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# PATTERNS OF MULTIPLE PATERNITY IN FRUITS OF *MIMULUS RINGENS* (PHRYMACEAE)[1]

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Multiply sired fruits provide unambiguous evidence that pollen from two or more donors was deposited on a stigma and successfully fertilized ovules. Such multiple paternity within fruits can have important consequences for both parental and offspring fitness, but little is known about the frequency of multiple paternity or the mechanisms causing it. In this study we quantify the extent of multiple paternity in replicate experimental arrays of *Mimulus ringens* (square-stem monkeyflower) and use observations of pollinator behavior to infer mechanisms generating multiply sired fruits. In each array, floral displays were trimmed to two, four, eight, or 16 flowers per plant to span the range of display sizes observed in nature. In our sample of 204 fruits, more than 95% had two or more outcross pollen donors. The number of sires per fruit averaged 4.63 ± 0.10 (mean ± 1 SE), including selfs, and did not vary significantly with floral display treatment. Patterns of bumble bee foraging, combined with limited pollen carryover, suggest that observed levels of multiple paternity cannot be fully explained by single probes that deposited mixed pollen loads. Multiple probes to flowers, each delivering pollen from 1–3 different sires, are more likely to have caused the observed patterns. These sequential visits may reduce the potential for pollen competition and female choice based on pollen tube growth rate.

**Key words:** *Bombus*; monkeyflower; multiple paternity; multiply sired fruits; paternity analysis; pollen carryover; pollination.

Angiosperm fruits often contain multiple seeds. These seeds may be sired by a single pollen donor, resulting in a full-sib family, or several pollen donors, resulting in a mixture of full and half-siblings within the same fruit. An increase in the number of sires per fruit decreases the genetic relatedness among siblings (Ritland, 1989) and increases the genetic variance in the progeny (Falconer, 1981). Since the occurrence of multiply sired fruits may have important consequences for both parental and offspring fitness (Ellstrand and Marshall, 1986; Marshall, 1991; Marshall and Folsom, 1991; Karron and Marshall, 1993; Paschke et al., 2002), a greater understanding of both the mechanisms generating multiple paternity within fruits and the occurrence and frequency of this phenomenon are needed.

There are two mechanisms through which mixed pollen loads and multiply sired fruits might arise (Ellstrand, 1984; Schemske and Pautler, 1984; Marshall and Ellstrand, 1985; Bernasconi, 2003). Under the first mechanism (''simultaneous deposition'') pollen from several donors is deposited by a single pollinator probe. This mixed load of pollen results from pollen carryover. A second mechanism (''sequential deposition'') involves deposition of pollen from different donors during several distinct pollinator probes. The two mechanisms are not mutually exclusive: a stigma may receive several probes, each depositing pollen from multiple donors (Dudash and Ritland, 1991). An important difference between the two mechanisms is that simultaneous deposition of a mixed load allows pollen of several donors to begin growing at the same time, while sequential deposition gives a head start to the pollen of just one or a few donors, whose pollen was deposited during the earliest floral probe. Because this head start decouples pollen tube growth rate from the order of arrival at ovules, sequential deposition limits the scope for pollen competition and/or female choice based on pollen tube growth rate (Marshall and Ellstrand, 1985; Snow, 1986).

The relative importance of these two mechanisms and the overall rate of multiple paternity in fruits should be influenced both by pollinator behavior and plant characteristics. For example, multiple paternity through simultaneous deposition is more likely when pollen carryover is extensive (Campbell, 1998). In contrast, multiple paternity through sequential deposition is more likely if the amount of pollen deposited during a single probe is insufficient to fertilize all ovules and the time between probes is short compared to the time required for pollen tubes to reach ovules (Dudash and Ritland, 1991; Campbell, 1998).

The extent of multiple paternity might also be influenced by the floral display. The number of flowers probed consecutively on a plant is often positively correlated with display size (Klinkhamer et al., 1989; Dudash, 1991; Robertson, 1992; de Jong et al., 1993; Mitchell et al., 2004). As the number of flowers probed on each plant increases, a larger fraction of the pollen load deposited during a single probe may be geitonogamous self-pollen (Barrett et al., 1994; Harder and Barrett, 1995, 1996; Snow et al., 1996; Karron et al., 2004). In addition, if the rate of pollen carryover is independent of floral display size, an increase in number of consecutive probes on each plant may lead to a reduction in number of outcross donors represented in a single pollen load (Campbell, 1998).

