Pollinator visitation patterns strongly influence among-flower variation in selfing rate

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INTRODUCTION

In many animal-pollinated Angiosperms, the proportion of seeds generated by self- and cross-fertilization varies dramatically among closely related species, among populations within species, and even among individuals within populations (see Barrett and Eckert, 1990; Barrett, 2003; Karron et al., 2004; Goodwillie et al., 2005; Medrano et al., 2005, and references therein). This variation has been attributed both to (a) plant traits, such as floral morphology (Humphreys and Gale, 1974; Epperson and Clegg, 1987; Motten and Antonovics, 1992; Karron et al., 1997; Herlihy and Eckert, 2007) and floral display size (Harder and Barrett, 1995; Snow et al., 1996; Karron et al., 2004; Williams, 2007); and (b) ecological factors, such as plant spatial distribution (Smyth and Hamrick, 1984; Warwick and Thompson, 1989; Karron et al., 1995a), pollinator identity and abundance (Brunet and Sweet, 2006), patterns of herbivory (Ivey and Carr, 2005) and the effects of competition for pollination (Bell et al., 2005). Selfing rates may even vary markedly on finer spatial scales (Barrett et al., 1994; Carromo and Hamrick, 2005). For example, studies of Mimulus ringens have documented striking variation in the mating system of adjacent flowers on the same daily floral display, often ranging from predominant selfing ($s > 0.80$) to predominant outcrossing ($s < 0.20$; Karron et al., 2004). These among-flower differences in the genetic composition of sibships increase the likelihood that self progeny will compete with other inbred siblings, potentially influencing the expression of inbreeding depression (Ritland, 1989; Schmitt and Ehrhardt, 1990). Although among-flower variation in mating patterns has important implications for theoretical research on the evolutionary stability of mixed mating systems (Holsinger, 1991; Lloyd, 1992; Goodwillie et al., 2005), the mechanisms responsible for this variation remain poorly understood.

We hypothesize that among-flower variation in selfing rates may reflect patterns of pollinator visitation. When a pollinator approaches a multiflower display (Fig. 1), the first flower probed receives outcross pollen and autogamous (within-flower) self pollen, but does not receive geitonogamous (among-flower) self pollen. However, if pollen carryover is limited, successive flowers probed on this display will receive increasing amounts of geitonogamous self pollen, resulting in increasing levels of self-fertilization (Barrett et al., 1994; Karron et al., 2004). Here the first direct experimental test of this hypothesis is described.
The novel experimental design combines detailed pollinator observation with unambiguous assignment of paternity in order to quantify selfing rates of individual M. ringens fruits precisely. This species is especially appropriate for these studies because pollen carryover is very limited (Holmquist, 2005), increasing the likelihood that later flowers in the pollinator visitation sequence will primarily receive geitonogamous self pollen. The following questions are addressed. (a) Do selfing rates differ on the first, second, third and fourth flowers probed on multiflower displays? (b) Does the order of floral visitation influence the proportion of self-fertilization attributable to geitonogamy?

**Materials and Methods**

**Study species**

*Mimulus ringens* (Phrymaceae) is a perennial herb native to wet meadows of central and eastern North America. Plants produce showy displays of large (2 cm) blue, zygomorphic flowers that are scattered across several indeterminate flowering stems. Each stem has only one or two open flowers. Flowers open simultaneously at dawn and last for half a day (Karron et al., 2004). Anthers dehisce and the stigma is receptive at the time of anthesis. At peak flowering, mean daily floral display size in five natural populations from north central USA ranges from 1-9 to 22-5 flowers, with an overall mean of 8-7 flowers (R. J. Mitchell, unpubl. res.).

At our field site in SE Wisconsin, USA, *Mimulus* flowers are primarily pollinated by workers of five bumble-bee species (*Bombus fervidus*, *B. griseocollis*, *B. impatiens*, *B. nevadensis* and *B. vagans*; Mitchell et al., 2004). The relative abundance of these pollinators fluctuates widely among years. During 2002, the year of the present study, *B. fervidus* was the principal pollinator of *M. ringens*.

