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# Effect of Pollination and Postpollination Processes on Selfing Rate

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EFFECT OF POLLINATION AND POSTPOLLINATION PROCESSES ON SELFING RATE  
ON *MIMULUS RINGENS*

by  
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Partial Fulfillment of the  
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## ABSTRACT

### EFFECT OF POLLINATION AND POSTPOLLINATION PROCESSES ON SELFING RATE ON *MIMULUS RINGENS*

by

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The University of Wisconsin-Milwaukee, 2016  
Under the Supervision of Professor Jeffrey Karron

Selfing rates vary widely within and among populations of self-compatible flowering plants. This variation is often attributed to differences in the amount and timing of self and outcross pollen deposition on stigmas, as well as to the influence of postpollination mechanisms that control pollen siring success. This study explores the relative importance of pollination and postpollination processes in determining selfing rates in monkeyflower, *Mimulus ringens*. We hand-pollinated flowers on each of 17 unrelated mothers with pollen from one of three experimental treatments intended to replicate field conditions: 1) Simultaneous deposition of 50% self pollen and 50% outcross pollen from 5 unrelated donors. 2) Self pollen followed 15 min later by application of an equal amount of outcross pollen from 5 unrelated donors. 3) Outcross pollen from 5 unrelated donors followed 15 min later by application of an equal amount of self pollen. We genotyped 757 progeny at 8 polymorphic microsatellite loci and used paternity exclusion to determine whether each seedling was self or outcross. When self and outcross pollen arrived simultaneously, observed proportions of self and outcross progeny did not deviate from the expected 1:1 ratio. However, when outcross pollen was applied 15 min prior to self pollen, there was a significant excess of outcross progeny. Selfing rate in *Mimulus ringens* is influenced by the timing of pollen arrival, but not by non-random postpollination sorting.

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## INTRODUCTION

Selfing rates of animal-pollinated flowering plants often vary widely within and among populations (Cruzan and Barrett, 1996). This variation has been attributed to pollination events that affect the amount and timing of self and outcross pollen deposition on stigmas (Williams and Mazer, 2016) and to postpollination events that influence pollen siring success (Cruzan and Barrett, 2016). Both pollination and postpollination events can influence offspring quality and population genetic structure (Karron et al., 2012), but they are rarely studied concurrently and therefore their relative contributions to variation in selfing rate remain largely unexplored.

Pollination events, mediated by interactions between plants and pollinators, influence the amount and proportion of self and outcross pollen deposited on stigmas (Mitchell et al., 2004, 2013; Devaux, 2014) and the order of self and outcross pollen arrival (Epperson and Clegg, 1987; Spira et al., 1996; Burkhardt et al., 2009). Earlier arriving pollen typically has a siring advantage over late-arriving pollen, and this difference is often positively associated with the time interval between pollinator visits to a flower (Epperson and Clegg, 1987; Burkhardt et al., 2009).

Postpollination events can also affect the realized selfing rate (Snow and Spira, 1991; Kruszewski and Galloway, 2006). Selfing rates are often lower than would be expected based on the relative amounts of self and outcross pollen deposited on stigmas (Johnston, 1993; Cruzan and Barrett, 2016). This discrepancy may be the result of competitive interactions amongst pollen grains, or of interactions between male gametophytes and stylar tissue. These postpollination processes may influence both the relative fertilization success of self and outcross pollen, and also the relative success of pollen from different outcross donors (Marshall and Ellstrand, 1986; Marshall and Folsom 1991; Rigney et al., 1993; Mitchell and Marshall 1998). For instance, if outcross pollen tubes grow more rapidly than self pollen tubes, then they may sire a disproportionate number of ovules, even if deposited well after the arrival of self pollen grains on the stigma (Lloyd, 1992; Spira et al., 1996; Nemeth and Smith-Huerta, 2002). Competition favoring outcross pollen may result in a decreased proportion of self progeny, reducing the

expression of inbreeding depression and altering population genetic structure (Baker and Shore, 1995; Karron et al. 2012; Lankinen et al., 2016).

