RESEARCH ARTICLE

American Journal of Botany

The opportunity for outcrossing varies across the geographic range of the primarily selfing *Clarkia xantiana* ssp. *parviflora*

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Manuscript received 13 February 2020; revision accepted 22 February 2020.

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Citation: Ruane, L. G., S. M. Mangum, K. M. Horner, and D. A. Moeller. 2020. The opportunity for outcrossing varies across the geographic range of the primarily selfing *Clarkia xantiana* ssp. *parviflora*. *American Journal of Botany* 107(8): 1199–1207.

doi:10.1002/ajb2.1510

PREMISE: The timing of self-fertilization has potentially important consequences for the trajectory of mating system evolution, the opportunity for outcrossing, and the maintenance of genetic variation in populations. For primarily selfing taxa, it remains poorly understood as to how floral variation influences the opportunity for outcrossing and whether those attributes vary among populations across geographic ranges.

METHODS: We examined variation in floral traits (herkogamy, protandry, flower size, stigma stage at anthesis, timing of stigma receptivity) in seven populations of *Clarkia xantiana* ssp. *parviflora*, a primarily selfing taxon, spanning from the western to eastern margins of its distribution. We also performed experimental emasculations and pollinations (followed by stigma severing) to quantify the extent of opportunities for outcrossing across flower development.

RESULTS: There was marked among-population variation in all floral traits, particularly between far eastern and western populations. Emasculation experiments showed that the eastern populations had minimal autonomous selfing, but western populations had high rates of selfing within 24 h after anthesis. Population variation in autofertility was significantly predicted by floral trait variation, especially protandry and petal size.

CONCLUSIONS: Greater protandry both extended the time over which outcrossing could potentially occur and reduced the probability of autonomous selfing, suggesting that there may be a tradeoff that results in fitness loss when pollinator visits are not common. The east-west pattern of differentiation in some floral traits parallels that of postglacial range expansion, suggesting that selection on the mating system may have been strong in the process of range expansion.

KEY WORDS breeding systems; geographic variation; inbreeding; mating system evolution; mixed mating strategy; pollination; range expansion; sexual selection; Onagraceae.

Mating system variation is prevalent in flowering plants, with considerable variation among lineages and floras in the extent to which they reproduce via outcrossing versus selfing (Stebbins, 1974; Barrett and Harder, 2017; Moeller et al., 2017). Meta-analyses have indicated that outcrossing rates vary continuously despite significant modes to the distribution at high and low outcrossing rates (Goodwillie et al., 2005; Moeller et al., 2017; Whitehead et al., 2018). For primarily outcrossing taxa, with frequent pollinator-mediated reproduction, the relationships between trait variation and mating patterns are generally well-characterized. However, for primarily selfing taxa, the influence of floral trait variation on the timing of self-pollen deposition, and thus opportunities for outcrossing, has seldom been explored despite its potential importance for the long-term maintenance of genetic variation (reviewed in Glémin et al., 2019).

Classic papers by Lloyd (1979, 1992) described how the floral mechanisms that determine the timing of selfing can influence the dynamics of mating system evolution. For example, floral mechanisms that promote autonomous selfing following opportunities for outcrossing (delayed selfing; e.g., Kalisz et al., 1999) are selected under a broader range of conditions than those that promote selfing prior to or coincident with opportunities for outcrossing (prior and competing selfing, respectively). This may occur, in part, because individuals with delayed selfing may benefit from the 50% transmission advantage of mixed maters (Fisher, 1941). Alternatively, delayed selfing may be favored because of reproductive assurance when it allows for an opportunity to produce outcrossed offspring, which often have higher fitness (i.e., due to lower inbreeding depression), while still ensuring high fecundity via selfing when pollinators and/or mates are unavailable (Goodwillie and Weber, 2018). Similar to delayed selfing, prior/competing selfing can ensure reproduction when pollinators or mates are scarce. However, this mode of selfing results in greater pollen and seed discounting that limits the potential for pollen export via pollinators (and male fitness) when pollinators are available (Holsinger and Thomson, 1994; Chang and Rausher, 1998; Briscoe Runquist et al., 2017); therefore, prior/competing selfing can minimize the genetic transmission advantage of selfing. It remains unclear, however, as to whether these different modes of selfing are similarly effective at maximizing selfed-seed production when pollinators are absent or scarce. Moreover, the causal relationship between the evolution of different modes of selfing and their function in current pollination environments remains poorly understood.

A variety of floral traits can mediate the probability and timing of selfing and ultimately influence the mating system. The physical proximity of the anthers and stigma (herkogamy) and the timing of pollen release in relation to stigma receptivity (dichogamy) are among the most commonly considered and best understood floral mechanisms that influence the mating system (Lloyd and Webb, 1986; Bertin and Newman, 1993; Opedal, 2018, 2019). In addition to morphological and developmental traits that influence when pollen is deposited, flowering plants can also control the timing of fertilization by suppressing pollen germination on the stigma. Delayed stigma receptivity is a mechanism whereby the process of pollen deposition is temporally decoupled from pollen germination via chemical suppression on the stigma surface (Willson and Burley, 1983; Galen et al., 1986; Armbruster et al., 2002). In mixed mating taxa, delayed stigma receptivity could promote outcrossing either by preventing pollen adherence to the stigma or by delaying the germination of self-pollen grains that may have preemptively fertilized ovules (Armbruster and Rogers, 2004). In addition, delayed stigma receptivity has also been considered a floral mechanism that promotes competition among male gametophytes (Willson and Burley, 1983; Galen et al., 1986; Skogsmyr and Lankinen, 2002; Lankinen and Madjidian, 2011). In combination, dichogamy, herkogamy, and delayed stigma receptivity modulate the probability and timing of self-pollination in many self-compatible flowering plants.