When probing *M. ringens* flowers, bumble-bees contact the stigma and then the anthers with their tongues and faces (Mitchell et al., 2004). A single *Bombus* probe typically deposits 3000–7000 pollen grains (Flanagan et al., 2009) onto the papillose stigma. Nearly all flowers produce seed capsules (Karron et al., 2004). Each flower has approx. 6000 ovules, and seed set following a single *Bombus* probe is typically 1600–2300 seeds (Karron et al., 2006; Flanagan et al., 2009). Usually <400 of these seeds result from autogamous self-fertilization (Karron et al., 2004, 2006). Seed set for flowers receiving three probes is significantly (43.6 %) higher than seed set for flowers receiving a single probe (Karron et al., 2006). This suggests that stigmas often do not receive a saturating load of pollen during an initial probe.

Pollen carryover is often highly restricted in species pollinated by intensively grooming pollinators, such as *Bombus* (Thomson, 1986; Rademaker et al., 1997; Castellanos et al., 2003). In *M. ringens*, >90 % of the realized pollen dispersal from a donor flower is to the next three flowers in the visitation sequence (Holmquist, 2005). Despite this low level of pollen carryover, nearly all fruits are multiply sired (Mitchell et al., 2005). Flowers receiving a single probe average 3-12 outcross sires per fruit, indicating that pollen from multiple donors is often deposited at one time (Karron et al., 2006).

**Propagation of genets with unique marker genotypes**

In order to link floral visitation sequences with selfing rates of individual flowers, 20 replicate linear arrays were established, each composed of seven genets with unique multilocus combinations of homozygous genotypes at four unlinked allozyme loci [acid phosphatase (Acp-1, EC 3-1-3-2), aconitase (Aco-3, EC 4-2-1-3), glutamate oxaloacetate transaminase (Got-1, EC 2-6-1-1) and shikimate dehydrogenase (Skd-1, EC 1-1-1-25)]. These plants are a sub-set of 16 *M. ringens* genets that have been used extensively in previous paternity studies since they afford unambiguous assignment of paternity to all sampled offspring (Karron et al., 1995a, b, 1997, 2004, 2006). Each of these 140 plants (20 ramets of each of seven genets) was transplanted into 30 cm pots.

**Experimental plot and linear arrays**

The experimental plot was located at the University of Wisconsin-Milwaukee Field Station (Saukville, WI, USA). The plot contained a diversity of old field vegetation, including several species that are pollinated by bumble-bees. Since no other *Mimulus* plants were present and the nearest natural population is 15 km away, pots with flowering *Mimulus* plants were placed at this site so that bumble-bees would become familiar with the floral morphology of this species.

To ensure that flowers in each linear array were not visited prior to pollinator observations, all 140 *Mimulus* plants were placed into one of two pollinator-free screened enclosures on the evening prior to each observation day. These screened enclosures were also positioned on two opposite sides of the plot to increase the likelihood that bumble-bees would forage systematically along a linear array (similar to Morris et al., 1994).

**Observation of foraging sequences**

The linear arrays were set up on eight fair-weather days during August, 2002. At dawn, floral displays were trimmed to four flowers on six of seven genets in each array. The first plant in each array was not trimmed, and had 15–23 flowers. This larger display served to attract bees to the linear array.
Ten linear arrays were sequentially exposed to pollinators from 0830 h to 1130 h each day. A single wild bumble-bee was permitted to forage on each array and the foraging sequence was videotaped using a Sony Video 8 Handycam with ×12 optical zoom. Immediately following the foraging sequence, the array was returned to the greenhouse to protect the flowers from further visitation.

If a bee failed to probe all four flowers on each genet sequentially, or if a second bee landed on the array, the foraging sequence was discarded. Videotaped foraging sequences were reviewed to verify that pollinators sequentially probed all four flowers on each genet, and to determine the order of floral visits on each display. In six of the 80 foraging sequences a single bee sequentially probed all four flowers on each genet. Each of these six foraging sequences were by *B. fervidus* workers.

Stigmas of the visited flowers closed within 90 min following pollination. Approximately 3 h following pollination, labelled plastic tags were tied to pedicels of each flower on genets three to seven. Flowers on the second plant in each array were not tagged because we wanted to ensure that flowers assessed for selfing rates had potentially received out-cross pollen from at least two different donors. Once fruits had matured, all 120 fruits (4 flowers × 5 genets × 6 foraging sequences) were harvested.

**Genotyping progeny**

The 120 progeny arrays were germinated in separate 15 cm pots placed in a 20 °C plant growth chamber. When seedlings were 2 weeks old, they were transplanted into individual cells in plastic flats, and left to grow for an additional 3 weeks in a greenhouse. Twenty-five randomly selected seedlings from each progeny array were genotyped using the tissue extraction and electrophoretic methods of Karron et al. (2004). Seed germination rates were high (>80 %) and seedling mortality was minimal. In earlier work (J. D. Karron, R. J. Mitchell, K. G. Holmquist and J. M. Bell, unpubl. res.) no evidence was found for inbreeding depression at early stages of the life cycle. Therefore, it is unlikely that our selfing rate estimates are biased due to early mortality of inbred zygotes (Farris and Mitton, 1984).