Previous studies of *Mimulus ringens* suggest that pollinator behavior strongly affects mating patterns (Mitchell et al., 2004; Karron et al., 2004). For example, pollinators often probe more flowers sequentially on large floral displays (geitonogamy; Mitchell et al., 2004; Eckert et al., 2009), leading to a significant increase in the rate of self-fertilization (Karron et al., 2004; 2009). Likewise, the potential for postpollination processes to affect selfing rates in *Mimulus* is strong because pollinators often deposit mixtures of self and outcross pollen (Karron et al., 2009). However, the influence of postpollination processes on selfing rate in *M. ringens* remains largely unstudied.

The present study explores the relative importance of pollination and postpollination processes in determining selfing rates in *Mimulus ringens*. Our experimental design closely matches patterns of pollen delivery in the wild (Karron et al., 2004, 2006; Holmquist et al., 2012). By altering the order of arrival of pollen deposition on stigmas, this study addresses two questions: (1) Do self and outcross pollen differ in siring success? (2) Do selfing rates depend on the order of arrival of self and outcross pollen on stigmas?

## **MATERIALS AND METHODS**

**Study species**—*Mimulus ringens* L. (Phrymaceae) is a diploid perennial herb native to wet meadows of central and eastern North America. The showy purple hermaphroditic flowers open before dawn and last for half a day. Populations typically have selfing rates of 25-40% (Karron and Mitchell, 2012; Karron et al., unpublished). Controlled single-donor self and outcross hand pollinations with abundant pollen do not differ in number of seeds per fruit, germination rate, or seedling survival (Karron et al., unpublished data).

When a bumble bee visits a *Mimulus* flower, 6,000-10,000 pollen grains representing 2-5 outcross pollen donors are typically deposited on the stigma (Karron et al., 2006; Flanagan et al., 2009). Most flowers receive a second bee visit within 15 min of the initial probe (Karron et al., 2006). Flowers receiving a second probe produce more outcross seeds and have higher mate diversity than flowers

receiving a single probe, indicating that pollen deposited during sequential probes can contribute to siring success (Karron et al., 2006).

***Experimental design***—We grew pollen donor and pollen recipient plants from seed in the University of Wisconsin-Milwaukee greenhouse. All of the plants were the product of crosses between pairs of unrelated plants derived from a single large natural population in Akron, Ohio. The 5 pollen donors and 17 pollen recipients in the experimental crosses shared no parents in common.

While flowers were still in bud, we emasculated 3 flowers on each of the 17 pollen recipients to prevent autonomy. The following morning we hand-pollinated each flower with an equal mixture of self and outcross pollen applied in one of three ways (experimental treatments): 1) Simultaneous deposition of 50% self pollen and 50% outcross pollen from 5 unrelated donors. This 1:1 mixture was applied twice, separated by a 15 min interval. 2) Self pollen followed 15 min later by application of an equal amount of outcross pollen from 5 unrelated donors. 3) Outcross pollen from the same 5 unrelated pollen donors followed 15 min later by application of an equal amount of self pollen. The total amount of pollen applied to stigmas was consistent across treatments.

We prepared fresh mixtures of outcross pollen each morning, immediately prior to controlled pollinations. We used a microspatula to scoop all pollen from an equal number of anthers from each of the 5 donors onto a microscope slide. Since the extracted pollen was often clumped, we used a probe to mix the pollen thoroughly. We applied a mean of  $10,094 \pm 337.9$  pollen grains (N=15) during each pollen application using a calibrated microspatula. The left half of both lobes of the stigma always received the first pollen load, while the right half of both lobes received the second pollen load. For the simultaneous treatment, equal quantities of self and outcross pollen were mixed together and applied simultaneously.