Variation in floral traits is typically correlated with transitions to self-compatibility and the propensity for selfing vs. outcrossing. Correlations between floral traits and the magnitude of selfing have been documented among-taxa (Routley et al., 2004; Kalisz et al., 2012), among-populations (Holtsford and Ellstrand, 1992; Moeller, 2006; Brys et al., 2013; Luo and Widmer, 2013), and among-individuals (Karron et al., 1997). Studies of geographic variation in key floral traits have been particularly insightful for understanding the causes of mating system evolution. Such interpopulation variation has implicated pollination environments and abiotic environments as potential causes of mating system evolution (Herrera et al., 2006; Moeller, 2006; Evans et al., 2011). Populations at the leading or trailing margins of species' range expansions may also experience differential selection on traits that affect the mating system (Levin, 2012). This collection of studies illuminates where and why primarily outcrossing populations with large floral displays shift to higher rates of selfing and the mechanism by which selfing occurs. For primarily selfing taxa, considerably less work has aimed to quantify and understand interpopulation variation in the mechanism and timing of selfing and therefore potential opportunities for outcrossing.

In this paper, we examined the extent and timing of self-fertilization among populations across the geographic range of *Clarkia xantiana* ssp. *parviflora* (Eastw.) Harlan Lewis & P. H. Raven (Onagraceae) (hereafter ssp. *parviflora*), a taxon that is predominantly selfing but where some outcrossing can occur (Eckhart and Geber, 1999; Runions and Geber, 2000; Moeller, 2006; Pettengill et al., 2016). Prior work on ssp. *parviflora* has suggested that autonomous self-fertilization occurs readily without the intervention of pollinators (Runions and Geber, 2000; Moeller, 2006) and that ssp. *parviflora* populations have considerably lower pollinator availability than populations of its sister outcrossing subspecies (Fausto et al., 2001; Moeller, 2006). Phylogeographic analyses have suggested that ssp. *parviflora* diverged in allopatry to the east of its outcrossing ancestor (ssp. *xantiana*), and its range subsequently expanded to a zone of secondary sympatry (Pettengill and Moeller, 2012b). This range expansion occurred from east to west across the range since the last glacial maximum. We were particularly interested in examining divergence in floral mechanisms that influence opportunities for outcrossing, which may have occurred in the process of range expansion.

We focused on four primary questions: (1) To what extent do floral traits (e.g., petal size, herkogamy, protandry) differ among populations of *Clarkia xantiana* ssp. *parviflora*? (2) Does variation in these floral traits influence the probability and timing of self-pollen deposition and thus the opportunity for outcrossing? (3) To what extent does delayed stigma receptivity further modulate the probability of self-fertilization following self-pollen deposition? (4) Did range expansion since the last glacial maximum result in an east-to-west geographic pattern of floral differentiation?

MATERIALS AND METHODS

Study system and population sampling

Clarkia xantiana ssp. *parviflora* is an annual plant endemic to the eastern portion of the southern Sierra Nevada foothills (Eckhart and Geber, 1999). Subspecies *parviflora* is a monophyletic group derived from *Clarkia xantiana* ssp. *xantiana*, a primarily outcrossing but self-compatible taxon (Pettengill and Moeller, 2012a). Phylogeographic studies suggest that the taxa diverged in allopatry approximately 65,000 years before present and have since come into secondary sympatry (Pettengill and Moeller, 2012b). The earliest-diverging lineages of ssp. *parviflora* are found in the far eastern portion of ssp. *parviflora*'s range and there is a roughly east-west pattern of population genetic differentiation (Pettengill and Moeller, 2012b; Pettengill et al., 2016). Population outcrossing rates are on average 0.26 (range: 0–0.4) based on a survey of eight natural populations (Pettengill et al., 2016) and 0.22 (range: 0–0.33) based on individuals in an experimental population in the field (Briscoe Runquist et al., 2017). In addition, individual ssp. *parviflora* populations contain significantly lower DNA sequence diversity than those of ssp. *xantiana*, consistent with patterns observed across other sister outcrosser-selfer pairs (Pettengill and Moeller, 2012a; Pettengill et al., 2016). The two subspecies' ranges are largely parapatric but overlap in a narrow region (~5–10 km wide) centered on Lake Isabella, California.

We sampled seven populations spanning nearly the full east to west range of the taxon (Fig. 1). The two most eastern (allopatric) populations, E-89A and E-47A, occur in the Chimney Peak Wilderness, an area adjacent to the Mojave Desert; E-47A occurs at a higher elevation than E-89A. Three additional allopatric populations—C-66A, C-53A, and C-56A—are arrayed between the extreme eastern and western populations. The two most western populations—W-77S and W-69S—are found in the portion of the range sympatric with ssp. *xantiana*. While the W-77S population occurs in a site where both subspecies can be found within meters to tens of meters of one another, the W-69S population is isolated from its sister subspecies albeit within the sympatric region. Seeds were sampled haphazardly from plants across most of each population in an effort to capture possible within-population genetic variation in phenotypes.

Plant growth environment

Seeds collected from the field were grown for one generation in a greenhouse common garden at the University of Minnesota (Minneapolis, Minnesota, USA) to standardize maternal environmental effects. Seeds collected from greenhouse-grown plants were then planted at Christopher Newport University (Newport News, Virginia, USA) for the experiments reported in this paper. In total, 330 plants were grown (7 populations × 42–50 plants per population). A subset of these plants (23–30 per population) was used to measure floral traits and the propensity for autonomous selfing. The remaining 17–20 plants per population were used to quantify temporal patterns of stigma receptivity. Only a single progeny was grown per maternal family.

