were low and not significantly different from zero, indicating that the population was predominantly outcrossing, a finding consistent with the paternity analysis detailed below which indicated very low levels of selfing.

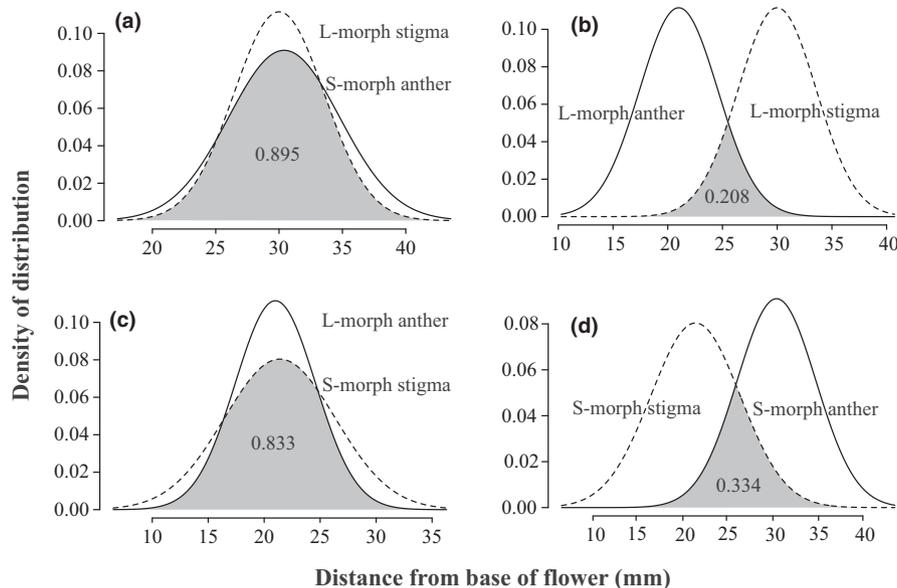


Fig. 2 The spatial distribution and overlap of sexual organs in the floral morphs of *Luculia pinceana*. The solid and dotted lines indicate normal fitted density distributions of anthers and stigmas, respectively. The grey area indicates the proportion of overlap between female and male sexual organs. (a) Stigmas of the L-morph and anthers of the S-morph; (b) stigmas and anthers of the L-morph; (c) stigmas of the S-morph and anthers of L-morph; (d) stigmas and anthers of the S-morph.

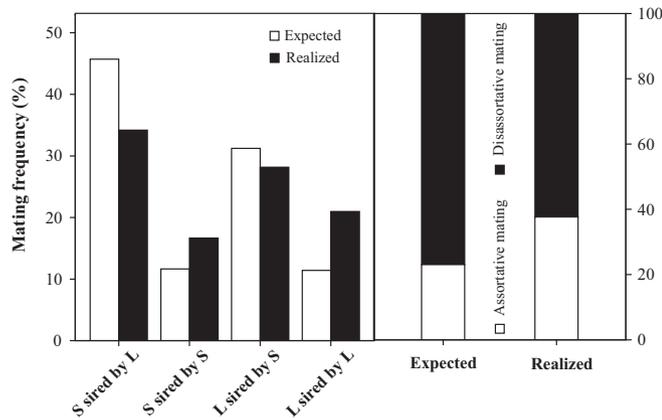


Fig. 3 Expected and realized mating patterns between and within the morphs in the population of *Luculia pinceana*. The results for expected mating patterns were obtained from the spatial overlap of sexual organs adjusted for differences between morphs in pollen production (see the Materials and Methods for details). The observed results are from paternity analysis conducted in CERVUS 3.0 (80% confidence criterion).

