

EFFECTS OF FLORAL DISPLAY SIZE AND BIPARENTAL INBREEDING ON OUTCROSSING RATES IN *DELPHINIUM BARBEYI* (RANUNCULACEAE)¹

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Floral display size represents a tradeoff between the benefits of increased pollinator visitation and the quantity of pollen received vs. the costs of increased self-pollination and reduced pollination quality. Plants with large floral displays often are more attractive to pollinators, but pollinators visit more flowers per plant. Intraplant foraging movements should increase self-pollination through geitonogamy, lowering outcrossing rates in large plants. Local genetic structure should also increase inbreeding and decrease outcrossing estimates, if pollinators move between neighboring, related plants. These predictions were tested in a population of larkspurs (*Delphinium barbeyi*) in Colorado. Allozymes were used to estimate outcrossing rates of plants varying in display size. Floral displays varied widely (2–1400 flowers; 1–26 inflorescences per plant), and outcrossing rate decreased significantly with increasing display size. Large, multistalked plants self over twice as frequently as single-stalked plants (46 vs. 21%). Local population structure is significant, and biparental inbreeding depresses outcrossing in plants surrounded by genetically similar neighbors. Protandry, coupled with stereotypical bottom-up pollinator foraging, reduces self-fertilization by autogamy or geitonogamy within inflorescences. Selfing is predominantly (>60%) by geitonogamy between inflorescences in large plants. Geitonogamy may be a significant cost to plants with large floral displays if inbreeding depression and/or pollen and ovule discounting results. If so, floral display size, particularly inflorescence number, may be under contrasting selection for pollination quantity vs. quality.

Key words: Colorado; *Delphinium barbeyi*; geitonogamy; genetic structure; inflorescence size and number; pollinator behavior; Ranunculaceae; selfing rate.

Floral display size—the number of flowers per inflorescence, plant, or genet—presents an evolutionary dilemma (Charlesworth and Charlesworth, 1987a; Klinkhamer and de Jong, 1993; Finer and Morgan, 2003). In animal-pollinated species, large floral displays may increase pollinator visitation, and thus pollen receipt and donation (Willson and Rathcke, 1974; Klinkhamer et al., 1989; Mitchell, 1994; Emms et al., 1997; Ohashi and Yahara, 2001). However, increased attractiveness of large floral displays is often associated with an increased frequency of intraplant pollinator movements during each visit (Geber, 1985; Hessing, 1988; Vaughton and Ramsey, 1998; Ohashi and Yahara, 2001; Galloway et al., 2002; Karron et al., 2004; Huang et al., 2006). Sequential moves between flowers on the same plant increases the potential for geitonogamy, the transfer of self-pollen between flowers on an individual plant. Such geitonogamous selfing can incur a number of fitness costs in both self-compatible and self-incompatible species (de Jong et al., 1993; Klinkhamer and de Jong, 1993; Harder and Barrett, 1995, 1996; Snow et al., 1996). If selfed offspring are less fit than those resulting from

outcrossing, then geitonogamy will result in inbreeding depression (Darwin, 1876; Charlesworth and Charlesworth, 1987b; Husband and Schemske, 1996). Deposition of self-pollen on stigmas may interfere with outcross pollen deposition, germination, growth, and fertilization, usurping ovules that might otherwise be outcrossed (Lloyd, 1992; de Jong et al., 1993; Klinkhamer and de Jong, 1993; Harder et al., 2001). Finally, self-pollen deposited on stigmas is unavailable for male reproductive success through outcrossing, resulting in pollen discounting (Charlesworth, 1980; Holsinger et al., 1984; Lloyd, 1992; Harder and Barrett, 1996). In addition, whereas within flower selfing (autogamy) may confer ecological advantages of reproductive assurance in low or variable pollinator environments, geitonogamy, by requiring pollinators, contributes little to reproductive assurance but retains the costs of showy floral display (Lloyd, 1992; Johnston, 1998; Kalisz and Vogler, 2003; Ashman et al., 2004; Morgan and Wilson, 2005). Thus, the evolution of floral display size depends on the tradeoffs between the benefits of attraction in terms of pollination quantity and the costs of geitonogamy, including its effects on pollination quality.

While floral display size influences pollinator behavior in ways that promote geitonogamy, quantifying the relationship between floral display, outcrossing rate, and modes of selfing has only begun. By comparing similar species (Sun and Ganders, 1988; Williams et al., 2001), populations within species (Brunet and Sweet, 2006a), or groups of individuals within populations (Appendix), the effects of display size variation on mean selfing rates have been examined at the population or group level. Because large sample sizes are required to accurately estimate individual outcrossing rates (Ivey and Wyatt, 1999; Ritland, 2002), outcrossing rate variation among individual plants differing in display size

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within a population remains poorly known. To date, intrapopulation studies have generally manipulated display size in artificial arrays and used plants homozygous for allozyme markers to detect outcrossing (Crawford, 1984; Snow et al., 1996; Karron et al., 2004; but see Vrieling et al., 1999 for an alternative approach). Studies examining natural populations used floral emasculations and genetic markers to dissect the components of the mating system (Schoen and Lloyd, 1992; Leclerc-Potvin and Ritland, 1994; Eckert, 2000; Herlihy and Eckert, 2004; Brunet and Sweet, 2006b). I am aware of only one study that examined the relationship between outcrossing and floral display size in a natural population of unmanipulated plants (Brunet and Eckert, 1998; in *Aquilegia caerulea*). All but one (Herlihy and Eckert, 2004) of these previous studies have supported a negative relationship between display size and outcrossing rate (Appendix). Yet, in most of these studies, display size varied over a relatively narrow range (<20 flowers per plant open per day). Some plants produce much larger floral displays with hundreds or even thousands of flowers open simultaneously, and little is known about the fitness tradeoffs of pollination quantity vs. quality in such species (de Jong et al., 1992; Barrett et al., 1994; Snow et al., 1996). In particular, plants that produce multiple flowering stalks of the same genet, including clonal species, may undergo high levels of self-fertilization (Handel, 1985; Eckert, 2000).