We germinated seeds in 315 cm^3 containers (Deepots, Stuewe and Sons, Tangent, Oregon, USA), which were filled with Metromix 360 (Sun Gro Horticulture, Agawam, Massachusetts, USA) and randomly assigned to a rack and a location within a large growth chamber (4.8 × 2.3 × 2.4 m; BDW series, Conviron, Manitoba, Canada). During germination and seedling development, plants were exposed to a constant temperature of 13°C and a 10 h photoperiod provided by metal halide and halogen incandescent lamps. Once the first set of true leaves was produced, seedlings were thinned to one per pot. Two weeks after germination, plants received water in the mornings and quarter-strength Hoagland's solution in the evenings (Hoagland and Arnon, 1938). After three weeks of growth, the photoperiod was increased to 11 h and the temperature was increased to 16°C/17°C (night/day); two weeks later, the photoperiod and temperature were further increased to 12 h and 21°C/21°C (night/day), respectively. Once plants began flowering, care was taken to maintain physical separation between plants to prevent unsupervised pollinations. Racks were rotated within the growth chamber every other day to minimize position effects due to microenvironmental variation within the growth chamber.

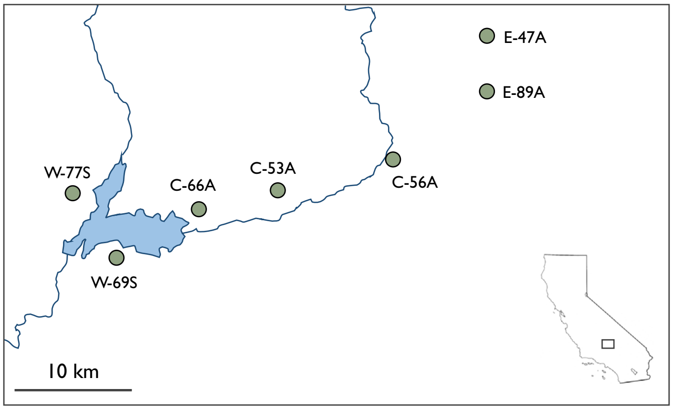

FIGURE 1. Map of *Clarkia xantiana* ssp. *parviflora* population locations near Lake Isabella in Kern County, California, USA.

Floral traits

The first flower to open on the main stem was used to quantify stigma stage at anthesis, potandry, herkogamy, and petal area. Once flower buds developed, each plant was observed every 12 h over five weeks, and the date and time of anthesis of its first flower were recorded. Anthesis refers to the timing of flower opening; in ssp. *parviflora*, the small, inner whorl of anthers dehisce pollen at anthesis (the timing of the outer whorl of anthers varies among genotypes and populations). The developmental stage of the stigma was recorded at the time of anthesis on a scale from 1–4. Stigma stage was classified as 1 if the four arms of the stigma were tightly closed, 2 if the four stigma arms had started to unfold but were less than 50% unfolded, 3 if the stigma arms were more than 50% unfolded but not fully unfolded, and 4 if the four stigma arms had unfolded completely as indicated by a flat stigma surface.

Protandry (i.e., the temporal separation between the maturation of the stigma and anthers) was zero when a stigma was at stage 4 at the time of anthesis. When a stigma was not fully flat at anthesis (i.e., stage 1, 2, or 3), then the stigma of that flower was revisited every 12 h and protandry was recorded as the number of hours from anthesis until the stigma surface was completely flat (stage 4). Herkogamy (i.e., the spatial separation between the anthers and stigma) was quantified by measuring the distance between the stigma and the closest anther as soon as the stigma became completely flat (stage 4). After stigma stage, protandry and herkogamy were recorded, one bottom petal was removed from the flower and taped to a piece of paper. Pages of taped petals (along with scale bars) were scanned and imported into ImageJ software to quantify petal area (Schneider et al., 2012).

Autofertility

The degree of autonomous selfing was assessed by leaving plants undisturbed while three consecutive flowers on the main stem opened then senesced. The three autonomously set fruits per individual were harvested when ripe and stored in separate coin envelopes. Once dry, the fruits were dissected and the number of developed seeds was counted. Autofertility of each individual was calculated by dividing the *number of seeds per fruit produced by undisturbed flowers* by the *number of seeds per fruit produced when supplemental self-pollen was applied to stigmas*. For this calculation, the number of seeds produced by undisturbed flowers was the average of three fruits per individual.

In addition to assessing population variation in the propensity for autonomous selfing, we also examined variation in the timing of self-pollination by emasculating flowers at different timepoints.

For two adjacent flowers per plant (both on the main stem), the anthers were removed either 6 h or 24 h after anthesis (e.g., Vaughton and Ramsey, 2010; Fan and Li, 2012). These emasculations were performed carefully by severing filaments with microscissors in such a way that prevented pollen deposition during anther removal. To minimize the potential effects of flower position, we rotated which flower along the main stem was emasculated at each time point. Seeds resulting from these flowers had to come from autonomous self-pollination events that occurred prior to the emasculation treatment (i.e., within either 6 h or 24 h of anthesis). To compare the levels of autonomous self-pollination among populations, the fruits produced following each emasculation treatment were harvested when ripe and dissected once dry to quantify the number of seeds. Autofertility was calculated by dividing the *number of seeds per fruit produced by flowers in each emasculation treatment* by the *number of seeds produced when self-pollen was added to stigmas*.

Stigma receptivity schedules

The receptivity of stigmas at various developmental stages was quantified by staining stigmas for the presence of peroxidase enzymes, which peak in their concentration when stigmas are most receptive to pollen (Galen and Plowright, 1987; McInnis et al., 2006). Bud emasculations were performed on four consecutive flowers along the main stem to ensure stigmas were free from pollen when harvested for staining. Following bud emasculations, the developmental stage of each stigma was monitored closely every 4 h when chamber lights were on. Stigmas were harvested for staining at the following four developmental stages: newly flat, 6 h after becoming newly flat, 24 h after becoming newly flat, and 48 h after becoming newly flat. At all of these stages, pollen readily adheres to the stigma following both natural and artificial pollen transfer. To minimize the potential effects of flower position, we rotated which flower along the main stem was used for each developmental stage.