Table 1 Number of observed alleles (*A*), observed and expected heterozygosities (H_O and H_E), paternity exclusion probability (PEP), and inbreeding coefficient (F_{IS}) at each locus, for 120 adult individuals of *Luculia pinceana*

Locus	<i>A</i>	H_O	H_E	PEP	F_{IS}^a
LP54	2	0.392	0.355	0.145	-0.104 ^{ns}
LP198	5	0.250	0.225	0.101	-0.111 ^{ns}
LP45	6	0.725	0.647	0.418	-0.122 ^{ns}
LP162	4	0.817	0.698	0.273	-0.171 ^{ns}
LP4	2	0.083	0.080	0.038	-0.039 ^{ns}
LP107	3	0.075	0.104	0.051	0.277*
LG11	4	0.308	0.300	0.156	-0.029 ^{ns}
LG26	4	0.308	0.302	0.163	-0.019 ^{ns}
LG33	12	0.633	0.586	0.367	-0.082 ^{ns}
Multilocus	4.67	0.399	0.3662	0.900	-0.090 ^{ns}

^aSignificance levels were determined by 1800 randomization tests.

*, $P < 0.05$; ns, not significant.

Using CERVUS, the paternity assignments were similar using the SI and SC models. In the SI model, a single most likely male parent was assigned to 348 (33.1%) of the 1051 offspring, with 80% confidence. However, a single most likely father was found for an additional 13 offspring (11 for the S-morph and two for the L-morph) at the same confidence level when selfing was allowed. The most likely father assigned was rejected for 703 (66.9%) of the offspring, including 11 selfed offspring of the S-morph because pollination studies indicate that the S-morph is self-incompatible (Ma *et al.*, 2009). Therefore, in total 348 offspring were used to estimate realized mating patterns.

Realized mating patterns

Goodness-of-fit *G*-tests revealed that the frequency of the four mating types deviated significantly from random mating ($\chi^2 = 25.36$, $df = 3$, $P < 0.001$). The proportion of offspring

Table 2 Number of progeny (*n*), number of observed alleles (*A*), observed and expected heterozygosities (H_O and H_E), and inbreeding coefficient (F_{IS}) at each locus, for progeny individuals of *Luculia pinceana*

Locus	<i>n</i>	<i>A</i>	H_O	H_E	F_{IS}^a
LP54	1051	4	0.364	0.347	-0.050 ^{ns}
LP198	1051	8	0.386	0.362	-0.067 ^{ns}
LP45	1047	8	0.715	0.593	-0.206 ^{ns}
LP162	1051	4	0.725	0.724	-0.002 ^{ns}
LP4	1051	2	0.129	0.121	-0.069 ^{ns}
LP107	1050	6	0.073	0.075	0.023 ^{ns}
LG11	1050	4	0.290	0.306	0.051*
LG26	1051	9	0.278	0.283	0.017 ^{ns}
LG33	1044	11	0.614	0.684	0.102*
Multilocus	–	6.22	0.397	0.388	-0.023 ^{ns}

^aSignificance levels were determined by 1800 randomization tests.

*, $P < 0.05$; ns, not significant.

resulting from disassortative mating was significantly higher than from assortative mating ($\chi^2 = 21.13$, $df = 1$, $P < 0.001$). Among the 348 offspring for which only one male parent was determined, the frequency of disassortative mating was 62.3%, including 119 and 98 seeds sired by the L- and S-morphs on S- and L-morph maternal parents, respectively. The proportion of seed resulting from assortative mating in L- and S-morph families was 20.9% ($n = 73$) and 16.6% ($n = 58$), respectively (Fig. 3). Thus, of the total offspring sampled 55.2% were sired by the L-morph and 44.8% by the S-morph ($\chi^2 = 3.72$, $df = 1$, $P = 0.053$).