Pollinator movements between plants are usually spatially restricted (Levin and Kerster, 1974; Pyke, 1978; Waser, 1982), and neighboring plants are often more genetically related than random (Heywood, 1991; Williams, 1994; Williams et al., 2001). Pollen transfer between such genetically related near-neighbors results in biparental inbreeding (Uyenoyama, 1986) and will bias estimates of the selfing rate upward (Waller, 1993; Griffin and Eckert, 2003). Thus, autogamy, geitonogamy, and biparental inbreeding all may influence the selfing rate estimated from genetic markers (Ritland, 1984; Schoen and Lloyd, 1992; Eckert, 2000; Herlihy and Eckert, 2004). All three forms of inbreeding may affect the evolution of plant mating systems and the stability of intermediate outcrossing rates, although in different ways. For instance, autonomous selfing (autogamy without pollinator visitation) is often selectively advantageous because it confers reproductive assurance, while facilitated autogamy (requiring pollinator visitation) is almost never advantageous because of gamete discounting. Likewise, geitonogamy is disadvantageous because of severe gamete discounting and may be an unavoidable consequence of selection for outcross pollination (Lloyd, 1992; de Jong et al., 1993; Herlihy and Eckert, 2004; Goodwillie et al., 2005). Biparental inbreeding, by reducing both the genetic (transmission) cost of outcrossing and the fitness difference between self and outcross matings (lowering inbreeding depression), can either limit or facilitate selection for selfing (Lloyd, 1979; Uyenoyama et al., 1993; Waller, 1993).

The potential for biparental inbreeding depends on the local density of related flowering plants, their degree of relatedness, and the relative size of their floral displays. This combination of factors determines a plant's local mating environment (LME). A neighboring plant with many flowers may contribute more pollen than a similarly related small neighbor. Moreover, plants surrounded by many related pollen sources (high floral density) should appear more selfed because of their higher proportion of biparental inbreeding. Hence, large plants in dense neighborhoods of related individuals should have the highest levels of inbreeding because of the combined effects of

geitonogamy and biparental inbreeding. The LME should therefore be associated with the selfing rate of individual plants, and the proportions of uniparental vs. biparental inbreeding making up the total effective selfing rate should depend on plant size and its LME.

In an earlier study (Williams et al., 2001), the effects of different floral display size between two sympatric larkspur species, *Delphinium barbeyi* and *D. nuttallianum* was investigated. *Delphinium barbeyi* has multiple inflorescences with many flowers ($\bar{X} = 450$) per plant, while *D. nuttallianum* has a single flowering stalk with few flowers per plant ($\bar{X} = 4.7$). It was found that bumblebees and to some extent hummingbirds, the two primary pollinators, visited more flowers per plant as flower number increased. On larger plants, bees visited multiple inflorescences. *Delphinium barbeyi* had significantly lower outcrossing rates than *D. nuttallianum*. Given these floral and mating system differences lead to more restricted gene flow, fine-scale genetic structure was much more pronounced in *D. barbeyi*. It was hypothesized that the differences in outcrossing rates seen between these species should also be found within *D. barbeyi* populations, among individual plants differing in flower and inflorescence number.

To investigate the effects of floral display size and the LME on outcrossing rate variation, I estimated outcrossing rates of individual *D. barbeyi* plants from a natural, unmanipulated population in Colorado. In particular, I tested the following predictions: (1) large plants, with more flowers or inflorescences, have lower outcrossing rates than small plants, (2) between inflorescence geitonogamy is the predominant form of self-fertilization in large, multistemmed plants, and (3) high local density of related flowering plants increases biparental inbreeding and decreases estimates of individual plant outcrossing rates.

MATERIALS AND METHODS

Species and study site—*Delphinium barbeyi* Huth (Ranunculaceae) is a long-lived, herbaceous perennial native to subalpine meadows in Colorado (Warnock, 1997). Individual plants form distinct clumps, reaching diameters of up to 1 m. Plants produce 1–40+ inflorescences ($\bar{X} = 16$), each with approximately 25 flowers. Total flower number per plant ranges from <10 to >1400 ($\bar{X} = 450$; Williams et al., 2001). The main pollinators are bumblebees (particularly *Bombus appositus* and *B. flavifrons*) and hummingbirds (*Selasphorus platycercus*, *S. rufus*, and *Stellula calliope*) (Waser, 1982; Williams et al., 2001). Bees and hummingbirds preferentially visit plants with larger floral displays, and visit more flowers on large vs. small plants (Williams et al., 2001). A *D. barbeyi* population near the present study site had a multilocus outcrossing rate (t_m) of 0.55 (± 0.04 SE). In a survey of genetic structure within 12 populations using spatial autocorrelation, there was significant local genetic similarity among neighboring plants (Williams et al., 2001).

Field work for this study was conducted from 24 July to 10 August 2002 in a population of *D. barbeyi* located immediately south of the Rocky Mountain Biological Laboratory (RMBL, 2900 m a.s.l.) in southwestern Colorado, USA. Physical and floristic characteristics of the site are described elsewhere (Langenheim, 1962; Williams et al., 2001). The population contained several hundred flowering plants of all sizes. All flowering plants ($N = 144$) in a 14×26 m area were tagged and mapped to the nearest 10 cm.

Collection of plant material and electrophoresis—For each tagged plant in the plot, the number of flowering stalks (inflorescences) per plant and the number of flowers and fruits per inflorescence were counted. Fresh leaf tissue was collected from each plant for immediate electrophoretic analysis of adult genotypes. As fruits matured, 1–6 infructescences were collected from each plant. Infructescences were placed in paper bags and sealed for several days in

TABLE 1. Allele frequencies, maternal fixation index (F), population-level, single-locus outcrossing rates (t_s), and correlation of selfing (r_s -families) among families in a population of *Delphinium barbeyi*. Estimates are based on genotypes of 144 plants for adult frequencies, and 1003 seeds from 51 maternal families for pollen and ovule frequencies, fixation index, outcrossing rates, and correlations. Standard errors are shown in parentheses after the estimates.

Allele	Adult	Pollen	Ovule	F	t_s	r_s -families
<i>Pgi-1</i>	0.646	0.630	0.598	0.210 (± 0.125)	0.803 (± 0.044)	0.044 (± 0.084)
<i>Pgi-2</i>	0.090	0.087	0.088			
<i>Pgi-3</i>	0.264	0.283	0.314			
<i>Pgm-1</i>	0.128	0.184	0.127	0.127 (± 0.126)	0.481 (± 0.044)	0.428 (± 0.237)
<i>Pgm-2</i>	0.674	0.634	0.706			
<i>Pgm-3</i>	0.198	0.182	0.167			
<i>Acp-1</i>	0.701	—	—	—	—	—
<i>Acp-2</i>	0.299					

plastic garbage bags with moth crystals (p -dichlorobenzene) to kill any seed predators. Fruits were then allowed to air dry in a warm greenhouse for several weeks. Bulk samples of seeds were collected from each infructescence for electrophoresis and outcrossing estimation. Seeds used for outcrossing analysis were therefore sampled from several fruits per infructescence.