We collected mature fruits 45 days following pollination. We separately germinated seeds from all sampled fruits and harvested leaf tissue from 8 week old seedlings. Seedling survival exceeded 95%. A total of 757 progeny, 14-15 from each of 3 treatments on 17 maternal plants, were genotyped at 8



microsatellite loci (Miri24, Miri27, Miri40, Miri46, Miri58, Miri78, Miri82 and Miri95), following the protocols of Nunziata et al. (2012). Each locus had 4-9 alleles. Progeny genotypes were compared to genotypes of the 17 maternal plants and 5 pollen donors. We used simple paternity exclusion to determine whether each seedling was the product of self- or cross-fertilization. For 78% of the outcross progeny, we were also able to unambiguously determine the identity of the male parent.

To quantify the mean number of pollen grains per flower for each of the 5 pollen donors we placed 2 anthers into a 1mL 70% ethanol solution, and counted samples on a depression slide. Three 8 $\mu$ L samples of suspended pollen were quantified for each flower. Since flowers have 4 anthers, pollen production per flower was calculated as mean number of grains /  $\mu$ L x 2000  $\mu$ L. Pollen production averaged 158,996 grains per flower, with plant means ranging from 121,332 to 193,010 grains.

**Data analysis**—For each of the 3 experimental treatments, we performed a  $\chi^2$  test to determine whether there was a significant deviation from the expected 1:1 ratio of self and outcross progeny. For the subset of outcross progeny where we were able to unambiguously determine the identity of the male parent, we also performed a  $\chi^2$  test to determine if the observed outcross sire ratio was significantly different than the expected ratio based on relative proportions of outcross pollen in the mix.

## RESULTS

**Siring success of self and outcross pollen**—When equal quantities of self and outcross pollen were applied simultaneously to the stigma, the mean selfing rate ( $\pm$  SD) across mothers was  $49.9 \pm 18.7$  % (Fig. 1). Thus, self and outcross pollen were equally successful at fertilizing ovules, matching the expected 1:1 ratio based upon proportions of self and outcross pollen on the stigma ( $\chi^2 = 0$ , df = 1,  $P > 0.05$ ). When self pollen was applied to the stigma 15 min prior to application of outcross pollen, the observed selfing rate was  $54.8 \pm 17.9$  %, which did not differ significantly from the expected 1:1 ratio ( $\chi^2 = 2.29$ , df = 1,  $P > 0.05$ ). When outcross pollen was given a 15 min head start, the observed selfing

rate was  $39.6 \pm 17.4$  % and was significantly different than the expected 1:1 ratio ( $\chi^2 = 11.19$ ,  $df = 1$ ,  $P < 0.001$ ).

***Siring success of outcross pollen donors***—Across all treatments, the proportion of seeds sired varied markedly amongst the five pollen donors (Fig. 2). Since some of the paternity share variation may reflect differences in pollen production, we adjusted the expected paternity shares of each donor by the relative number of pollen grains per flower. Even with this adjustment, observed outcross sire ratios were significantly different than expected sire ratios ( $\chi^2 = 42.48$ ,  $df=4$ ,  $P < 0.001$ , Fig. 2). For example, pollen donor D sired 3 times as many seeds as donor C, even though the donors had similar pollen production per flower.

## DISCUSSION

Variation in selfing rates within and among flowering plant populations has been attributed to pollination events that influence the amount and timing of pollen deposition on stigmas, and postpollination events that affect the relative siring success of self and outcross pollen (Medrano et al., 2012; Cruzan and Barrett, 2016; Lankinen et al., 2016; McCallum and Chang, 2016). Although pollination and postpollination events can markedly affect offspring quality and population genetic structure, these processes have rarely been studied concurrently to determine their relative contribution to selfing rate variation. The findings of the present study suggest that for *M. ringens*, selfing rates are largely influenced by the order of arrival of self and outcross pollen grains, with minor contributions of postpollination sorting.