**Data analysis**

Paternity was unambiguously assigned to all 3000 genotyped progeny, and we were therefore able to characterize selfing rates of each of the 120 fruits with a high degree of precision. Each fruit was classified as the first, second, third or fourth flower probed on a floral display. Sample sizes were equal (n = 30 fruits) in each class. Analysis of variance (ANOVA; JMP version 7.02; SAS Institute, 2007) was used to test for differences in selfing rate among the four visitation sequence classes, with individual ramets nested within bee foraging bouts. Bee foraging bout was treated as a random factor, and therefore its significance was tested over a denominator mean square of the sequence × bout interaction.

Selfing in the first flowers probed on each display is entirely attributable to autogamy. Assuming that the rate of autogamy is similar in the other three visitation sequence classes, the rate of geitonogamous selfing in these visitation classes can be estimated as follows: [geitonogamous selfing = (observed selfing rate) – (autogamous selfing rate in the first flowers probed)].

To quantify the proportion of self-fertilization attributable to geitonogamy, the ratio of geitonogamous selfing to total selfing in each visitation class was calculated.

**RESULTS**

The mean rate of self-fertilization varied significantly among the four visitation sequence classes (Table 1; Fig. 2). The mean selfing rate for initial probes (± s.e.) was 0.21 ± 0.03, for second flowers was 0.42 ± 0.03, for third flowers was 0.57 ± 0.04, and for fourth flowers was 0.78 ± 0.04 (Fig. 2). The selfing rate did not vary significantly among individual bee foraging bouts (Table 1; non-significant bout effect), and the effect of visitation sequence class was consistent across bouts (non-significant interaction). However, some ramets tended to have more selfing than others (significant ramet (bout) effect; ramet mean selfing rates varied from 0.25 to 0.88). We could not directly test for effects of genet because genets were visited in the same order in every bout, therefore confounding genet identity with position in the foraging bout. However, there was relatively little difference in selfing rate among genet–position combinations (selfing rate means

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**TABLE 1. Analysis of variance for the effects of visitation sequence class (whether the flower was the first, second, third or fourth sequentially probed flower on a plant) on selfing rate**

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>SS</th>
<th>F</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Visitation sequence class</td>
<td>3</td>
<td>5.277</td>
<td>95.375</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Bee foraging bout</td>
<td>5</td>
<td>0.510</td>
<td>1.440</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>Ramet (bee foraging bout)</td>
<td>24</td>
<td>1.907</td>
<td>4.309</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Visit sequence class × bee foraging bout</td>
<td>15</td>
<td>0.147</td>
<td>0.53</td>
<td>0.9</td>
</tr>
<tr>
<td>Error</td>
<td>72</td>
<td>1.328</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Model *R*² = 0.855. A bee foraging bout is a random effect, so its effect is tested over the interaction with visitation sequence class. Significant effects are highlighted in bold.

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**FIG. 2.** Effect of the pollinator visitation sequence on selfing rates in *Mimulus ringens*. Each data point shows the selfing rate for a single fruit, based on unambiguous assignment of paternity to 25 progeny per fruit. *n* = 30 for each visitation class.
ranging from 0.34 to 0.59 across the five different categories of genet–position combination).

Of the total among-fruit variation in selfing rate, 57% was attributable to the sequence of floral visitation within displays, while 20% was attributable to variation among ramets. On all 30 displays the selfing rate of the fourth flower probed was higher than the selfing rate of the first flower probed.

Estimated rates of geitonogamous selfing in the four visitation classes were: initial probes 0.00, second probes 0.21, third probes 0.37, fourth probes 0.57. Therefore, geitonogamous selfing accounted for nearly half of all selfing in second probes, and nearly three-quarters of all selfing in fourth probes.

**DISCUSSION**

The order of pollinator probes on *M. ringens* floral displays strongly and significantly influences selfing rates of individual fruits. Fruits resulting from initial probes are predominantly outcrossed, with a low rate of autogamous selfing. Since pollen carryover is very limited, successive flowers receive increasing amounts of geitonogamous self pollen. By the fourth flower probed, stigmas primarily receive geitonogamous self pollen, resulting in a high rate of self-fertilization. The implications of these findings are explored below.