In this study we quantify the extent of multiple paternity in *Mimulus ringens* (square-stem monkeyflower) and use observations of pollinator behavior to infer mechanisms generating multiply sired fruits. We address the following questions: (1) How many mates sire the progeny of individual fruits? (2)

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Does floral display size affect the number of sires per fruit? (3) What mechanisms produce multiply sired fruits?

## MATERIALS AND METHODS

***Study system***—*Mimulus ringens* L. (Phrymaceae) is a perennial herb native to wetlands of central and eastern North America. This species is self-compatible and has a mixed mating system (Karron et al., 1995a, 2004). The purple, zygomorphic, 2 cm deep flowers each last a single day. The anthers dehisce before dawn, and the bilobed stigmas usually close within 90 min after pollination. Stigma closure is nearly complete by midday, by which time flowers have typically received 2–4 pollinator probes (Mitchell et al., 2004). *Mimulus ringens* flowers are primarily pollinated by workers of three bumble bee species (*Bombus fervidus*, *B. griseocollis*, and *B. impatiens* (Karron et al., 1995a, b; Mitchell et al., 2004). When visiting flowers, bumble bees first contact the stigma and then the anthers with their faces. *Mimulus ringens* stigmas are papillose, and the small (12 μm diameter) pollen grains are frequently deposited in layers, each with several thousand grains (Bell et al., in press). Nearly all flowers produce capsules, which contain 700–6000 seeds.

Questions concerning multiply sired fruits in *M. ringens* were addressed as part of a larger study examining the influence of floral display on selfing rates and reproductive success (Karron et al., 2004; Mitchell et al., 2004). We briefly summarize the overall methodology of that work below and then explain how we use those data to determine the extent of multiple paternity within *Mimulus* fruits.

***Estimates of multiple paternity***—To facilitate assignment of paternity to all sampled progeny, we bred a set of 16 *M. ringens* genets with unique multilocus combinations of homozygous genotypes at four allozyme loci (Karron et al., 1995a, 1997, 2004). We clonally replicated these genets and planted them in four separate experimental arrays of 36 plants at the University of Wisconsin–Milwaukee Field Station. To minimize gene dispersal between arrays, gardens were separated by 75 m of old field vegetation containing several unrelated bumble bee-pollinated taxa. Gene flow from natural populations of *M. ringens* was also unlikely since the nearest population is >15 km away.

Each array contained 36 ramets planted in a square grid with 0.8 m spacing between plants. Single ramets of each of 15 different genets were planted in the center of the array (see Fig. 1a in Karron et al., 2004). These ''central genets'' were planted in a different random order in each array. These unique genotypes were then surrounded by a buffer row composed of 21 ramets of a 16th genet (genet ''D'' in all arrays).

On 11 August 2000, we used scissors to trim floral displays for each ramet to two, four, eight, or 16 flowers. We trimmed the four ramets of each genet to a different floral display, and each array had a mixture of all four floral displays (see Fig. 1 in Karron et al., 2004). Manipulations were performed in the early morning hours, before pollinators became active. Pollinators foraged freely in the arrays throughout the remainder of the day.

Two teams of 2–3 observers began recording patterns of pollinator movement in the four arrays at 0620 hours. During 20-min observation periods we recorded the exact sequence of floral probes and plant visits by each bee, noting each visited plant's identity and spatial position and the number of flowers probed. We followed individual bees for as long as possible to obtain complete visitation sequences within an array. The two teams rotated observations among the four arrays until 1100 hours, when nearly all stigmas had closed and pollinator activity had noticeably declined. Bees probed a mean (±1 SE) of 18.2 ± 2.36 flowers ($N = 72$ foraging bouts) before they departed an array. At the end of the day we tagged up to four flowers on each central genet and 1 mo later collected the ripened fruits.