Using fresh leaf tissue, I genotyped each flowering plant on the grid ($N = 144$) for three polymorphic enzyme loci; phosphoglucose isomerase (*Pgi*; three alleles); phosphoglucomutase (*Pgm*; three alleles), and acid phosphatase (*Acp*; three alleles). Electrophoretic techniques, grinding, and running buffers were as described for *D. nuttallianum* in Williams and Waser (1999). Several tissue samples from different sides of large plants (clumps) were genotyped to confirm they were a single plant. Only *Pgi* and *Pgm* had activity in seeds, so outcrossing rates were estimated based on only two polymorphic loci. Progeny arrays segregated as expected for all maternal genotypes, and all potential paternal alleles were detected in outcrossed progeny.

Mating system analysis—Using bulk seed samples from 51 plants that spanned the range of display size variation, I estimated outcrossing rates for (1) the population as a whole, (2) groups of plants with similar display sizes, and (3) individual plants. From 12–40 ($\bar{X} = 19.7$) seeds from each maternal plant had scorable enzyme activity ($N = 1003$ total progeny). All mating system analyses were performed using the computer program MLTR (ver. 3.1, revised September 2004; Ritland, 2002) on progeny arrays with known maternal genotypes. Standard errors of mating system parameter estimates were calculated from the standard deviation of 1000 bootstrap estimates where progeny arrays were the unit of resampling (population and group level analyses), or 100 bootstraps per family where individuals within families were resampled (individual plant level analysis). Ninety-five percent confidence intervals (CIs) of the estimates were calculated as 1.96 times the standard error.

Population level—Population level single- (t_s) and multilocus (t_m) maximum likelihood outcrossing rates were estimated for the entire sample of 51 plants, regardless of size. In addition, the maternal fixation index (F) and the correlation of selfing (r_s) among families and among loci were estimated using the Newton–Raphson method. Because only 51 plants from a large population of potential pollen donors were sampled, pollen and ovule allele frequencies (p) were not constrained to be equal (i.e., t , p , and F were jointly estimated). However, estimates of pollen and ovule allele frequencies did not differ significantly (Table 1) and constraining them to be equal does not alter the results.

Group level—Maximum likelihood outcrossing rates and the correlation of selfing among loci were jointly estimated for the 51 plants grouped by size, under the mixed mating model assumption that pollen pool allele frequencies are equal for all groups (Ritland, 2002). Plants were grouped in two different ways: five groups spanning the range of plant sizes (1, 2–3, 4–7, 8–11, and 12–26 inflorescences) and two groups splitting the size distribution in half (1–8 inflorescences vs. 9–26 inflorescences). Because group sizes are small (Table 3) pollen and ovule gene frequencies were constrained to be equal. This increases statistical efficiency because one less parameter is estimated. Using the alternative assumption of unequal pollen and ovule gene frequencies does not alter the results.

Individual plant level—Maximum likelihood estimates of individual plant outcrossing rates were calculated for each seed family, holding pollen allele

frequencies constant across families. Ritland (2002) recommends using the method-of-moments estimation procedure (Cruzan and Arnold, 1994) as being less upward biased (Ivey and Wyatt, 1999) for individual family outcrossing estimates. Multilocus, method-of-moments family level estimates were computed in MLTR for comparison to maximum likelihood estimates. Both methods gave similar, correlated outcrossing estimates ($r = 0.742$). Because the standard errors for the method-of-moments estimates ($\bar{X} = 0.235$) were larger than for the maximum likelihood estimates ($\bar{X} = 0.172$), I report only the latter results. As can be seen, standard errors of individual plant outcrossing rate estimates can be large, so I also estimated the minimum outcrossing rate (t_{min}) for each plant. This was calculated as the proportion of unambiguously outcrossed seeds (containing a nonmaternal allele) in each progeny array. Although such measures underestimate the true outcrossing rate (because of biparental inbreeding and limited marker variation), a strong correlation between t_m and t_{min} indicates that their rank orders are correct.

Biparental inbreeding—I used two methods to estimate the amount of biparental inbreeding at the population and group levels. Because multilocus estimates of outcrossing (t_m) are less sensitive to biparental inbreeding than single-locus estimates (t_s), their difference, $t_m - t_s$, is frequently used to estimate its magnitude (Shaw et al., 1981; Ritland, 2002). However, $t_m - t_s$ tends to greatly underestimate the true magnitude of biparental inbreeding, especially when estimates are based on few loci (Leclerc-Potvin and Ritland, 1994; Ritland, 2002; Griffin and Eckert, 2003; Herlihy and Eckert, 2004). The correlation of selfing among loci (r_s) provides an alternative measure of the degree of biparental inbreeding contributing to the overall “effective selfing” rate (true selfing plus biparental inbreeding; Ritland, 1984, 2002). When estimates of r_s are < 1.0 , some loci in an individual progeny’s multilocus genotype indicate outcrossing, while other loci appear selfed as the result of biparental inbreeding. When selfing rates are low ($1 - t = s < 0.2$), r_s corresponds to the proportion of s due to true uniparental selfing, with the remainder of the selfing rate due to biparental inbreeding (Ritland, 2002). With higher selfing rates, r_s should still give a relative measure of the importance of biparental inbreeding in groups of plants of different sizes.

Analysis of spatial genetic structure and the local mating environment—Patterns of genetic similarity as a function of distance between plants were estimated using multilocus spatial autocorrelation (Smouse and Peakall, 1999) with the program GenAlEx (Peakall and Smouse, 2001). The multilocus genetic distance among individuals was used to estimate the genetic correlation (r) as a function of distance among all 144 mapped, flowering adults. The 95% CIs of the genetic correlations were estimated from 999 permutations of the genetic and geographic distance matrices. A correlogram was generated for genetic correlations in 2.5 m distance classes.

If local genetic structure facilitates biparental inbreeding, then detectable outcrossing should depend on a plant’s relative size and genetic similarity with its neighbors. An index of the local mating environment (LME) around each plant was devised to attempt to account for these effects on individual plant outcrossing rates. For each plant (target), all other flowering plants within a 2-m radius (neighborhood) were identified. For each neighbor, the number of alleles at the *Pgi* and *Pgm* loci that differed from the target were found (0–4) and multiplied by the number of flowers on the neighboring plant. For example, if the target plant has the multilocus genotype *Pgi-11*, *Pgm-23*, then a neighbor with the genotype *Pgi-22*, *Pgm-22* would differ by two alleles detectable as

TABLE 2. Maternal fixation index (F), average single-locus (t_s) and multilocus (t_m) outcrossing rates, the correlation of selfing among families ($r_{s-families}$), and two measures of biparental inbreeding, $t_m - t_s$ and the correlation of selfing among loci (r_{s-loci}), in a population of *Delphinium barbeyi*. Estimates are based on genotypes of 1003 seeds from 51 maternal families. Standard errors are shown in parentheses after the estimates.