Once a stigma had reached its assigned developmental stage, it was removed from the plant and submerged in a 200 μl solution, which was made by adding one Macherey-Nagel (Bethlehem, Pennsylvania, USA) Peroxtesmo Ko test paper (15 mm × 15 mm) to 1 mL distilled water (Dafni and Motte Maués, 1998). This stain prompts receptive areas of the stigma to produce bubbles and change color from white to blue (Motten, 1982; Sullivan, 1984). Stigmas were removed from the solution after 3 min, then viewed under a dissecting microscope to record the percentage of stigma surface area that exhibited the color change (Kearns and Inouye, 1993). For ssp. *parviflora*, the blue stain radiated over time from the outer edges of the four-lobed stigma towards the center of the stigma, indicating that the outer edges of the stigma became receptive before the center of the stigma. Leaving stigmas in the solution for 6 min did not increase the amount of stigma surface that changed color; thus, 3 min was ample time for stigmas to fully respond to the solution.

In addition to staining stigmas at sequential developmental stages, we also performed experimental pollinations to assess the contribution of stigma receptivity to the opportunity for outcrossing. Specifically, self-pollen was added to bare, newly flat stigmas in the morning, then stigmas were separated from flowers by severing the top of the styles 6 h after pollen grains were deposited (e.g., Elle et al., 2010). Results from a previous experiment revealed that some pollen grains have the capacity to germinate within 5 h of stigma contact; thus, removing the stigma 6 h after the experimental self-pollination allowed pollen grains that had already germinated the opportunity to sire seeds but prevented any pollen grains that had not yet germinated from accessing ovules. Flowers in this treatment were emasculated the day before anthesis to ensure stigmas were free from autonomously deposited pollen grains. The number of seeds sired by self-pollen grains that germinated within 6 h of stigma contact was compared to the number of seeds sired by self-pollen grains deposited on stigmas that were not severed. These two experimental self-pollination treatments (severed stigma vs. not severed stigma) were performed on adjacent flowers on the main stem. To minimize the potential effects of flower position, we rotated which flower received the severed stigma treatment. The resulting fruits from both experimental self-pollinations were collected when ripe and later dissected to quantify the number of seeds sired. The fraction of maximum seed sired by self-pollen grains when given 6 h to germinate was calculated by dividing the number of seeds produced in the severed stigma treatment by the number of seeds produced in the treatment where stigmas were not severed. A value of 1 would indicate that all of the pollen grains that sired seeds germinated within 6 h of reaching stigmas.

For all experimental pollinations, pollen grains were collected from newly open flowers (<24 h old) located at the tip of the main stem or the tip of a branch, and the entire stigmatic surface was coated with a layer of pollen grains.

Statistical analyses

The effect of population on floral traits was tested using an ordinal regression for categorical traits (stigma stage at anthesis) and one-way analysis of variance (ANOVA) for continuous traits (protandry, herkogamy, petal area). Population is considered a fixed effect in all our analyses. Protandry, herkogamy, and petal area were log transformed to achieve normally distributed residuals and homogeneity of variances (Sokal and Rohlf, 1995); when necessary, a small constant (0.5 for protandry and 0.1 for herkogamy) was added to all replicates prior to the transformation. To determine if differences among population means were statistically significant, Tukey's honestly significant difference (HSD) tests were performed after each ANOVA. We also performed nonparametric correlations with the four floral traits (petal area, herkogamy, protandry, and stigma stage at anthesis) to reveal how these variables are correlated with each other.

We tested whether autofertility differed through time and among populations using an ANOVA including emasculation treatment (no emasculation, emasculation 6 h post-anthesis, and emasculation 24 h post-anthesis), population (fixed effect), and their interaction. The individual plant was included as a random effect in this model. We then used preplanned contrasts to test whether emasculation treatments differed from one another within populations. Simple linear regressions were performed to test whether floral trait variation (petal area, herkogamy, protandry, and stigma stage at anthesis) significantly predicted population variation in autofertility.

We tested whether stigma receptivity varied through time and among populations using an ANOVA that included stigma developmental stage (newly flat stigmas, 6 h, 24 h, and 48 h), population (fixed effect), and their interaction. Individual was included as a random effect in this model. The overall test determines whether the time course of stigma receptivity varied significantly among populations. Beyond this overall test, we had an a priori interest in testing whether stigma receptivity differed significantly among stages within each population; thus, we conducted this subset of contrasts using least square differences among stages within populations.

The effect of population on the fraction of maximum seed sired by self-pollen when given 6 h to germinate was analyzed using a one-way ANOVA. A Tukey's HSD test was then performed to determine if differences among population means were statistically significant.

Analyses were performed using JMP (ver. 9.0; SAS Institute, Cary, North Carolina, USA).

RESULTS

Floral traits

All four floral traits differed significantly among populations. For petal area, genotypes from the two most eastern populations, E-47A and E-89A (0.78 and 0.66 cm^2/petal, respectively), had larger petals than the remaining western populations (range: 0.39–0.48 cm^2/petal; $F_{6,186} = 69.7, P < 0.001$). The western populations did not differ significantly from one another (Fig. 2A).

The significant effect of population on herkogamy ($F_{6,185} = 4.8$, $P < 0.001$) was largely due to the fact that the high elevation, eastern population (E-47A) had mean anther-stigma distance (1.5 mm) approximately 3–50× greater than all remaining populations (range: 0.03–0.55 mm; Fig. 2B). The remaining populations did not differ significantly from one another.