The majority (68%, $n = 43$ families) of maternal families were composed of offspring resulting from a mixture of disassortative and assortative mating (L-morph 64.7%; S-morph 72.4%; Fig. 4). However, 12 of the 34 L-morph families and 8 of the 29 S-morph families resulted from a single mating class (L-morph: 6 disassortative and 6 assortative; S-morph: all disassortative). The frequency distribution of observed vs potential mating, based on paternity analysis and mating pair distances, respectively, was significant for disassortative mating events (Mann–Whitney *U*-test; $W = 174.5$; $P < 0.05$). There was a much higher observed frequency of disassortative mating among potential mates at shorter distances (Fig. 5a). By contrast, the frequency of assortative mating was unaffected by mating distance and randomly distributed among the various distance classes (Fig. 5b). Thus, there was no significant difference in observed and predicted patterns of assortative mating ($W = 187.5$; $P > 0.05$).

Based on the results of our paternity assignments, we estimated that the number and frequency of the four classes of mating events were: $q_{LS} = 119$, $q_{SL} = 98$, $q_{LL} = 73$, $q_{SS} = 58$ ($q_{LS} : q_{SL} : q_{LL} : q_{SS} = 0.34 : 0.28 : 0.21 : 0.17$, respectively; Fig. 3). These values satisfy the conditions for the stability of stylar dimorphism predicted by the Lloyd & Webb (1992b) pollen transfer model for both the pollen limitation and no pollen limitation cases.

Discussion

The current study of *L. pinceana* is the first to employ highly polymorphic genetic markers to measure mating patterns

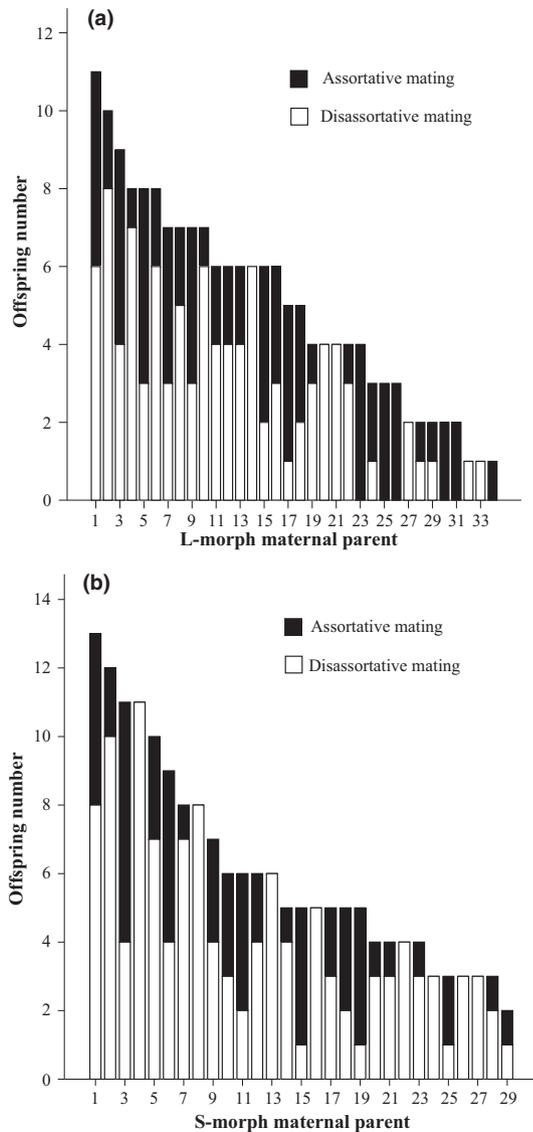


Fig. 4 Distribution of disassortative and assortative mating events among families of the (a) L-morph and (b) S-morph of *Luculia pinceana*. The results are based on the paternity analyses conducted in CERVUS 3.0 (80% confidence criterion).

through male function in a distylous species. Three novel findings were revealed by our paternity analyses: (1) the amount of disassortative mating was sufficient to maintain distyly, although just over one-third of all fertilizations involved assortative mating (Fig. 3); (2) the spatial location of individuals in the population influenced levels of disassortative mating, whereas proximity had no effect on assortative mating (Fig. 5); (3) in the absence of intramorph incompatibility, reciprocal herkogamy in *L. pinceana* functioned to promote disassortative mating, a finding consistent with Darwin's hypothesis on the adaptive significance of heterostyly. Below we consider the potential reproductive and ecological factors influencing mating patterns in *L. pinceana* and consider the relevance of our findings to hypotheses concerned with the evolution and function of heterostyly.