F	t_s	t_m	$r_{s-families}$	$t_m - t_s$	r_{s-loci}
0.173 (± 0.093)	0.661 (± 0.034)	0.685 (± 0.036)	0.197 (± 0.090)	0.024 (± 0.010)	0.507 (± 0.173)

outcrosses (at *Pgi*) from the target. The local mating index for the target plant is calculated as the sum of the number of different alleles \times flowers on all surrounding plants, divided by the total number of alleles \times flowers (including those on the target plant) within its neighborhood. The LME index varies from 0 to ~ 1 . Low values indicate that few unique alleles are found in the neighborhood, making it difficult to detect outcrossing (thus, biparental inbreeding would be high). High values of the LME index indicate that neighboring plants are genetically distinct from the target and that outcrossed pollen will be more easily detected in the target's progeny (and biparental inbreeding would be low).

Effects of floral and ecological characteristics on the mating system—Population and group level estimates of mating system parameters were tested for significance by the nonoverlap of their 95% CIs. The significant deviation of estimates from zero or one and the difference between estimates for different floral display size groups were tested with two-tailed tests. The relationships among floral characters (inflorescence and flower number), the LME index, and individual plant outcrossing rates were examined using linear regression in Statview for Windows (ver. 5.0.1, SAS Institute, Cary, North Carolina, USA). The joint effects of plant size and LME on outcrossing rate were tested with multiple regression in Statview.

RESULTS

Distribution of plant size—The size of flowering displays ranged from 1–26 inflorescences, with a median size of four flowering stalks ($\bar{X} = 6.1 \pm 0.48$ SE, $N = 144$). The total number of flowers per plant ranged from 2–1413, with a median of 71 ($\bar{X} = 169.2 \pm 23.3$ SE, $N = 122$). There was a strong correlation between flower number (Fls) and inflorescence number (Infls) per plant ($r = 0.89$), as well as between inflorescence number and plant diameter ($r = 0.74$). The 51 plants used in the mating system analysis had a size distribution similar to the population as a whole, with a slight oversampling of large plants (median Infls = 6.0, range 1–26; median Fls = 111, range 2–1382).

Outcrossing rates—**Population level**—Pollen and ovule allele frequencies did not differ significantly from each other or from the allele frequencies of the 144 sampled adult plants in

the population (based on overlapping CIs, not shown; Table 1). The multilocus outcrossing rate (t_m) for the population was 0.685 (± 0.036 SE), indicating a mixed mating system (Table 2). The correlation of selfing among families ($r_{s-families} = 0.197$) was low, indicating high variance in outcrossing rates among plants. Although the single-locus outcrossing estimates for *Pgi* and *Pgm* differed substantially, the average of single locus estimates ($t_s = 0.661 \pm 0.034$) was only slightly lower than t_m (Tables 1 and 2). Two measures of biparental inbreeding gave differing results (Table 2). The difference between $t_m - t_s$ was low (0.024 ± 0.010), suggesting little mating among relatives. In contrast, the low correlation of selfing among loci ($r_{s-loci} = 0.507 \pm 0.173$) suggests that as much as half of the apparent selfing occurring in the population is the result of biparental inbreeding.

Individual plants—The range of multilocus outcrossing rate (t_m) estimates for individual plants varied from 0.16 to 1.20 ($\bar{X} = 0.635 \pm 0.033$). The estimated maternal fixation index (F) was 0.203 (± 0.009). Estimates of t_m slightly exceeded unity (1.025–1.200) for four families with doubly heterozygous maternal genotypes. These high “out-of-bound” estimates were truncated to 1.0, and truncated t_m estimates for individual plants averaged 0.629 (± 0.031). Minimum outcrossing estimates (t_{min}) averaged only 0.383 (± 0.028). There was a moderately strong correlation between t_m and t_{min} ($r = 0.766$), indicating that the rank order of outcrossing rates among plants is a good estimate of their true relative magnitudes.

Relationship of floral display size with outcrossing rates and biparental inbreeding—**Group level**—When plant size is used as a categorical variable, small plants (1–8 inflorescences) outcross at a significantly higher rate ($t_m = 0.763 \pm 0.039$) than large plants (>8 inflorescences, $t_m = 0.542 \pm 0.046$; Table 3A). There is a trend for larger plants to self at higher rates when plants are grouped into five categories (Table 3B). However, smaller group sample size and higher standard errors lead to overlap of the 95% CIs among all groups. Estimates of t_{min} also

TABLE 3. Effects of floral display size (number of inflorescences) on outcrossing rates and biparental inbreeding. Maximum likelihood, multilocus (t_m) and average single-locus (t_s) outcrossing rates, their difference ($t_m - t_s$), averages of individual plant minimum outcrossing rates (t_{min}), and correlation of selfing (r_{s-loci}) among loci for 51 *Delphinium barbeyi* plants grouped into (A) two size classes or (B) five size classes. The maternal fixation index (F) for both analyses was 0.193 (± 0.093). Standard errors are shown in parentheses after the estimates.

No. of inflorescences	No. of plants	t_m	t_s	$t_m - t_s$	t_{min}	r_{s-loci}
A.						
1–8	29	0.763 (± 0.039)	0.717 (± 0.038)	0.045 (± 0.012)	0.432 (± 0.041)	0.137 (± 0.154)
9–26	22	0.542 (± 0.046)	0.547 (± 0.053)	–0.005 (± 0.016)	0.319 (± 0.032)	1.000 (± 0.190)
B.						
1	9	0.787 (± 0.075)	0.745 (± 0.075)	0.042 (± 0.025)	0.475 (± 0.077)	0.139 (± 0.298)
2–3	9	0.752 (± 0.099)	0.727 (± 0.098)	0.025 (± 0.016)	0.377 (± 0.090)	0.482 (± 0.287)
4–7	10	0.730 (± 0.047)	0.663 (± 0.041)	0.066 (± 0.026)	0.409 (± 0.061)	0.001 (± 0.141)
8–11	10	0.547 (± 0.088)	0.555 (± 0.095)	–0.008 (± 0.026)	0.311 (± 0.055)	1.000 (± 0.317)
12–26	13	0.574 (± 0.053)	0.579 (± 0.071)	–0.006 (± 0.021)	0.335 (± 0.037)	1.000 (± 0.251)