Protandry differed significantly among populations ($F_{6,184} = 30.0$, $P < 0.001$) with the two far eastern, allopatric populations plus the far western population (sympatric with outcrossing sister taxon) having significantly greater protandry than the remaining central populations (Fig. 2C). The central populations each averaged less than 24 h of protandry (range: 14.5–22.3 h) while W-77S, E-89A, and E-47A plants averaged 41.0, 46.3, and 120.5 h of protandry respectively.

Stigma stage at anthesis also differed significantly among populations. In three of the four central populations, stigmas were over 50% open at the time of anthesis (more than half of the way to having a flat, open stigma; Fig. 2D); for the remaining populations, stigmas were either completely closed (i.e., E-47A) or less than 50% unfolded (i.e., W-77S, C-66A and E-89A) at anthesis (Fig. 2D).

The stigma stage at anthesis was not indicative of how quickly the stigma unfolded following anthesis. Although stigmas of plants derived from W-77S and C-66A were of similar stages at anthesis (Fig. 2D), those on plants from C-66A became flat within 22.3 h, on average, while those from W-77S took nearly twice as long to become flat (41 h; Fig. 2C). Similarly, stigmas that were more tightly closed at anthesis did not necessarily require more time to unfold; for example, the stigmas of W-77S and E-89A plants required a similar amount of time to become flat (Fig. 2C), even though E-89A stigmas were more tightly closed than W-77S stigmas at anthesis (Fig. 2D).

Most pairs of floral traits were not strongly correlated (Spearman's rho range: 0.18–0.51), except for protandry and stigma stage at anthesis (0.70; Appendix S1).

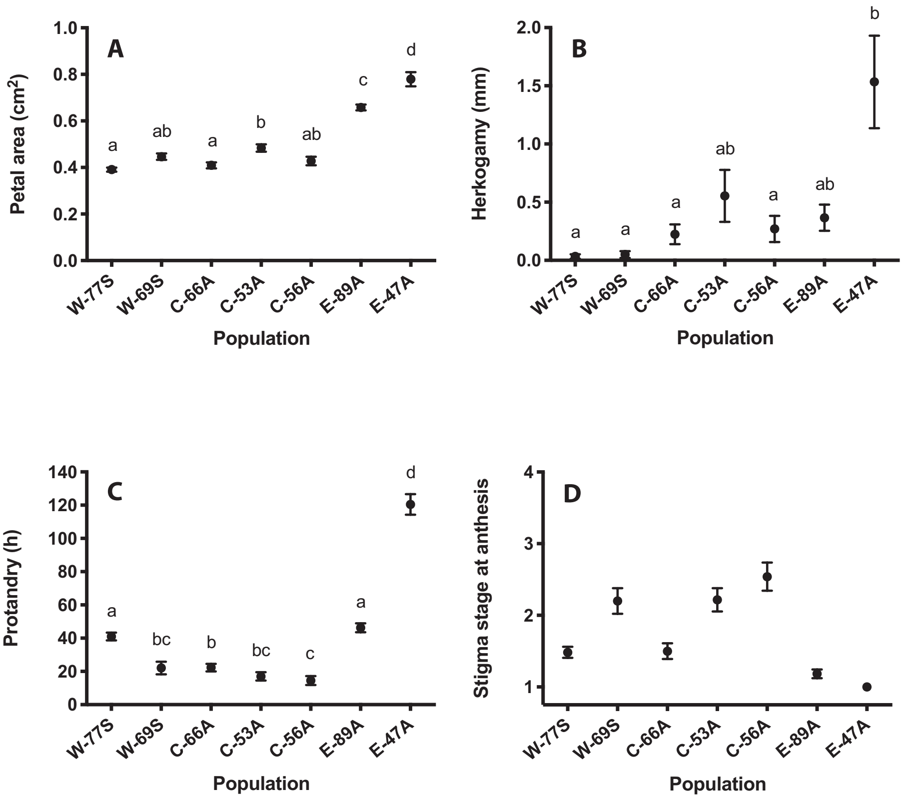

FIGURE 2. Comparisons of floral traits among seven populations of *Clarkia xantiana* ssp. *parviflora* when grown in a common growth chamber environment. Means and standard errors are presented for (A) petal area, (B) herkogamy, (C) protandry, and (D) stigma stage at anthesis. Means sharing letters are not significantly different ($P > 0.05$).

Autofertility

The magnitude of autonomous seed set differed significantly among populations ($F_{178.5} = 24.8$, $P < 0.001$). When flowers were left undisturbed (i.e., not jostled or emasculated) in the growth chamber, the W-69S and the three central populations (C-66A, C-53A, and C-56A) had the greatest propensity for selfing, with autofertility ranging from 0.71–0.87. Plants in the far western population (W-77S) and the two far eastern populations (E-89A and E-47A) were significantly less likely to set seed autonomously, with autofertility ranging from 0.08–0.48.

The degree to which emasculation treatment affected autofertility varied among populations, as indicated by the significant interaction between population and time treatment in the two-way ANOVA ($F_{351.8} = 6.7, P < 0.001$). Removing anthers 6 h after anthesis reduced the average autofertility as little as 39% in one of the central populations (C-56A) and as much as 98% in one of the far eastern populations (E-89A; Fig. 3). When anthers were removed 24 h after anthesis, plants from W-69S and C-56A only had a 4-6% reduction in autofertility; however, plants from E-89A still experienced an 83% reduction (Fig. 3).