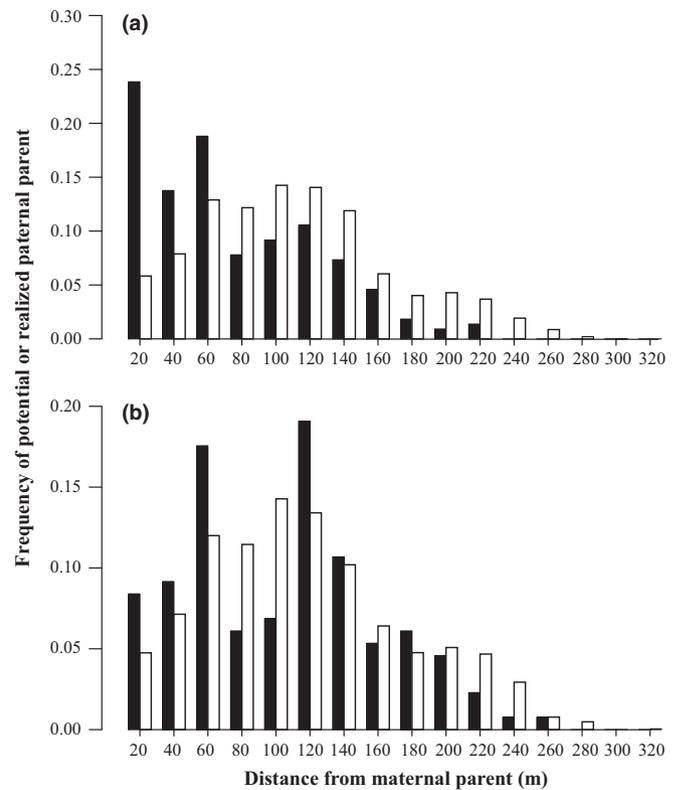


Fig. 5 Comparison of the potential (open bars) and realized (closed bars) male parent distributions as a function of the female parent in *Luculia pinceana*. (a) Disassortative mating events and (b) assortative mating events.

Potential and realized mating patterns

Darwin (1877) proposed that the reciprocal positioning of female and male sex organs in the floral morphs of heterostylous species function to promote cross-pollen transfer through segregated pollen deposition on the bodies of animal pollinators. This idea has led to measurements of the degree of reciprocal herkogamy in heterostylous species (Richards & Koptur, 1993; Eckert & Barrett, 1994; Sanchez *et al.*, 2008), and efforts to use morphology to predict pollen transfer and mating outcomes (Lloyd & Webb, 1992b; Barrett *et al.*, 2004). We examined the spatial matching of stigma and anthers in the floral morphs of *L. pinceana* using a modification of a method developed by Lau & Bosque (2003) involving calculation of the degree of overlap in the distributions of sex organs. This analysis revealed a limited amount of overlap between nonreciprocal sex-organ heights (Fig. 2), leading to the prediction that if morphology largely governs pollen transfer in *L. pinceana*, disassortative mating should predominate in our study population. Consistent with this prediction we found that disassortative mating was the most common form of outcrossed mating and, following the models of Lloyd & Webb (1992b), that the level we measured was sufficient to maintain distyly in the population. Clearly, because our study involved a single population, further analyses of the type we have conducted would be required to determine the generality of this result for other populations with equal morph ratios.

The realized mating patterns of both floral morphs of *L. pinceana* included more assortative mating than predicted based on pollen transfer alone. For example, the ratio of disassortative to assortative mating was substantially less ($(q_{LS} + q_{SL}) / (q_{LL} + q_{SS}) = 1.63$), than from expectations from sex-organ overlap ($(o_{LS} + o_{SL}) / (o_{LL} + o_{SS}) = 3.26$), or when we also adjusted by pollen production ($(e_{LS} + e_{SL}) / (e_{LL} + e_{SS}) = 2.33$). The difference between expectations based on morphology vs the realized patterns of mating estimated by genetic markers represents a reduction of 15% in the amount of disassortative mating due to assortative mating.