Because each central genet had a unique multilocus genotype, we used paternity exclusion based on allozyme genotypes (Ellstrand, 1984; Karron et al., 1995a) to unambiguously determine paternity for up to 10 seedlings from each of the resulting 204 fruits (total $N = 1949$ seedlings; 9.5 ± 0.8 seedlings per fruit). Because *M. ringens* fruits typically contain thousands of seeds (Karron et al., 2004), our 10-seedling samples can provide only minimum estimates of multiple paternity. To investigate whether observed levels of multiple paternity are a function of the number of seedlings sampled per fruit, we genotyped an additional 10 seedlings from each flower on the two-flowered displays. This enabled us to compare the number of sires observed in samples of 10 seeds and 20 seeds from each fruit.

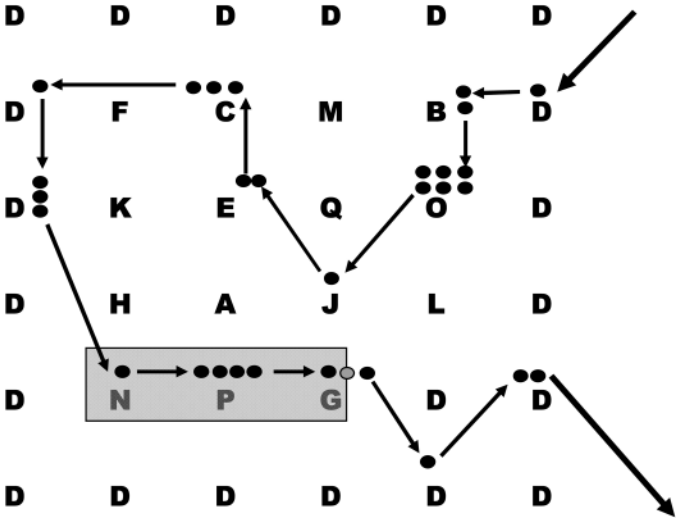


Fig. 1. Diagram of an experimental array, illustrating the sliding window of pollinator visitation used to estimate pollen loads deposited on flowers. Letters indicate individual ramets of each genet. The arrows and dots indicate the foraging route for one representative *Bombus fervidus* bout, which occurred in array number 4 at 1004 hours. Dots indicate individual flowers probed on each plant, and arrows indicate the bee's flight path. The shaded box outlines the six-flower window for the second flower probed on genet ''G'' (that flower is indicated by a gray dot).

***Pollen carryover window***—To determine whether observed levels of multiple paternity could result solely from deposition of mixed pollen loads during single probes, we generated pollinator-based expectations for the number of sires per fruit. These approximations of the composition of individual pollen loads were generated from (1) observations of pollinator behavior during this study and (2) data on patterns of pollen carryover in *M. ringens*. Holmquist et al. (2002; K. G. Holmquist, unpublished data) estimated pollen carryover by establishing linear arrays composed of distinct marker genets, each with a single unvisited flower. More than 99% of the seedlings sired by each donor flower were represented in fruits from the first five flowers probed in sequence following the visit to the donor flower (Holmquist et al., 2002; K. G. Holmquist, unpublished data). Therefore, we chose to use a six-flower pollen carryover window as a generous estimate of the pool of potential pollen donors for any given recipient flower.

We applied this carryover window to all foraging bouts observed on this day to estimate pollen loads deposited by a pollinator as it probed each flower. For example, in the bout shown in Fig. 1, the bee initiated foraging on a border plant, probed two flowers on genet ''B,'' six on Genet ''O,'' one on Genet ''J,'' two on Genet ''E,'' and so forth. Given the limited pollen carryover in this species, any given flower should receive the great majority of pollen from the most recently probed six flowers, and we estimated this as follows: Consider the second flower probed on Genet ''G'' (lower middle of the diagram). Here, the shaded box indicates the most recently probed six flowers, which include one self-flower, four flowers on genet ''P,'' and one flower on genet ''N.'' Thus, this focal flower had two outcross donors in the six-flower window. If paternity is determined solely by the composition of pollen deposited during a single probe, and the six-flower carryover window adequately describes the pollen available for deposition, we would expect this fruit to have two different outcross donors (plus self). For the next (third) flower probed on genet ''G,'' only one outcross donor is represented in the six-flower window. We used this procedure to quantify the number of outcross donors in the six-flower window for all flowers in the visitation record. We