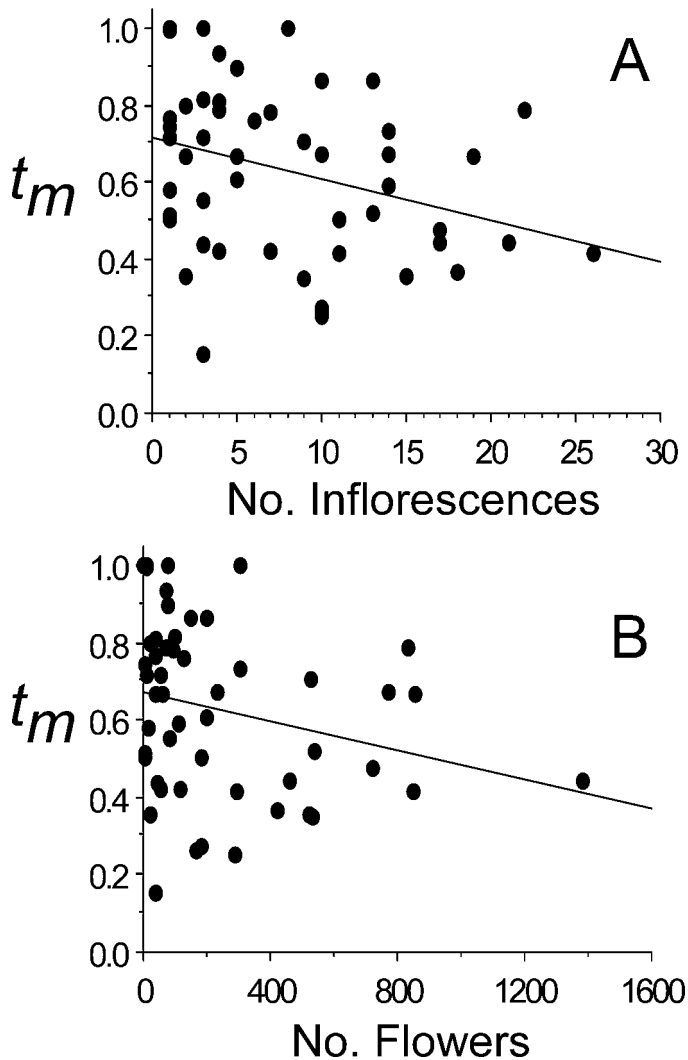


Fig. 1. Individual plant multilocus outcrossing rates (t_m) as a function of (A) the number of inflorescences and (B) the number of flowers per plant among 51 *Delphinium barbeyi* plants. There was a significant regression of outcrossing on inflorescence number ($P = 0.026$, $r = 0.312$), while the relationship with flower number was only marginally significant ($P = 0.073$, $r = 0.253$).

suggest that small plants outcross more frequently than larger plants, but the differences among groups are not significant (Table 3).

Both $t_m - t_s$ and the correlation of selfing among loci (r_s) indicate that significant biparental inbreeding occurs (Table 3). Biparental inbreeding appears to contribute more strongly to the selfing rate of small than large plants. For the two-group comparison, only the estimates for small plants differ significantly from the null expectation of no biparental inbreeding ($t_m - t_s > 0$; $r_s < 1.0$). The trend for decreasing biparental inbreeding with display is not significant in the five-group comparison. The two estimation methods indicate that very different magnitudes of biparental inbreeding contribute to effective selfing ($1 - t_s = 0.283$) in the population. The difference $t_m - t_s$ accounts for only 16% ($0.045/0.283$) of the effective selfing rate, while the correlation of selfing method

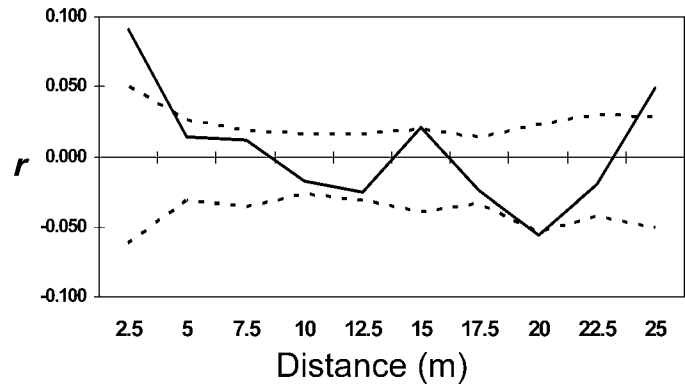


Fig. 2. Multilocus correlogram for the degree of genetic similarity (r) among *Delphinium barbeyi* plants in 2.5-m distance classes. Upper and lower 95% confidence intervals for the correlations are indicated by the dashed lines. Genetic similarity is based on genotypes at three polymorphic enzyme loci for 144 flowering plants. Plants growing within 2.5 m of one another are significantly more genetically similar than random.

estimates that biparental inbreeding contributes up to 86% ($1 - r_s = 0.863$) of effective selfing in small plants.

Individual plant level—There was a significant negative association between individual plant size and outcrossing rate, albeit with considerable scatter in the data. The regression of t_m on inflorescence number is significant, although the correlation is fairly weak ($t_m = 0.714 - 0.011 \times \text{Infls}$, $P = 0.026$, $r = 0.312$; Fig. 1A). The negative relationship between t_m and total flower number per plant was only marginally significant ($t_m = 0.68 - 0.0002 \times \text{Fls}$, $P = 0.073$, $r = 0.253$; Fig. 1B). Floral display size was not as good a predictor of the minimum outcrossing rate ($t_{\min} = 0.444 - 0.008 \times \text{Infls}$, $P = 0.074$, $r = 0.252$; $t_{\min} = 0.417 - 0.0001 \times \text{Fls}$, $P = 0.145$, $r = 0.207$).

Population structure and its effects on outcrossing rates and biparental inbreeding—**Spatial autocorrelation**—Significant spatial genetic structure was seen among closely neighboring plants in the mapped population. Plants growing within 2.5 m of one another were significantly genetically correlated (i.e., the estimated genetic correlation exceeded the 95% CI; Fig. 2). The distance at which the correlogram first crosses the x-axis is often interpreted as the average patch diameter of genetically related individuals. In this population of *D. barbeyi*, this distance is approximately 8 m. Genetic similarity did not decline monotonically, additional positive and negative peaks were seen at greater distances.

Index of local mating environment—The LME surrounding the 51 plants for which outcrossing rates were estimated was generally low ($\bar{X} = 0.282 \pm 0.025$; Fig. 3) and varied from 0.0–0.87. Low LME indicates that most plants are surrounded by genetically similar neighbors, supporting the spatial autocorrelation results. There was only a marginally significant positive association between individual plant outcrossing rates (t_m) and LME ($t_m = 0.538 + 0.323 \times \text{LME}$, $P = 0.069$, $r = 0.257$; Fig. 3A). However, LME was strongly associated with minimum outcrossing estimates per plant ($t_{\min} = 0.176 + 0.735 \times \text{LME}$, $P < 0.0001$, $r = 0.660$; Fig. 3B).