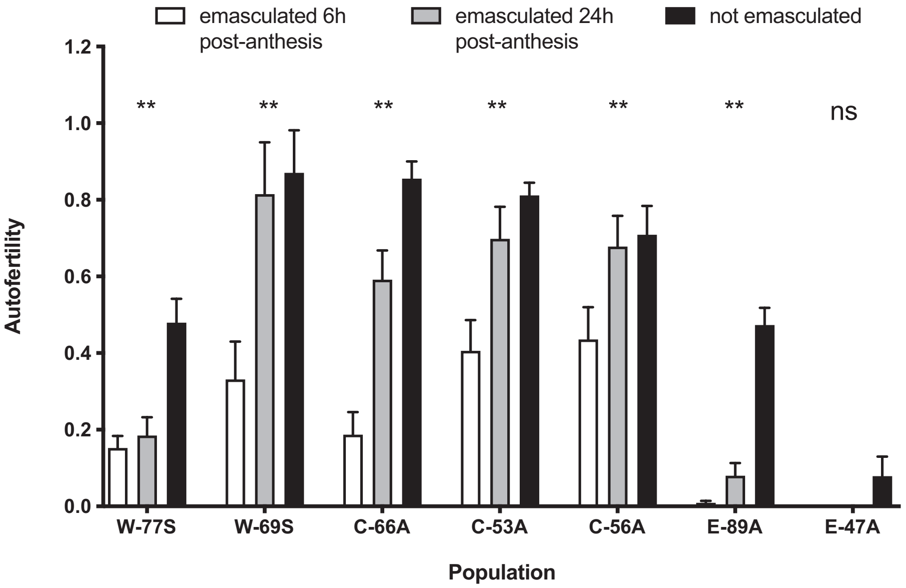

FIGURE 3. Degree of autofertility when anthers were removed 6 h after anthesis (i.e., self-pollination had to occur within 6 h of anthesis), when anthers were removed 24 h after anthesis (i.e., self-pollination had to occur within 24 h), and when anthers were not removed (i.e., self-pollination could occur over the entire life of the flower). Means and standard errors are presented for seven populations of *Clarkia xantiana* ssp. *parviflora* when grown in a common growth chamber environment. Asterisks indicate that emasculation treatment significantly affected autofertility within that population (** $P < 0.0005$), according to a post hoc ANOVA.

Population variation in autofertility was significantly predicted by petal area and protandry ($P < 0.05$ and $P < 0.01$, respectively; Fig. 4A, C) and marginally significantly predicted by herkogamy and stigma stage at anthesis ($P = 0.05$ and $P = 0.06$, respectively; Fig. 4B, D).

Stigma receptivity

Stigma age significantly affected the percentage of stigma area that was receptive ($F_{487.1} = 278.2$, $P < 0.0001$); however, this effect varied across populations ($F_{487.1} = 6.0$, $P < 0.0001$). In six of the seven populations, the percentage of stigma area that was receptive when stigmas were newly flat was relatively low (ranging from 6.5–25.3%) and gradually increased over the next 48 h (Fig. 5). Plants from C-56A, however, had nearly fully receptive stigmas (i.e., 82% of the stigma surface was receptive) as soon as they became flat (Fig. 5).

The magnitude of stigma receptivity differed significantly among populations ($F_{505.2} = 50.3$, $P < 0.0001$) and ranged from 38.7% (C-53A) to 84.3% (C-56A), when averaged across all stigma developmental stages. Despite differences in stigma receptivity patterns among populations, self-pollen grains—when deposited on newly flat stigmas and given only 6 h to germinate—sired a similar quantity of seeds across all populations (Fig. 6). For example, even though C-56A had the greatest receptivity for young stigmas (Fig. 5), self-pollen grains from this population did not sire disproportionately more seeds when placed on newly flat stigmas and given 6 h to germinate (Fig. 6).

DISCUSSION

Floral mechanisms that mediate the opportunity for outcrossing

The opportunity for outcrossing is determined by a collection of traits that occur either before, during, or after pollination (Koski et al., 2018). The presence of multiple traits that act in succession can prevent a single trait that occurs early in the reproductive process from entirely determining an individual's mating system. For example, individuals that lack herkogamy or protandry cannot be assumed to be highly selfing. Traits that affect post-pollination processes, such as the timing of stigma receptivity, provide a downstream mechanism to prevent quickly deposited self-pollen grains from usurping ovules before outcross pollination has the opportunity to occur (Galen et al., 1986; Murdy and Carter, 1987; Skogsmyr and Lankinen, 2002; Lankinen and Madjidian, 2011).

In this study, we found that autofertility varied among populations and was predicted significantly by petal area and protandry (Fig. 4A, C), and marginally significantly predicted by herkogamy

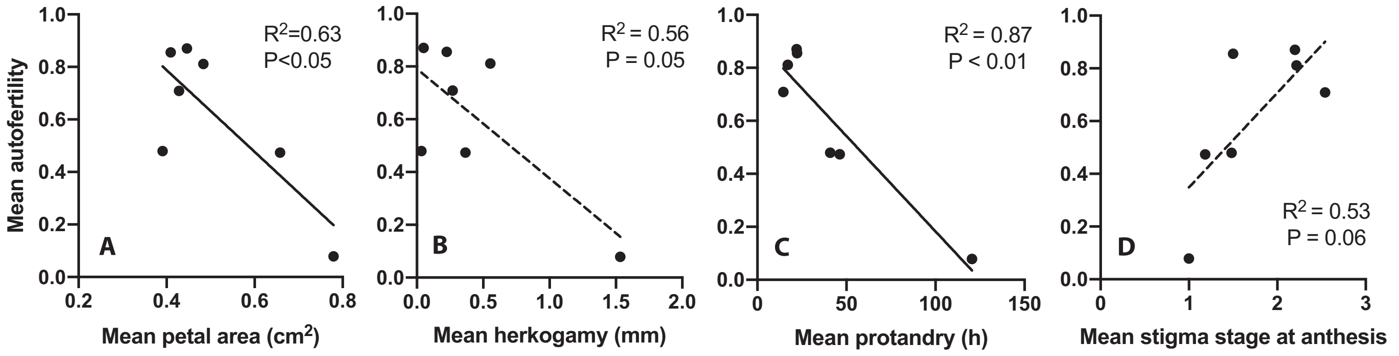

FIGURE 4. Correlations showing the relationships between autofertility and (A) petal area, (B) herkogamy, (C) protandry, and (D) stigma stage at anthesis in *Clarkia xantiana* ssp. *parviflora* when grown in a common growth chamber environment. Dots represent population means for each variable. The solid line indicates a statistically significant relationship ($P < 0.05$); dashed lines indicate relationships that were nearly statistically significant.