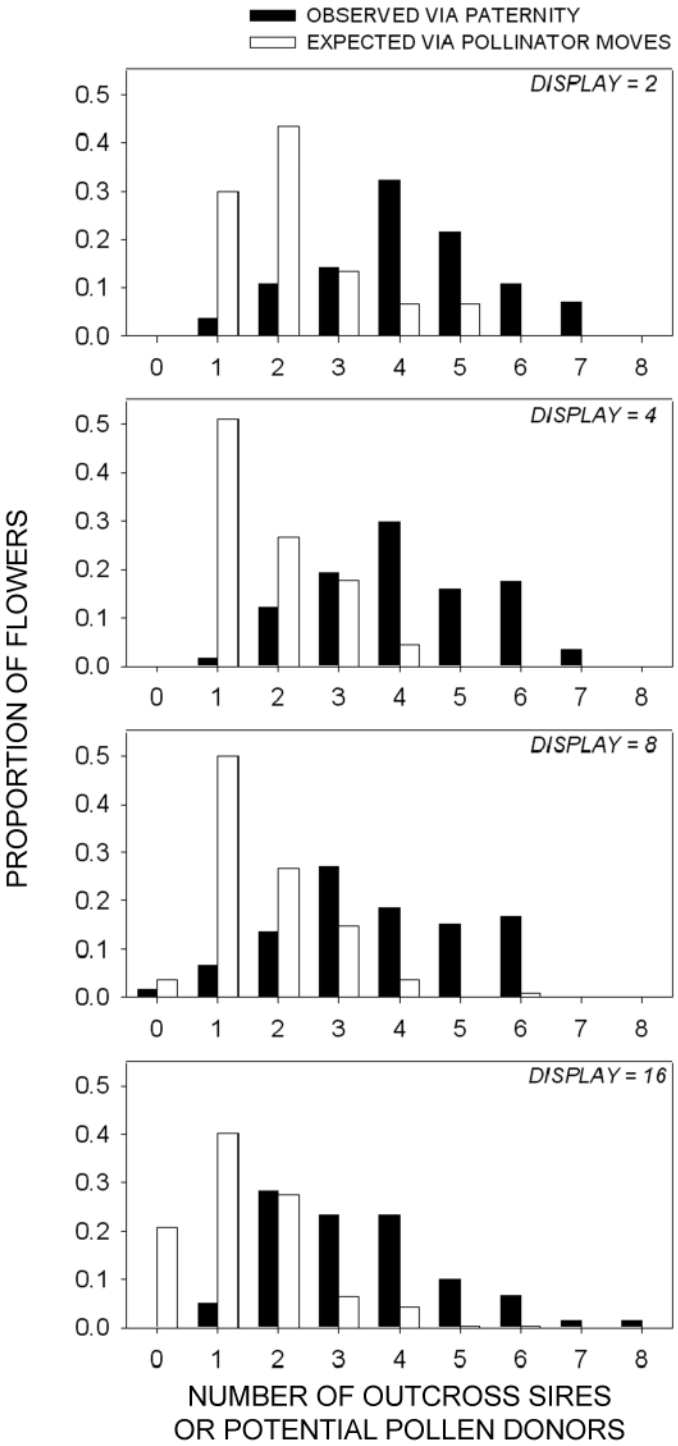


Fig. 2. Comparison of the distribution of the expected number of pollen donors represented in a single pollen load and the observed number of sires per fruit across four floral display classes.

excluded the first six flowers probed during a foraging bout, since we had no data on the flowers previously probed at that point, and only applied the sliding window to flowers on central genets.

***Analysis***—To test for differences in levels of multiple paternity in fruits among display classes we used the Generalized Linear Model, with Poisson errors and a log link function (Proc GENMOD; SAS Institute, 2000), with floral display as the independent variable. This method is a generalization of ANOVA that relaxes the assumption of continuous and normal error distributions and is therefore appropriate for our integer count data on number of sires per fruit (Agresti, 1996). Our conclusions are unchanged if we instead use standard ANOVA.