Multivariate predictors of outcrossing and biparental inbreeding—Both floral display size (number of inflorescences) and the local mating environment (LME) were significant determinants of the mating system in *D. barbeyi*. Jointly these two factors still only explain a relatively small proportion of the variance in outcrossing rates of individual plants. The multiple regression explains only 14% of the variance in t_m ($t_m = 0.631 - 0.009 \times \text{Infls} + 0.254 \times \text{LME}$, $P = 0.030$, $r = 0.370$), only marginally better than the simple regression on display size alone ($r = 0.312$). Likewise, the multiple regression only slightly better explains the variation in t_{min} ($t_{min} = 0.214 - 0.004 \times \text{Infls} + 0.707 \times \text{LME}$, $P < 0.0001$, $r = 0.671$) over the simple regression on LME ($r = 0.660$). Floral display size was the better predictor of multilocus outcrossing estimates (t_m), while LME was the stronger predictor of t_{min} .

DISCUSSION

Large floral display size is predicted to increase levels of self-fertilization as the result of more intraplant pollinator movements and higher levels of geitonogamy (Geber, 1985; de Jong et al., 1993; Snow et al., 1996). This prediction was strongly supported in a population of *D. barbeyi*, where plants with large floral displays outcross significantly less frequently than plants with small displays. Local genetic structure also facilitates mating among relatives, further lowering the outcrossing rate. Three functional modes of inbreeding—autogamy, geitonogamy (Lloyd and Schoen, 1992), and biparental inbreeding (Uyenoyama, 1986)—all appear to contribute to the total effective selfing rate (Ritland, 1984), and their relative contributions vary with floral display size and local genetic structure. However, none of these factors explain more than 14% of the variation in outcrossing rates among plants. In the following sections, I examine the functional explanations for these patterns and their potential evolutionary significance.

Mechanism of selfing—Floral display size (number of flowers and inflorescences) varies widely among *D. barbeyi* plants, and individuals with large floral displays outcross at significantly lower rates than do those with small displays. On average, plants with a single inflorescence produce 79% outcrossed seeds, while the largest plants with >8 flowering stalks outcross only 54% of the time. Inflorescence number was a better predictor of individual plant outcrossing rate than flower number. This is expected if selfing occurs predominantly by geitonogamy between flowers on different inflorescences rather than among flowers within an inflorescence or within individual flowers (autogamy). Eckert's (2000) investigation of modes of self-fertilization in *Decodon verticillatus* is the only other study to specifically document the effect of multiple flowering branches on the mating system. He found that multi-branched plants selfed at higher frequencies than plants with a single inflorescence, and that the number of flowers on other branches was negatively correlated to outcrossing rates (Appendix). Geitonogamy, particularly between-branch crossing, was the predominant (82%) mode of selfing (Eckert, 2000). Reducing the synchrony of flowering among different branches of multi-stemmed plants may limit this form of geitonogamy but at the potential cost of reduced pollinator attraction.

The flowers of *D. barbeyi*, like its sympatric congener *D.*

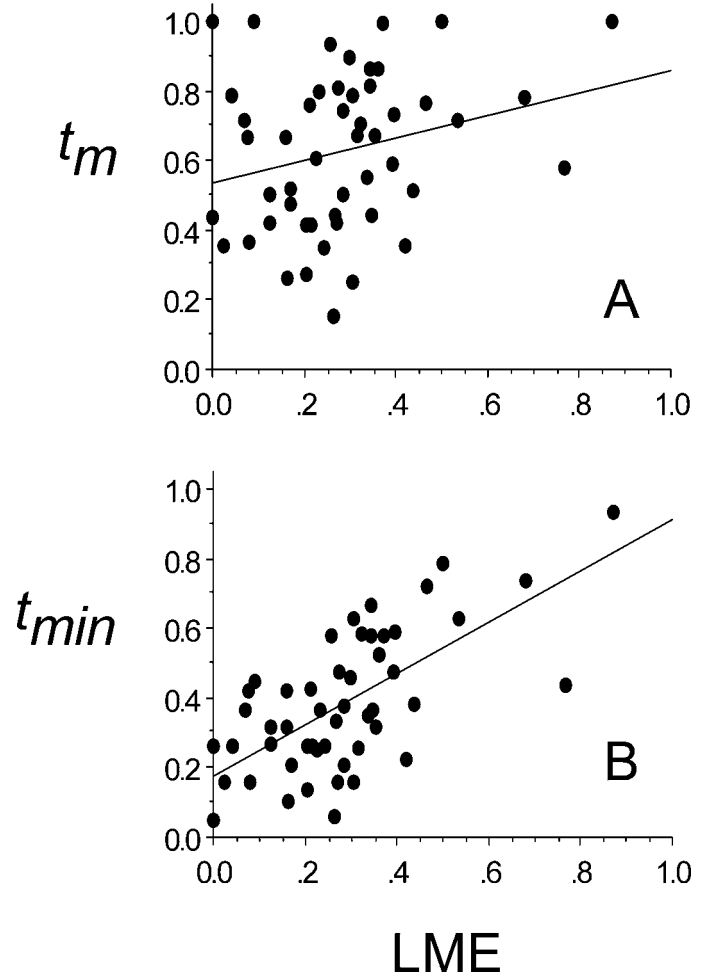


Fig. 3. Outcrossing rates as a function of the local mating environment (LME)—a measure of the size and genetic similarity of plants within a 2-m radius. (A) The multilocus outcrossing rate (t_m) of individual plants is only weakly associated with LME, while (B) minimum outcrossing estimates (t_{min}) show a strong correlation with LME.

nuttallianum, are protandrous—anthers mature before stigmas—and flowers mature from the base of the inflorescence upward (Pyke, 1978; Williams et al., 2001). Bumblebees tend to forage from the bottom to the top of vertical, spikelike inflorescences (Pyke, 1978; Waddington and Heinrich, 1979; Best and Bierzychudek, 1982), thus visiting female phase flowers first. These coupled patterns of floral development and pollinator foraging behavior in plants with vertical inflorescences should minimize both autogamy and within branch geitonogamy (Darwin, 1877; Pyke, 1978; Harder et al., 2000; Williams et al., 2001). Indeed, the high outcrossing rates in *D. barbeyi* plants with a small, single inflorescence (79%), that have no opportunity for between-branch pollinator movement, resemble those found in its single-stalked congener *D. nuttallianum* (88–97%; Williams et al., 2001) further suggesting that between-branch geitonogamy is the main mode of selfing in multi-stemmed plants.