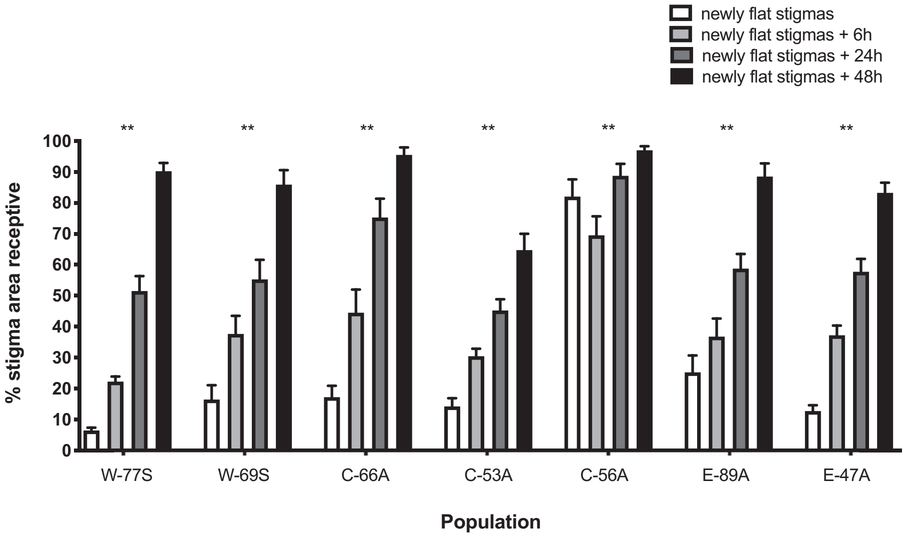

FIGURE 5. Receptivity schedules of stigmas harvested at four developmental stages (newly flat stigmas, 6 h after stigmas became flat, 24 h after stigmas became flat, and 48 h after stigmas became flat). Means and standard errors are presented for seven populations of *Clarkia xantiana* ssp. *parviflora* when grown in a common growth chamber environment. Asterisks indicate that developmental stage significantly affected stigma receptivity within that population (** $P < 0.0001$), according to a post hoc ANOVA.

and stigma stage at anthesis (Fig. 4B, D). The two far eastern populations (E-89A and to a lesser extent E-47A) had the greatest number of traits that prolong the time period over which outcrossing could occur (e.g., enhanced protandry) and maximize the likelihood of outcross pollination via pollinator attraction (i.e., larger petal size). These populations also had the lowest rates of autonomous selfing, suggesting that the more pronounced delayed selfing in these populations may come at a cost; thus, delayed selfing is not quite a "best-of-both-worlds" mating system (Goodwillie and Weber, 2018). By contrast, C-56A had the fewest mechanisms to promote outcrossing. Minimal herkogamy and protandry permitted quick self-pollen deposition, and those self-pollen grains had the potential to germinate immediately, because 82% of the surface of newly flat stigmas was receptive (Fig. 5). In the six remaining populations, the receptivity of newly flat stigmas was lower (i.e., 5–25%), providing a stronger mechanism to delay selfing and promote the potential receipt of outcross pollen. Even though plants from C-56A had the most receptive young stigmas, self-pollen grains from this population did not sire disproportionately more seeds when placed on newly flat stigmas and given 6 h to germinate (Fig. 6). It is possible that by waiting 14.5 h (on average) for C-56A stigmas to become flat after anthesis before transferring pollen, we missed the window of opportunity to detect a functional consequence of early stigma receptivity in this population. Future studies examining stigma receptivity at earlier stages of development would provide insight into whether the opportunity for outcrossing is reduced in this population compared to the others.

Population differentiation in mating system traits

We found fine-scale geographic variation in floral traits of ssp. *parviflora* that are known to influence the mating system. Populations have diverged considerably in some traits despite close proximity. For example, the E-47A population exhibited strongly divergent protandry, herkogamy, and flower size from E-89A despite their close geographic proximity (<5 km). Similarly, the C-56A population exhibited a strongly divergent timing of stigma receptivity from nearby populations (10.0 and 10.3 km away). These results suggest either contrasting patterns of selection in different populations or substantial isolation and genetic drift (or some combination of the two). Our prior population genetic studies and field surveys have indicated that ssp. *parviflora* populations are strongly isolated from one another both genetically and spatially, which has resulted in the fixation of alternative alleles over fine spatial scales, and that populations experienced bottlenecks upon colonization of most sites (Pettengill et al., 2016). Taken together, our results suggest that the population biology of ssp. *parviflora* is marked by rare dispersal events among patches of suitable habitat and subsequent isolation, which may facilitate rapid divergence in floral traits.

