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

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• *Background and Aims* Adjacent flowers on *Mimulus ringens* floral displays often vary markedly in selfing rate. We hypothesized that this fine-scale variation in mating system reflects the tendency of bumble-bee pollinators to probe several flowers consecutively on multiflower displays. When a pollinator approaches a display, the first flower probed is likely to receive substantial outcross pollen. However, since pollen carryover in this species is limited, receipt of self pollen should increase rapidly for later flowers. Here the first direct experimental test of this hypothesis is described.
• *Methods* In order to link floral visitation sequences with selfing rates of individual flowers, replicate linear arrays were established, each composed of plants with unique genetic markers. This facilitated unambiguous assignment of paternity to all sampled progeny. A single wild bumble-bee was permitted to forage on each linear array, recording the order of floral visits on each display. Once fruits had matured, 120 fruits were harvested (four flowers from each of five floral displays in each of six arrays). Twenty-five seedlings from each fruit were genotyped and paternity was unambiguously assigned to all 3000 genotyped progeny.
• *Key Results* The order of pollinator probes on *Mimulus* floral displays strongly and significantly influenced selfing rates of individual fruits. Mean selfing rates increased from 21 % for initial probes to 78 % for the fourth flower probed on each display.
• *Conclusions* Striking among-flower differences in selfing rate result from increased deposition of geitonogamous (among-flower, within-display) self pollen as bumble-bees probe consecutive flowers on each floral display. The resulting heterogeneity in the genetic composition of sibships may influence seedling competition and the expression of inbreeding depression.

Key words: Autogamy, bee, *Bombus fervidus*, floral display, geitonogamy, mating system, monkeyflower, *Mimulus ringens*, paternity analysis, pollen carryover, pollinator visitation sequence, self-fertilization.

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.*, 1995*a*), 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; Carromero 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 ($s > 0{\cdot}80$) to predominant outcrossing ($s < 0{\cdot}20$; 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.

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FIG. 1. A bumble-bee approaching a floral display of *Mimulus ringens*.

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.*, 1995*a*, *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 screenhouse 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 outcross 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

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*

Source	d.f.	SS	*F*	*P*
Visitation sequence class	3	5·277	95·375	**<0·000001**
Bee foraging bout	5	0·510	1·440	<0·25
Ramet (bee foraging bout)	24	1·907	4·309	**<0·0001**
Visitation sequence class × bee foraging bout	15	0·147	0·53	0·9
Error	72	1·328		

Model $R^2 = 0{\cdot}855$. 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.

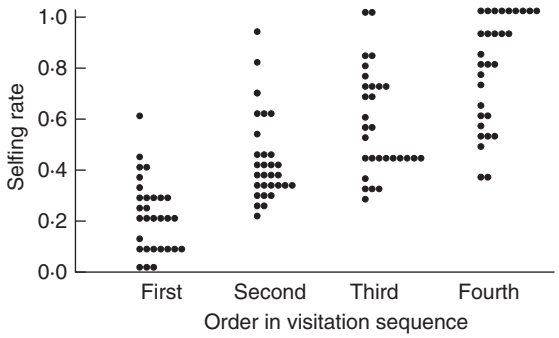

FIG. 2. Effect of the pollinator visitation sequence on selfing rates in *Mimulus ringens*. Each data point shows the selfing rate for a single fruit, based on unambiguous assignment of paternity to 25 progeny per fruit. $n = 30$ for each visitation class.

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 (Cruzan, 1998). However, Barrett *et al.* (1994) elegantly showed that selfing rates in *Eichhornia paniculata* increase from the bottom to the top of the indeterminate 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; Koelewijn, 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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