*Factors influencing among-flower variation in selfing rates*

Most mating systems studies pool seeds of an entire plant, ignoring heterogeneity at finer spatial scales (Cruanz, 1998). However, Barrett et al. (1994) elegantly showed that selfing rates in *Eichhornia paniculata* increase from the bottom to the top of the determinate inflorescences. They attributed these patterns of selfing to bumble-bee behaviour, noting that bees tend to forage from the bottom to the top of an *Eichhornia* inflorescence. Our results build on this pioneering study and demonstrate that patterns of bumble-bee foraging directly influence the extent of geitonogamous self-fertilization in *M. ringens*. Three factors are especially likely to promote among-flower variation in selfing rates: (a) large floral display size; (2) limited pollen carryover; and (3) few successive probes to individual flowers.

The tendency of pollinators to probe several flowers in sequence on large daily floral displays (Klinkhamer et al., 1989; Robertson and Macnair, 1995; Goulson et al., 1998; Mitchell et al., 2004) leads to a positive relationship between floral display size and mean rate of self-fertilization (Harder and Barrett, 1995; Snow et al., 1996; Karron et al., 2004). Since sequentially probed flowers receive increasing amounts of geitonogamous self pollen, the among-flower variance in selfing on large displays is likely to be higher than the among-flower variance on small displays.

The extent of geitonogamous self-fertilization also strongly depends on patterns of pollen carryover (Robertson, 1992; Harder and Barrett, 1996). In *M. ringens*, most pollen from a donor flower is dispersed to the next three flowers in the visitation sequence (Holmquist, 2005). While the first flower probed may be largely outcrossed, the fourth flower probed may primarily receive geitonogamous self pollen. In contrast, in a species with more extensive pollen carryover, only a small fraction of the pollen deposited on the fourth flower will be geitonogamous self pollen. Therefore, species with extensive pollen carryover may exhibit little among-flower variation in selfing rate.

Accumulation of pollen on stigmas following several separate pollinator foraging bouts may also influence among-fruit differences in selfing rate (Dudash and Ritland, 1991; Mitchell et al., 2004). For example, a particular flower might be the first probed on the plant during one pollinator’s visit (and therefore would receive mostly outcross pollen from this probe), and the last for a later pollinator (and therefore would receive mostly self pollen). Depending on the length of the interval between probes and how this interval affects paternity, the resulting fruit may have an intermediate selfing rate reflecting the weighted mean contribution of the two probes. Indeed, *M. ringens* stigmas often receive one or two additional probes within 15–60 min of the first (Karron et al., 2006), and pollen from these additional probes successfully fertilizes ovules (Karron et al., 2006). This provides an opportunity for later probes to even out the variation in selfing rate due to the order of the probe within displays. Yet selfing rates nonetheless vary markedly in open-pollinated *M. ringens* (Karron et al., 2004). One possible explanation for this surprising variation is that successive bees may probe flowers on a display in a similar order, and may therefore deposit similar proportions of self and outcross pollen onto stigmas (J. D. Thomson, University of Toronto, Toronto, pers. comm.)

*Sibling competition and inbreeding depression*

Wide among-flower variation in selfing within *M. ringens* floral displays has important implications for offspring fitness. Fruits of this species are gravity dispersed, and seeds frequently germinate in clusters likely to correspond to individual fruits. Although analyses of bulk seed samples may suggest that a plant has an intermediate selfing rate, the genetic composition of individual fruits may actually be highly heterogeneous. Since the expression of inbreeding depression may be influenced by seedling competition (Schmitt and Ehrhardt, 1990; Koolenwijn, 2004), it may manifest very differently across sibships. Seedling size hierarchies are likely to be much more pronounced in fruits with mixtures of self and outcross progeny, and much less pronounced in fruits that only contain self progeny.

**Conclusions**

Adjacent flowers on *M. ringens* floral displays often vary markedly in patterns of self and outcross pollen deposition, leading to considerable heterogeneity in the genetic composition of sibships. This variation is largely attributable to the order of pollinator probes on floral displays. Although the first flower probed primarily receives outcross pollen, successive flowers receive increasing quantities of geitonogamous self pollen, leading to increasing rates of self-fertilization. Future research is needed to determine whether pollinator visitation patterns cause fine-scale variation in the mating system of other self-compatible Angiosperms, especially species with large floral displays and limited pollen carryover.
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LITERATURE CITED