To compare the observed number of sires per fruit with that expected based on pollinator behavior, we used $\chi^2$ goodness of fit tests (Zar, 1999), pooling categories as needed to achieve expected values >5.

TABLE 1. Tests comparing observed and expected number of sires per fruit for each floral display treatment. Values shown are from a goodness of fit test for the hypothesis that the observed and expected distributions are the same. In the tests, observed values are based on actual paternity, and expected values are based on the number of pollen donors predicted to be deposited during a single bee visit, based on pollinator behavior and a six-flower pollen carryover window (see Fig. 2). Means for sires per fruit and for predicted number of donors are presented in the text. (Results: pollen carryover window).

| Display | $\chi^2$ | df | *P* |
|---|---|---|---|
| 2 | 10.3 | 2 | 0.006 |
| 4 | 28.4 | 3 | 0.00001 |
| 8 | 23.7 | 4 | 0.00009 |
| 16 | 26.6 | 5 | 0.0007 |

## RESULTS

***Estimates of multiple paternity***—Multiple paternity was very common in our study, with two or more outcross pollen donors detected in 95% (194/204) of the fruits. Including the self-parent as a sire, more than 99% of the fruits were multiply sired. Only one fruit out of 204 was completely selfed. Overall, the mean number of sires per fruit, including selfs, was 4.63 ± 0.10 sires ($N$ = 204, range = 1–9). The number of outcross sires in each fruit was 3.78 ± 0.10.

The total number of sires per fruit (including selfs) did not vary significantly with floral display treatment ($\chi^2_3 = 2.85$, $P > 0.40$). The number of sires per fruit in two-flower displays was 4.96 ± 0.29, in four-flower displays was 4.91 ± 0.18, in eight-flower displays was 4.47 ± 0.20, and in 16-flower displays was 4.37 ± 0.19. The slight (nonsignificant) tendency of many-flowered displays to have fewer sires may partly reflect the higher rate of selfing in large displays. Since fewer outcross seedlings were present in the 10-seed progeny arrays from large displays (Karron et al., 2004), there was reduced opportunity for detecting additional sires.

***Pollen carryover window***—Our observations of pollinator behavior suggest that the pollen deposited on stigmas by single probes usually includes very few outcross donors (Fig. 2, Table 1). In most cases only one to three donors were represented in the six most recently probed flowers, with the overall mean across displays = 1.5 (1 SE = 0.05; $N$ = 419 flowers with inferred visitation history). The number of potential outcross donors in the six-flower window varied significantly as a function of display treatment ($\chi^2_3 = 24$, $P < 0.0001$; Fig. 2). The number of potential donors in the window decreased steadily with increasing floral display (number of genets in the six-flower carryover window for two-flower displays was 2.17 ± 0.21, for four-flower displays was 1.76 ± 0.13, for eight-flower displays was 1.68 ± 0.10, and for 16-flower displays was 1.36 ± 0.07).

For all floral display size classes, the observed distribution

of sires per fruit was significantly greater than the expected distribution, which was based on the likely number of donors represented in pollen deposited during a single visit (Table 1, Fig. 2). Note the interesting downward shift in the distribution of the expected number of outcross pollen donors with increasing floral display. For 21% of the probes on 16-flower displays, nearly all of the pollen deposited during a single visit is likely to have been self-pollen. To examine whether the relationship between the extent of multiple paternity and the expected number of donors represented in a single pollen load depends on carryover properties, we repeated our analyses with larger carryover windows. Even with a carryover window as long as 10 flowers, the observed distribution of sires per fruit significantly exceeded the expected distribution of donors represented in a single pollen load for all floral display classes (results not shown).

Our estimates of outcross multiple paternity are minimum estimates because we sampled only 10 progeny from the thousands of seeds in a typical fruit. Sampling additional progeny would almost certainly increase the observed number of sires per fruit. We were able to directly test this prediction by genotyping an additional 10 seedlings for the 24 fruits on two-flowered displays. We then compared the number of sires per fruit for the original 10-seedling samples with our estimates for the 20-seedling sample.