Inter- and intraplant variation in floral reward and development may contribute to the relatively weak association ($r = 0.31$) between display size and outcrossing rate in this

population of *D. barbeyi*. Variance in flower size among plants (Ishii and Harder, 2006) and in nectar reward among flowers on a plant (Bernaskie and Cartar, 2004) affect intraplant pollinator movement and geitonogamy. Variation in the degree of protandry may also affect individual flower and whole inflorescence geitonogamy and outcrossing rates (Harder et al., 2000; Galloway et al., 2002; Routley and Husband, 2003). Upward foraging bumblebees strongly increased selfing in terminal flowers of nonprotandrous *Eichhornia paniculata* inflorescences (Barrett et al., 1994; Harder and Barrett, 1995; Harder et al., 2000). If the degree of protandry differs among individual *Delphinium* plants, then variation in autogamy and within-branch geitonogamy may contribute to the high variation in outcrossing rates among plants that cannot be explained by differences in display size.

Magnitude of display size effect on selfing—The range of floral display size among *D. barbeyi* plants is much greater than in previously studied species. However, the range of outcrossing rate variation is only slightly greater than that found in species with much smaller displays (Appendix). Nevertheless, species with a small range of display sizes also tended to have a smaller range of outcrossing rates (Appendix). Outcrossing rate estimates for the 51 individual *D. barbeyi* plants spanned a much wider range than group estimates, varying from 0.16–1.00 (Fig. 1). Other studies that have examined continuous variation in the relationship between floral display size and individual plant outcrossing rates found a similarly wide range of outcrossing rates among plants and fruits (Crawford, 1984; Brunet and Eckert, 1998; Vrieling et al., 1999; Karron et al., 2004).

Inflorescence number in *D. barbeyi* has an influence on outcrossing rate variation similar to that of flower number in single-stalked plants of other species (Appendix). However, both inflorescence and total flower number were rather weak predictors ($r^2 = 0.10$ and 0.06 , respectively) of individual plant outcrossing rates. A potential cause of the low predictive power of these variables is that they are measures of total, rather than daily, display size (de Jong et al., 1992; Barrett et al., 1994). Both the number of open flowers and the number of inflorescences with open flowers vary throughout the flowering season. Inflorescences do not mature synchronously (personal observation), and daily flower number is only a fraction of total flower number (Williams et al., 2001). Karron et al. (2004) have shown that outcrossing rates vary widely among fruits on the same plant and among plants with the same floral display size. The stochastic nature of pollinator visitation patterns (Mitchell et al., 2004, 2005) and pollen carryover (Barrett et al., 1994), combined with daily variation in floral display, likely contribute to the low explanatory power of total display size or inflorescence number for outcrossing estimates derived from bulk seed samples used in this study. Despite these limitations of the experimental design, the correlations of outcrossing rate to floral display size are still comparable to those in other studies that used daily rather than total flower number ($r^2 = 0.001$ – 0.34 ; Brunet and Eckert, 1998; Vrieling et al., 1999; Eckert, 2000; Herlihy and Eckert, 2004).

Local population structure and biparental inbreeding—Another factor potentially contributing to variation in outcrossing rates among plants is biparental inbreeding. Several lines of evidence suggest that biparental inbreeding is an important component of the effective selfing rate in *D. barbeyi*.

First, significant local population structure was detected by multilocus spatial autocorrelation in the smallest distance class (2.5-m radius) in this population (see also Williams et al., 2001). The additional, marginally significant peaks at greater distances may be due to a few patches of genetically similar or dissimilar individuals within the sampled 14×26 m plot, because this plot size is small relative to the 8-m mean patch diameter of genetically related plants. Stochastic variation among loci, when autocorrelation is averaged over few loci, may also lead to the nonmonotonic decline in genetic similarity seen in the correlogram (Slatkin and Arter, 1991; Williams, 1994).

Second, the local mating environment (LME) significantly affected outcrossing rates of individual plants. Plants surrounded by a high floral density of genetically related individuals (low LME) had lower outcrossing rates than those with genetically dissimilar neighbors (high LME). This was particularly true for minimum estimates of outcrossing (l_{min}), which should be more sensitive to biparental inbreeding than multilocus estimators (Shaw et al., 1981; Ritland, 2002; Griffin and Eckert, 2003). Eckert (2000) found a similar relationship in a clonal plant in that ramets with a higher density of clonal neighbors had higher selfing rates.

Third, single inflorescence plants had the highest outcrossing rates (0.787 ± 0.075), but these estimates were still significantly less than complete outcrossing. Single-stalked plants, which had a mean flower number of only 12.7, should experience little or no geitonogamy because of the typical bottom-up pollinator foraging behavior (discussed earlier). Selfing in these plants should occur because of autogamy and/or biparental inbreeding. Estimates of autonomous selfing rates are only 9% based on seed set in pollinator exclosures (Williams et al., 2001). Assuming that additional facilitated selfing in these protandrous flowers is negligible, then over half of the effective selfing rate of 21% in single-inflorescence plants may be due to biparental inbreeding.

Finally, the two estimators of biparental inbreeding, although differing widely in magnitude, suggest that mating between relatives occurs and is a more important component of effective selfing in small than large plants (Table 3). Larger plants, which experience more intraplant pollinator movements, should receive proportionally less outcross pollen and effectively self more through geitonogamy than biparental inbreeding. Small plants with little geitonogamy should receive proportionally more outcross pollen. Effective selfing and biparental inbreeding therefore should be more strongly influenced by LME in small plants. However, large plants with high rates of geitonogamy may have reduced male siring success because of pollen discounting (Harder and Barrett, 1995), making them less effective pollen donors and less likely to contribute to biparental inbreeding. This may act to counter some of the predicted effects of LME on biparental inbreeding and hence help explain the weak relationship between LME and outcrossing.

Contributions of autogamy, geitonogamy, and biparental inbreeding to selfing—The proportion of effective selfing due to geitonogamy in plants with different floral display sizes can be estimated by assuming that single-stalked plants do not self by geitonogamy (or do so at a very low rate), and that biparental inbreeding remains constant or decreases with increasing floral display size. As shown earlier, both of these assumptions are reasonable for *D. barbeyi*. The geitonogamy

TABLE 4. Estimates of the autogamy (a_n) and geitonogamy (g_n) fraction of total selfing (s_n) for *Delphinium barbeyi* plants with different numbers of inflorescences (n). Estimates of s_n are derived from the outcrossing estimates ($s = 1 - t_m$) in Table 3. The autogamy fraction $a_n = s_{n=1}(1 - s_n)/(1 - s_{n=1})$, and the geitonogamy fraction $g_n = s_n - a_n$ (Schoen and Lloyd, 1992). Both the autogamy and geitonogamy fractions include some effective selfing due to biparental inbreeding, but it is assumed that biparental inbreeding decreases in plants with larger floral displays (see text for details).