The floral morphs differed with respect to their potential vs realized mating patterns (Fig. 3). More pollen from the anthers of the L-morph was redirected from disassortative to assortative mating (9–12%) than occurred for pollen from the anthers of the S-morph (3–6%). The reciprocity analysis predicted that the S-morph should sire more seeds by intramorph mating than the L-morph ($a_{SS}/a_{LL} = 1.55$, and after adjustment for pollen production $e_{SS}/e_{LL} = 0.92$); however, the observed pattern was the reverse ($q_{SS}/q_{LL} = 0.80$). Of the total matings we identified, the L-morph was marginally more successful than the S-morph as a male parent, garnering 55.2% of all matings in our sample. The slight bias may be, in part, because the L-morph produces significantly more pollen than the S-morph. This pollen production difference characterizes the floral morphs of most distylous species (reviewed in Ganders, 1979) but has no influence on mating patterns in species with diallelic incompatibility. However, because of the occurrence of intramorph compatibility in *L. pinceana*, all cross-pollen transfer within and between the floral morphs are compatible and therefore pollen production differences may be functionally relevant to outcrossed siring success.

Role of pollination and post-pollination processes

Pollination processes probably contribute toward the significant amounts of assortative mating that we detected at the floral morph and individual levels (Fig. 4). Studies of pollen removal and deposition by pollinators visiting heterostylous plants consistently demonstrate that a substantial component of the pollen load of stigmas involves 'own form' (i.e. intramorph) pollen (reviewed in Ganders, 1979; Lloyd & Webb, 1992b; more recent studies on Rubiaceae include Stone, 1995; Ree, 1997; Paillet *et al.*, 2002; Lau & Bosque, 2003; Massinga *et al.*, 2005; Hernandez & Ornelas, 2007). Although segregated pollen deposition on the bodies of insects visiting heterostylous flowers has been demonstrated (Wolfe & Barrett, 1989), a variety of behaviours including pollen grooming by bees (Harder & Barrett, 1993) and proboscis coiling in Lepidoptera (Krenn, 1990) can disturb the original placement of pollen, resulting in the mixing of separate pollen pools and pollen transfer between nonreciprocal sex organs. The distribution of pollen on pollinators is therefore dynamic, resulting in stochastic variation in the composition of pollen loads triggered by the different foraging behaviours of pollinators (Harder & Wilson, 1998; Richards *et al.*, 2009). These aspects of the pollination process could increase pollen

carryover, resulting in a more random distribution of mating distances than might be predicted from morphology alone.

The frequency of disassortative mating was strongly influenced by the spatial proximity of mating partners, whereas, by contrast, patterns of assortative mating were randomly distributed among the various distance classes (Fig. 5a,b). Although we are unsure of the specific mechanism (s) that may be responsible for this result, the diverse factors disturbing pollen from its original placement on a pollinator's body become more likely with successive visits to flowers and this may increase the number of assortative transfers at greater mating distances. In addition, the occurrence of a low frequency of flowers in which the arrangement of sex organs deviated strongly from mean values, resulting in overlap between nonreciprocal organ heights (Fig. 2b,d), may have also contributed to the absence of a spatial signal for assortative mating.

At our study site the bee *Apis florea* commonly visited *L. pinceana*, but due to its short proboscis it was only capable of transferring pollen between long-level sex organs. However, our paternity analysis provided no evidence that preferential pollen transfer from the S- to the L-morph (q_{SL}) influenced mating patterns ($q_{SL}/q_{LS} = 0.82$) in the population. Other pollinators at our study site were longer-tongued *Bombus*, *Vespa* wasps, and butterflies, which each probed for nectar and were probably more effective at cross-pollinating both floral morphs.