In the additional 10 seedlings (9.5 scorable seedlings on average) sampled from fruits on two-flowered displays we detected a mean of $1.8 \pm 0.2$ new sires. The total number of sires in 20 seedling samples, including selfs, was 6.8 sires (6.0 sires excluding selfs). The new donors tended to be rare; only $2.8 \pm 0.3$ seedlings out of the 9.5 additional seedlings per fruit were sired by these donors—the other 6.7 seedlings were sired by donors already represented in the first 10 seedlings.

## DISCUSSION

Nearly all *Mimulus ringens* fruits were multiply sired, with a mean of 4.63 pollen donors per fruit. This is among the highest levels of multiple paternity reported in flowering plants. Low levels of multiply sired fruits were noted in studies of *Asclepias exaltata* (6.9% of fruits multiply sired) (Broyles and Wyatt, 1990) and *Phaseolus vulgaris* (5.8% and 8.2% of fruits multiply sired over 2 yr) (Ibarra-Perez et al., 1996). Moderate levels of multiple paternity were reported for *Raphanus sativus* (68–85% of fruits multiply sired over 3 yr; mean number of sires per fruit was 2.0 to 2.2) (Ellstrand, 1984; Ellstrand and Marshall, 1986), *Glycine argyrea* (60% of fruits multiply sired; mean number of sires per fruit was 1.7) (Brown et al., 1986) and *Yucca filamentosa* (minimum of 58.6% of fruits multiply sired; mean number of sires per fruit was 2.0) (Massey and Hamrick, 1999). High levels of multiple paternity have been reported for *Ipomopsis aggregata* (100% of fruits multiply sired; mean number of sires per fruit was 4.4) (Campbell, 1998); *Eucalyptus rameliana* (mean effective number of mates per fruit was 3.85) (Sampson, 1998); *Mimulus guttatus* (mean effective number of mates per fruit was 3.33) (Dudash and Ritland, 1991); and *Mimulus ringens* (present study).

Although we detected high levels of multiple paternity within fruits, the values we observed are likely to be underestimates for the following reasons: First, we genotyped only 10 out of more than 4000 seeds per fruit. Genotyping a larger sample of progeny in each fruit would undoubtedly reveal more fathers, especially those that are rare. When we doubled our sample of genotypes per fruit, the mean number of sires per fruit increased to 6.8. Second, the border ramets in each garden were genetically identical (all genotype ''D''), and we could not determine which ''D'' ramet sired offspring. Had additional genetic markers been available for these border plants, our estimate of the number of sires per fruit might have been somewhat higher. However, this underestimate in number of sires per fruit might have been modest, since we detected no difference in number of sires per fruit as a function of distance from the border row ($\chi^2_2 = 0.8$, $P > 0.6$). Third, our experimental arrays had 16 genotypes (36 individuals), setting a clear upper limit on the number of potential sires. Although natural populations of *Mimulus ringens* often have fewer than 50 individuals, this design constraint might underestimate the number of sires per fruit in some wild populations.

The high level of multiple paternity in *M. ringens* fruits is a major aspect of the mating system and is likely to play an important role in the reproductive biology of the species. Offspring in multiply sired fruits are less closely related than are offspring in singly sired fruits, and this may affect the extent and variability of sib competition among zygotes within fruits and among seedlings in the soil (Hamilton, 1964; Kress, 1981; Karron and Marshall, 1990, 1993). Indeed, studies of wild radish (*Raphanus sativus*) and other species have documented increases in seed set, fruit set, and seed size for multiply sired fruits (Ellstrand and Marshall, 1986; Marshall and Ellstrand, 1986; Marshall, 1991; Paschke et al., 2002), and these changes are likely to affect offspring vigor as well. Comparison of these traits for single and multiply sired fruits is necessary to determine whether these patterns also hold in *M. ringens*.

***Floral display and levels of multiple paternity***—Floral display did not influence the observed number of sires per fruit. One potential explanation for the lack of an effect of display size is that on both large and small displays pollinators frequently probe just one or two flowers before leaving the plant (Mitchell et al., 2004). Therefore, flowers on large displays may often receive pollen from nearly as many donors as do flowers on small displays.