No. of inflorescences	No. of plants	s_n	a_n	g_n	% g_n
1	9	0.213	0.213	0.000	0.0
2–3	9	0.248	0.204	0.044	17.7
4–7	10	0.270	0.198	0.072	26.7
8–11	10	0.453	0.148	0.305	67.3
12–26	13	0.426	0.155	0.271	63.6

fraction of total selfing for each floral display size class can be derived (Table 4) using the methods of Schoen and Lloyd (1992), where selfing rates ($s = 1 - t_m$) are calculated from the outcrossing rates of plants in different inflorescence size classes (Table 3). Because the total effective selfing rate (s_n) increases with floral display size, the fraction due to geitonogamy (% g_n) increases from zero in single-inflorescence plants to over 60% of the total inbreeding in the largest size class. Biparental inbreeding should contribute to effective selfing in both autogamous and geitonogamous fractions. However, because the magnitude of biparental inbreeding appears to decrease with increasing display size (see earlier), the proportion of selfing due to geitonogamy in large plants may be an underestimate.

Variations in plant floral display and the local mating environment affect components of the mating system in potentially complex ways. Between-inflorescence geitonogamy is the most important component of selfing in large, multi-inflorescence plants. Geitonogamy, whether within or between branches, should have little impact on selfing in small plants. The contribution of biparental inbreeding depends on the local mating environment and appears to decline with increasing plant size. These predictions await further testing employing floral manipulations (Schoen and Lloyd, 1992; Herlihy and Eckert, 2004) and more variable genetic markers (Ritland, 2002) to better tease apart the relative contributions of autogamy, geitonogamy, and biparental inbreeding and their associations with floral display in *Delphinium*.

Evolutionary implications—Geitonogamy may be an unavoidable consequence of selection for outcrossing in plants with large floral displays. As such, geitonogamy may help explain the prevalence of mixed mating systems (Lloyd, 1992; de Jong et al., 1993; Harder and Barrett, 1996; Eckert and Herlihy, 2004; Goodwillie et al., 2005; Brunet and Sweet, 2006b). The magnitudes of the potential fitness costs associated with high levels of inter-inflorescence geitonogamy in *D. barbeyi* (inbreeding depression, pollen and ovule discounting) and how they are balanced by the reproductive benefits of large floral display (pollinator attraction, pollen receipt and removal) are currently unknown. If geitonogamous selfing is costly, then selection should act to limit maximum display size and/or reduce the costs of geitonogamy by other means (de Jong et al., 1992). Evidence suggests that a history of inbreeding has affected the magnitude and timing of inbreeding depression in *D. barbeyi*. Early-acting inbreeding depression appears low, because selfing does not reduce seed set compared to outcrossing. However, adult plants are much less inbred than predicted from the mating system, suggesting that later acting inbreeding depression occurs (Williams et al.,

2001). A comparison of whole plant seed set, siring success, and seedling recruitment across a range of floral display sizes, as well as a better understanding of the magnitude and timing of inbreeding depression, will be necessary to evaluate the fitness costs and benefits of floral display in *D. barbeyi* and other species with multiple inflorescences.

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APPENDIX. Summary of intraspecific studies of the relationship of floral display size to outcrossing rate. Most studies examined variation in the number of open flowers on single inflorescences and used allozyme markers to estimate group or individual plant outcrossing rates. The latter three studies compared outcrossing rates in plants with different numbers of inflorescences or among plants in populations with different mean floral display sizes.

Species	Experimental design ^a	Range of floral display sizes	Range of outcrossing rates (r) ^b	Difference in outcrossing rates (r) ^b	Percent change in outcrossing ^b	Correlation of display size with r ^c	Reference
<i>Mimulus guttatus</i>	N, N, F	1–2 (2) ^d	0.83–0.75	0.08	9.5	—	Leclerc-Potvin and Ritland, 1994
<i>Aquilegia caerulea</i>	N, N, F	0.5–2.0 ^e (3)	0.59–0.47	0.12	20.3	r = –0.42	Brunet and Eckert, 1998
<i>Aquilegia canadensis</i>	N, N, F	1.0–2.8 ^f (2)	0.35–0.34 ^g	0.01	2.9	r = +0.03	Herlihy and Eckert, 2004
<i>Impatiens pallida</i>	N, M, F	1–≥3.0 ^f (2)	0.94–0.66	0.28	29.8	—	Schoen and Lloyd, 1992
<i>Eichhornia paniculata</i>	A, M, F	3–12 (4)	0.82–0.73	0.09	11.0	—	Barrett et al., 1994
<i>Eichhornia paniculata</i>	A, M, F	3–12 (4)	0.85–0.65	0.20	23.5	—	Harder and Barrett, 1995
<i>Hibiscus moscheutos</i>	A, M, F	3–12 (3)	0.75–0.52	0.23	30.7	—	Snow et al., 1996
<i>Mimulus ringens</i>	A, M, F	2–16 (4)	0.77–0.63	0.14	18.7	—	Karron et al., 2004
<i>Malva moschata</i>	A, N, F	5–25 (C)	0.88–0.45	0.43	47.1	—	Crawford, 1984
<i>Cynoglossum officinale</i>	A, N, F	1–94 (C)	0.97–0.43	0.54	55.6	r = –0.58	Vrieling et al., 1999
<i>Decodon verticillatus</i>	N, N, F ^h	0–59 (C)	—	—	—	r = –0.23	Eckert, 2000
<i>Decodon verticillatus</i>	N, N, I	1, ≥2 (2)	0.84–0.73	0.11	13.1	—	Eckert, 2000
<i>Delphinium barbeyi</i>	N, N, I	1–26 (5)	0.79–0.54	0.25	31.0	r = –0.31	this study
<i>Aquilegia caerulea</i>	N, N, P	2–14 ⁱ (C)	0.85–0.55	0.30	35.3	r = –0.75	Brunet and Sweet, 2006a

^a Experimental design describes three characteristics in sequence: Type of population studied (N = natural population, A = artificial array); type of display size variation (N = natural variation, M = manipulated floral display sizes); and aspect of display size studied (F = number of open flowers on a single inflorescence, I = number of inflorescences per plant, P = variation in flower number among populations).

^b Range listed from largest to smallest inflorescence size class examined.

^c Data shown for all studies reporting this relationship.

^d Number of floral display size classes compared (2–5), (C) = continuous range of sizes.

^e Mean number of male-phase flowers open simultaneously with female target flower.

^f Comparison of plants with a single vs. multiple flowers open simultaneously.

^g Mean of four population comparisons.

^h Total number of flowers on all nontarget branches.

ⁱ Mean display size in 10 populations.