***Importance of pollination events*** – Pollinator foraging patterns and number of pollinator probes have previously been shown to influence the selfing rate in *M. ringens* (Mitchell et al., 2004; Karron et al., 2004, 2006). Such pollination events can influence the order of arrival of self and outcross pollen on the

stigma, and time lags may then determine relative siring success of different pollen donors (Epperson and Clegg, 1987; Spira et al., 1996). For example, in *Ipomoea purpurea* pollen arriving 30 minutes following first pollinations sired 14% fewer ovules (Epperson and Clegg, 1987). Late arriving pollen also sired significantly fewer ovules in *Hibiscus moscheutos* (Spira et al., 1996). In the present study, we altered the order of arrival of self and outcross pollen loads with a time lag of 15 minutes that matches the interval between floral probes in nature. When applied first, outcross pollen had a statistically significant 11% siring advantage over self pollen. When self pollen was applied first, the selfing rate did not differ significantly from the null expectation. Since *Mimulus ringens* pollen tubes typically take 8-10 hours to fertilize ovules (Mitchell unpublished), a 15 minute head start corresponds to just 3% of the fertilization window.

Our estimate of the influence of pollination events is probably an underestimate because we applied the first and second pollinations adjacent to one another, rather than being layered as would occur following bee visitation. Layering is likely to reduce the success of later arriving pollen (Mitchell and Marshall, 1995), so effects of the timing of pollen delivery might well be stronger in the field.