The only other study to examine the effect of floral display on levels of multiple paternity within fruits was Campbell's (1998) research on *Ipomopsis aggregata*. Campbell developed a computer simulation of pollen deposition following hummingbird visits to *Ipomopsis* flowers. She concluded that an increase in mean floral display from seven to 13 flowers would only cause a slight reduction in the extent of multiple paternity within fruits. Since this hummingbird-pollinated species has a very high level of pollen carryover (Price and Waser, 1982; Waser and Price, 1984), a modest increase in number of probes on each plant would not substantially reduce the number of donors represented in single pollen loads deposited on stigmas.

***Mechanisms of multiple paternity***—The evolutionary implications of the observed high levels of multiply sired fruits in *Mimulus ringens* depend on the timing of deposition of pollen from each donor. In *Mimulus ringens* pollen of just one or two outcross donors is likely to be deposited during a single probe (Fig. 2). Therefore, the observed levels of multiple paternity cannot be attributed solely to a single probe that deposits a mixed load with numerous donors.

The mismatch between the observed patterns of multiple paternity within fruits and our estimate of the number of donors likely to be represented in a single pollinator probe is

largely due to the fairly restricted level of pollen carryover in *M. ringens* (<1% of pollen dispersed more than five flowers beyond a donor flower). However, an increase in the carryover window to 10 flowers would still not result in simultaneous deposition of pollen from as many donors as are represented in *M. ringens* fruits. Note that our comparison of the distribution of multiple paternity within fruits and the number of pollen donors likely to be represented in a single pollen load is a conservative test for two reasons: First, our experimental design is likely to have underestimated the number of sires per fruit. Second, our carryover window model assumes equal representation in the pollen load of all flowers in the window. Yet, the roughly exponential decline in pollen carryover (K. G. Holmquist, unpublished data) should cause pollen from early donors to be perhaps an order of magnitude less common than pollen from more recently probed flowers. Including such reduced carryover in the calculations above (by decreasing probability of siring seedlings by 50% with each step) decreases mean number of sires in the six-flower window by 20–40% (unpublished results from this study.)

Sequential deposition of pollen was quite plausible in our study. Since *Mimulus* flowers received 0.7 bee visits per flower per hour (Mitchell et al., 2004), flowers may have frequently received a second and perhaps a third visit prior to stigma closure. Visits in fairly rapid sequence (e.g., 20 min apart) may be especially likely to result in multiple paternity (Epperson and Clegg, 1987). Detailed studies linking the visitation history of individual flowers with patterns of paternity in individual fruits will be necessary to further confirm the potential for this mechanism.

Studies of *Raphanus sativus* (Marshall and Ellstrand, 1985) and *Ipomopsis aggregata* (Campbell, 1998) suggest that multiply sired fruits in those species could be explained solely by deposition of a single mixed pollen load. This mechanism does not appear to satisfactorily explain the high levels of multiple paternity observed in *Mimulus ringens*. Instead, we believe that multiple paternity in this species results from sequential probes, each carrying pollen from a small number of donors. Our findings are consistent with Dudash and Ritland's (1991) studies of the correlation of paternity in fruits of *Mimulus guttatus*, a species with flowers that frequently last 4 d. Dudash and Ritland (1991) experimentally removed corollas to prevent additional pollinator visits after 24 h. They estimated that the effective number of mates in fruits of unmanipulated flowers was 30% higher than in fruits of flowers open for just a single day. Therefore, Dudash and Ritland (1991) also concluded that sequential visitation contributes to multiple paternity.

Our findings suggest that multiply sired fruits in *Mimulus ringens* result from multiple probes to flowers, each probe delivering pollen from 1–3 different sires. This combination of the two mechanisms (simultaneous and sequential deposition) allows some potential for pollen competition and female choice, but also allows the order of arrival of pollen loads to play a role in determining paternity. The relative importance of these factors will depend on how often pollinators visit, the amount and composition of pollen deposited with each visit, and variation among pollen donors in pollen performance. Further experimental field work and controlled pollination experiments are necessary to explore these possibilities (Mulcahy et al., 1983; Marshall and Ellstrand, 1985; Snow, 1986; Mitchell and Marshall, 1998).

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