Controlled intramorph crosses of *L. pinceana* indicate that the species does not possess the typical diallelic incompatibility found in heterostylous plants (Ma *et al.*, 2009). Our studies of mating patterns confirmed that the species is intramorph compatible and within-morph cross-pollination results in viable seedlings. In several heterostylous species with self- and intramorph compatibility, cryptic incompatibility functions to promote disassortative mating (Weller & Ornduff, 1977; Casper *et al.*, 1988; Cruzan & Barrett, 1993; Eckert & Allen, 1997). Although cryptic incompatibility has been reported in the Rubiaceae (Wu *et al.*, 2010), the patterns of mating that were revealed by our paternity analysis provided no strong evidence that *L. pinceana* possesses the ability to discriminate between different classes of outcross pollen. However, the absence of significant selfing in the self-compatible L-morph could potentially result from inbreeding depression at the seed germination phase and/or post-pollination discrimination against self-pollen tubes resulting from cryptic self-incompatibility. In heterostylous *Decodon verticillatus* both intermorph and intramorph cross-pollen tubes outcompete self-pollen tubes in the style (Eckert & Allen, 1997). A similar form of cryptic self-incompatibility could possibly account for the near absence of selfing in the L-morph.

It seems probable that in common with most heterostylous species pollen loads of *L. pinceana* are composed of both intermorph and intramorph outcross pollen. As a result, maternal families should contain offspring originating from a mixture of disassortative and assortative mating, as occurred in 68% of the 43 families we assayed. It is worth noting that most families containing offspring of a single source (i.e. either disassortative or assortative) were composed of a small number of individuals (see

Fig. 4). It is therefore likely that by sampling larger family sizes more families would include progeny sired by both intermorph and intramorph outcross pollen.

Evolution and maintenance of floral dimorphism

Models for the evolution of distyly differ with respect to whether diallelic incompatibility is a precondition for the evolution of reciprocal herkogamy. In Lloyd & Webb's (1992b) model, reciprocal herkogamy evolves first, and a diallelic incompatibility system may or may not develop subsequently, depending on the genetic costs of self-fertilization. By contrast, in the model of Charlesworth & Charlesworth (1979) the evolution of diallelic incompatibility precedes the establishment of reciprocal herkogamy and is a prerequisite for the morphological polymorphisms to evolve. As yet there is no decisive evidence to fully reject either of these models and given the large number of independent origins of heterostyly across numerous plant families, it is quite possible that both pathways to heterostyly have occurred.

Rubiaceae offers outstanding opportunities for evaluating models of the evolution of heterostyly owing to the large number of heterostylous species in the family, the likelihood of multiple origins, and the wide variation in expression of incompatibility and morphology. It is not currently possible to interpret incompatibility expression in *L. pinceana* in a comparative context, because little is known about heterostyly or the presence of diallelic incompatibility in close relatives. The evolutionary status of self-incompatibility in *L. pinceana* is therefore difficult to interpret. Three possibilities can, however, be entertained: it represents an early stage in the evolution of diallelic self-incompatibility; it represents a late stage in the breakdown of diallelic incompatibility; or the system has evolved *de novo* and is unrelated to diallelic incompatibility. Regardless of which is true, our results are consistent with Lloyd and Webb's proposal that reciprocal herkogamy likely evolved before the establishment of incompatibility. In the population of *L. pinceana* we examined, reciprocal herkogamy can function in the absence of diallelic incompatibility to promote sufficient disassortative mating to maintain floral morph ratios at the expected isopleth equilibrium of 1 : 1. Future work in populations with L-morph-biased ratios would be valuable to determine whether morph-specific differences in rates of assortative mating or selfing may be a contributory factor.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Microsatellite loci analysed in *Luculia pinceana* with motif, allele size ranges, annealing temperature (T_a) and fluorescent dye

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