***Importance of postpollination events*** – When we pollinated flowers of *M. ringens* with an equal mix of self and cross pollen, the resulting selfing rate was 49.9%, suggesting that postpollination sorting does not discriminate against self pollen. However, studies of several other species suggest that selfing rates may often be strongly affected by postpollination processes (Cruzan and Barrett, 2016). Reduced siring success of self pollen has been attributed to competition amongst pollen grains as well as interactions between pollen and the style (Aizen et al., 1990; Snow and Spira, 1991; Nemeth and Smith-Huerta, 2002). For example, when equal quantities of self and outcross pollen were applied to *Campanulastrum americanum* stigmas, the percentage of self progeny ranged from 6 – 34% across maternal plants (Kruszewski and Galloway, 2006). Similarly, when self and outcross pollen were applied simultaneously to *Erythronium grandiflorum* stigmas, the selfing rate was 32% and significantly lower than expected (Rigney et al., 1993).

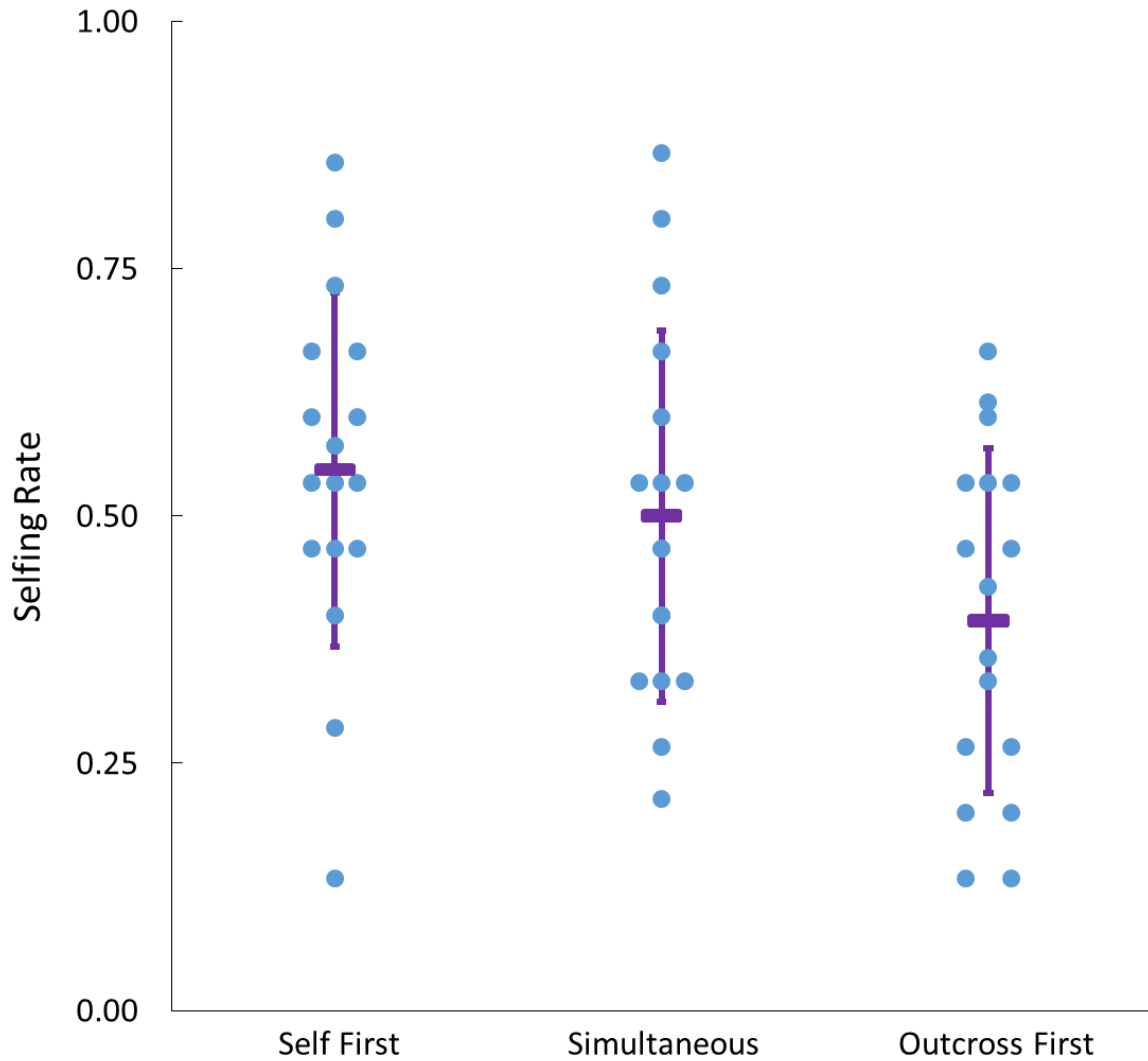
In *Mimulus ringens* self and outcross pollen arriving simultaneously on the stigma were equally successful in siring ovules. This suggests that in this species there should be a close correspondence between rates of self-pollination and self-fertilization. Indeed, this finding is consistent with our previous studies showing a close correlation between geitonogamous pollinator movements and geitonogamous self-fertilization (Karron et al., 2004; 2009).