Selfing taxa often exhibit greater population genetic structure relative to outcrossers (Hamrick and Godt, 1996; Vekemans and Hardy, 2004); however, comparatively less work has examined phenotypic variation across selfers' geographic ranges. Recent work on *Arabidopsis thaliana*, which is often considered to be universally high selfing, suggests that populations vary extensively in herkogamy and outcrossing rate, sometimes exceeding 60% outcrossing in experimental populations (Luo and Widmer, 2013). These patterns of fine-scale population divergence are intriguing given that selfers are

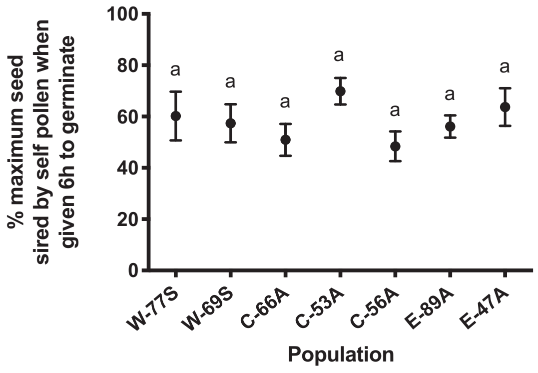

FIGURE 6. Fraction of maximum number of seeds sired by self-pollen grains that were placed on newly flat stigmas and given only 6 h to germinate. The fraction was calculated by dividing the number of seeds sired by self-pollen grains that were given 6 h to germinate (i.e., stigmas were severed 6 h after pollination) by the number of seeds sired by self-pollen grains that were allowed to germinate over the entire lifespan of the flower (i.e., stigmas were not severed). Means and standard errors are presented for seven populations of *Clarkia xantiana* ssp. *parviflora* when grown in a common growth chamber environment. Means sharing letters are not significantly different ($P > 0.05$), according to a Tukey's HSD test.

often considered to have a high capacity to colonize new sites, and therefore are considered "good dispersers" (Baker, 1955; Cheptou, 2012; de Waal et al., 2014). However, both population genetic and trait-oriented studies suggest that many selfing taxa rarely colonize new sites and have low rates of gene flow (Hamrick and Godt, 1996; Vekemans and Hardy, 2004). These population dynamics may thus facilitate divergence in floral traits either by random or deterministic processes.

The history of range expansion and floral evolution

We observed divergence of some floral traits between eastern and western ssp. *parviflora* populations. In particular, petal area and herkogamy have higher values in most eastern compared to western populations. Previous analyses suggest that the divergence of ssp. *parviflora* from ssp. *xantiana* occurred recently (~65,000 bp; Pettengill and Moeller, 2012b), that eastern ssp. *parviflora* populations (i.e., the ancestors of those lineages) were the earliest-diverging in the taxon, and that eastern populations exhibit the greatest genetic similarity to ssp. *xantiana* (Pettengill and Moeller, 2012a, b). Furthermore, population genetic structure shows a broad east to west pattern of divergence, consistent with species distribution models, which predicted a refugium during the last glacial maximum along the eastern extent of the taxon's range (Pettengill and Moeller, 2012a). The divergence of floral traits runs roughly parallel to these patterns of population genetic divergence; however, the causes of floral divergence are not yet clear. Field studies have revealed no evidence that pollinators are less abundant or diverse, or that pollen limitation is greater, in the western compared to eastern portion of the range (Moeller, 2006; Briscoe Runquist and Moeller, 2014). Therefore, it is more likely that progressive episodes of colonization and population bottlenecks (resulting in mate limitation) during westward range expansion could have caused repeated episodes of selection favoring a high propensity to autonomously self (Moeller and Geber, 2005).

Despite evidence of floral divergence between eastern and western populations, we also found protandry and autofertility were most similar between two far eastern (E-89A and E-47A) and the most western (W-77S) population, which is the only population in our study where ssp. *parviflora* individuals occur within meters to tens of meters of ssp. *xantiana* individuals. Past studies indicate that reproductive isolation is very strong between the taxa, even in sympatry, but not complete (Briscoe Runquist et al., 2014). Although we currently lack detailed information on the frequency of hybridization, some prior evidence from DNA sequence data suggests occasional admixture in sympatry. Thus, it is possible that alleles influencing floral traits have introgressed from ssp. *xantiana* to ssp. *parviflora* and influenced floral trait variation.

Implications of floral mechanisms for the evolution of selfing populations

Although ssp. *parvifora* is primarily selfing, some populations have a greater opportunity for outcrossing than others and vary in levels of DNA sequence variation (Pettengill et al., 2016). At a short timescale, outcrossing can provide a fitness advantage to offspring in cases where inbreeding depression would otherwise reduce the fitness of selfed offspring. Given that inbreeding depression persists in many selfing lineages despite purging, particularly at later life stages, outcrossing may have substantial positive fitness effects (Winn et al., 2011). Over long time periods, the presence of occasional outcrossing events may enhance effective population size (Nordborg, 2000), the rate at which multiple advantageous mutations can be fixed in populations (Hill-Robertson effect: Comeron et al., 2008; Hartfield and Glémin, 2016), and hinder the accumulation of mildly deleterious mutations (Heller and Maynard Smith, 1978; Lynch et al., 1995). Both of the inefficacy of selection and the accumulation of deleterious mutations have been proposed to potentially cause population extinction in highly selfing organisms (Takebayashi and Morrell, 2001; Hartfield and Glémin, 2016). Therefore, traits that promote occasional outcrossing in primarily selfing populations may be important for the maintenance of genetic variation and persistence of populations through time.

ACKNOWLEDGMENTS

The authors thank Leslie Clements, Diane Hunt, Kelsey Smith, and Morgan Wall for assisting with data collection and plant care throughout the experiment. We also thank the anonymous reviewers for providing very helpful comments on an earlier version of this manuscript. This project was funded by the CNU Summer Scholars Program and NSF DEB-1025004 to DAM (including ROA supplement).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Correlation matrix for the four floral traits in the seven populations of *Clarkia xantiana* ssp. *parviflora* included in this study.

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