***Siring success of outcross pollen donors*** – Postpollination events have also been shown to influence the relative fertilization success of different outcross pollen donors. For example, in a study of *Raphanus sativus* pollen donors differed significantly in pollen tube growth rate and number of seeds sired (Marshall and Diggle, 2001). In our study, there were significant differences amongst outcross pollen donors in the proportion of progeny sired (Fig. 2). Paternity shares of each of the five donors differed significantly from expected proportions, even following adjustment for differences in pollen production of each donor (Fig. 2). This suggests that postpollination processes may discriminate amongst sires even though mean performance of outcross pollen did not differ from mean performance of self pollen.

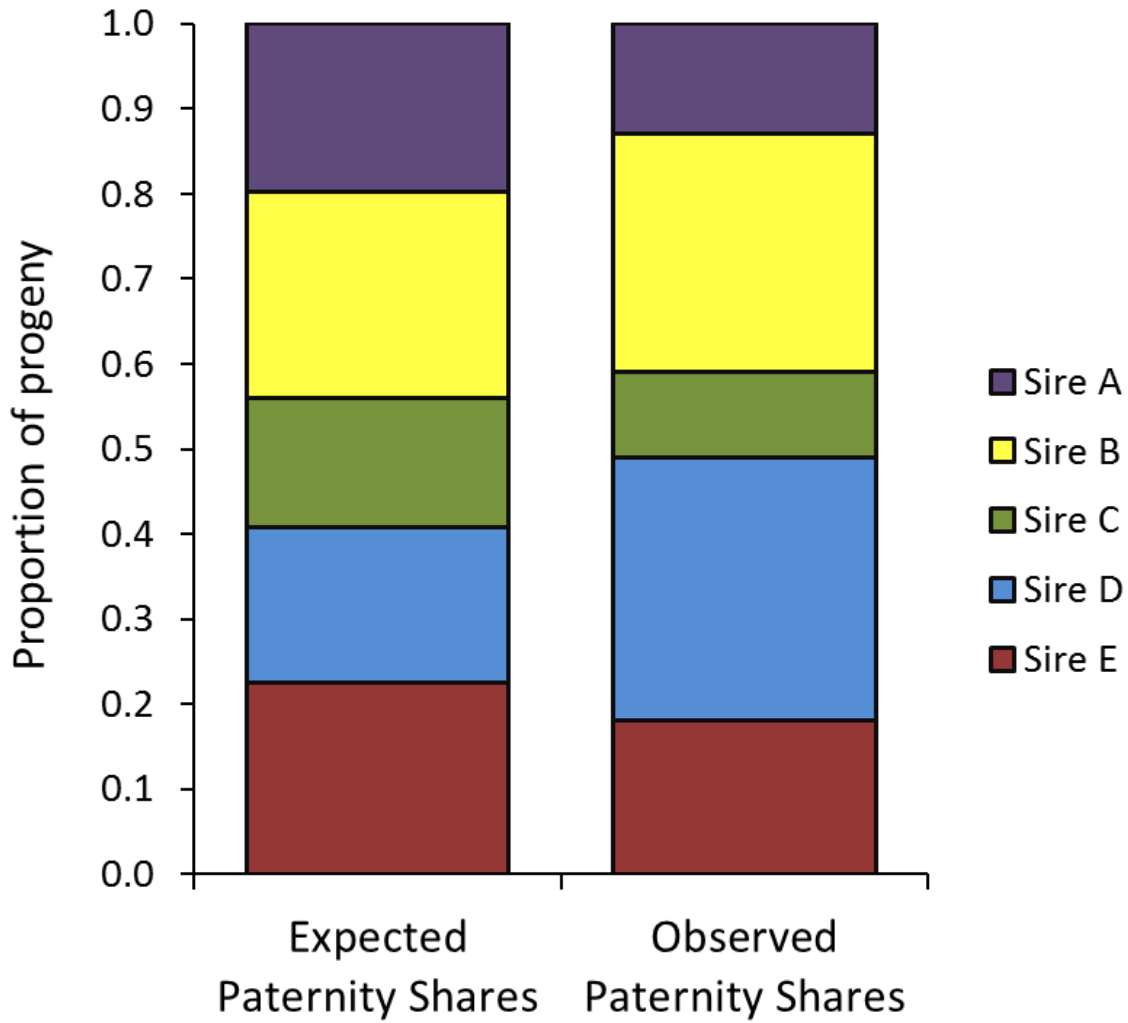
## CONCLUSIONS

In our study, order of arrival of self and outcross pollen is important in determining selfing rates in *Mimulus ringens*. When outcross pollen arrived first, the outcrossing rate was significantly higher than the expected 1:1 ratio. However, when applied simultaneously, self and outcross pollen were equally successful at siring seeds. This suggests that for *Mimulus ringens* selfing rates are influenced by pollination processes, but not by postpollination processes.

These results underscore how important the dynamics of the pollination process can be for plant mating systems. Subtle variation in the timing of self and outcross pollen arrival may lead to considerable differences in selfing rates among flowers, even when subsequent pollinator probes deliver pollen loads differing markedly in composition.



**Fig. 1.** Variation in selfing rates among mothers following 3 types of experimental pollinations: 1) self pollen deposition followed 15 min later by an equal application of outcross pollen; 2) simultaneous deposition of 50% outcross and 50% self pollen; 3) outcross pollen deposition followed 15 min later by an equal application of self pollen. For each treatment, blue data points represent the selfing rates for a single fruit from an individual maternal plant (N = 17). Mean is shown with purple horizontal bar, and SD is shown with purple vertical bar.



**Fig. 2.** Proportion of progeny sired by each of five unrelated outcross pollen donors (Sire A – E), pooled across pollination treatments. Expectations based on the relative pollen production of each pollen donor. The observed paternity shares differed significantly from the expected paternity ( $\chi^2 = 42.48$ ,  $df=4$ ,  $P<0.001$ ).

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