Title: Experimental and genetic analysis of selfing reveals no reinforcement in *Phlox cuspidata*

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Running header: Selfing in *Phlox cuspidata*

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Summary

- Reinforcement is the process through which prezygotic reproductive barriers evolve in sympatry due to selection against hybridization between co-occurring, closely related species. The role of self-fertilization in reinforcement and reproductive isolation is uncertain in part because its efficiency as a barrier against heterospecific mating can depend on the timing of autonomous selfing.
- To investigate whether increased autonomous selfing has evolved as a mechanism for reinforcement, we compared *Phlox cuspidata* populations across their native Texas range using both estimates of genetic diversity and experimental manipulation with morphological measurements. Specifically, we investigated patterns of variation in floral traits and timing of selfing between individuals from allopatric populations of *P. cuspidata* and from populations sympatric with the closely related species, *P. drummondii*.
- We infer intermediate rates of selfing across field-collected individuals with no significant difference between allopatric and sympatric populations. Among greenhouse grown plants, we find no differences in timing of selfing or other floral traits including anther dehiscence timing, anther-stigma distances, autonomous selfing rate and self-seed count between allopatric and sympatric populations. However, our statistical analyses indicate that *P. cuspidata* individuals sympatric with *P drummondii* seem to have generally larger flowers compared to allopatric individuals.
- Despite strong evidence of costly hybridization with *P. drummondii*, we find no evidence of trait divergence due to reinforcement in *P. cuspidata.* Although we document nearly complete autonomous self-seed set in the greenhouse, estimates of selfing rates from genetic data imply realized selfing is much lower in nature suggesting an opportunity for reinforcing selection to act on this trait.

Introduction

Determining how reproductive isolation (RI) evolves and is maintained between closely related species is key to understanding the speciation process (Coyne & Orr, 2004). RI is caused by the formation of multiple pre and post zygotic mechanisms. While these mechanisms vary among taxa, studies observing RI through time often note that prezygotic RI evolves with greater strength and faster in sympatry than in allopatry (Coyne & Orr, 1989; Noor, 1995). This pattern supports reinforcement as a broadly important process in species formation (Coyne & Orr, 1989; Tabah & Hiscock, 2003; Yukilevich, 2011). Reinforcement is the process through which prezygotic barriers to reproduction evolve in sympatry due to selection against costly hybridization between co-occurring, closely related species (Dobzhansky, 1940). Reinforcement often generates a pattern of reproductive character displacement (RCD), in which adaptive traits such as differences in reproductive timing or flower morphology evolve in sympatric populations but not allopatric populations (Levin, 1985; Knowlton, 1993). This trait divergence minimizes deleterious reproductive interactions between species that have come into secondary contact and increases RI in the process (Levin, 1985; Lowry et al., 2008; Hopkins, 2013; Pfennig & Rice, 2014; Baack et al., 2015). Most research on prezygotic isolation in plants has focused on how patterns of pollinator visitation and associated floral trait evolution can drive RI (Schemske & Bradshaw, 1999; Cardona et al., 2020). However, mating system shifts and associated floral differences can also drive prezygotic barriers between sympatric species, yet these processes have not been as extensively studied (Fishman & Wyatt, 1999; Wendt et al., 2002; Grossenbacher & Whittall, 2011; Castillo et al., 2016; Rausher, 2017).

Plants have evolved a wide range of mating system strategies that underly variation in outcrossing rates. For example, angiosperms have evolved genetic self-incompatibility (SI), herkogamy, dichogamy, dioecy, or even sterile flowers (i.e., nonsexual display that can enhance pollinator visitation) (Yampolsky & Yampolsky, 1886; Webb & Lloyd, 1986; Barrett & Harder, 1996; Barrett, 2010; Takayama & Isogai, 2005). Driven by this variation in mating system strategy, species fall along a continuous distribution of realized selfing rates (Igic & Kohn, 2006). At the highly-selfing extreme of this spectrum, flowering plants often evolve correlated suites of floral trait variation. Specifically, the “selfing syndrome” is characterized by smaller seed size, shorter bud developmental stages, reduced flower size, and reduced pollen to ovule ratio compared to their outcrossing relatives (Stebbins, 1950; Wyatt, 1984; Steiner, 1988; Ritland & Ritland, 1989; Sicard & Lenhard, 2011; Renner, 2014). These morphological characteristics consistently accompany transitions from outcrossing to selfing across angiosperm evolution.

The transition to autonomous selfing is one of the most frequent evolutionary transitions, with ~20% angiosperm species having evolved predominant selfing (autogamy) from outcrossing (Barrett, 2002). Frequent transitions towards selfing might seem surprising since selfers often incur the cost of inbreeding depression either through increased homozygosity of deleterious recessive alleles or through the loss of heterozygosity at loci exhibiting heterozygote advantage (Charlesworth, 1992; Possingham et al., 2001; Wright et al., 2013). If selfing occurs over enough generations, purging of deleterious recessive alleles by natural selection can effectively remove the harmful effects of inbreeding (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987). However, this purging can come at a cost of loss of genetic diversity and adaptive capacity. Despite this disadvantage, evolving autogamy provides reproductive assurance in situations where seed production is pollen limited (Lloyd & Schoen, 1992; Fishman & Wyatt, 1999; Busch & Delph, 2012; Toräng et al., 2017). Moreover, selfing plants transmit two copies of their genes to offspring compared to the one copy transmitted by outcrossing plants, resulting in a two-fold transmission advantage (Fisher, 1941).

Shifts in mating system can also lead to increased reproductive isolation between species in secondary contact (Brys et al., 2014; Cardona et al., 2020). For example, increased selfing can decrease hybridization by reducing heterospecific pollen transfer. Several case studies have found that increased selfing can evolve to avoid hybridization with competing species, a pattern consistent with reinforcement of speciation (Brown & Wilson, 1956; Antonovics, 1968; Levin, 1972; Fishman & Wyatt, 1999). Furthermore, macro-evolutionary studies indicate divergence in mating system is greater in sympatric sister species compared to allopatric sister species, suggesting the general importance of autonomous selfing for reproductive isolation (Grossenbacher & Whittall, 2011).

The efficiency of selfing as a barrier against heterospecific mating largely depends on the timing of autonomous selfing (Brys et al., 2016). For increased selfing to decrease hybridization, self-pollination must either occur before outcross pollination or outcompete heterospecific pollen when concurrently deposited on the stigma. Evolving an earlier developmental timing of autonomous self-fertilization can give priority to self-pollen and thus effectively cause reproductive isolation. Out of the three modes of autonomous selfing (prior, competing or delayed), prior (or earlier) autonomous selfing significantly reduces the possibility of successful outcross pollination and thus hybridization (Brys et al., 2016). If reinforcement were to favor a trait to decrease hybridization by increasing self-fertilization, then we hypothesize that the timing of selfing will be favored to evolve earlier in sympatric populations relative to allopatric populations. This pattern would ensure selfing prior to heterospecific pollen deposition and hybrid formation (Elle et al., 2010; Brys et al., 2016; Yang et al., 2018). To our knowledge, there has been only one study investigating the timing of selfing as a contributor to RI which did find a pattern consistent with reinforcement such that the selfing species exhibits earlier selfing in sympatric relative to allopatric populations (Randle et al., 2018).

The annual Texas wildflowers *Phlox drummondii* and *Phlox cuspidata* provide an ideal system to investigate the interaction between mating system evolution and reproductive isolation. These two species are partially sympatric and hybridize where they co-occur (Ferguson et al., 1999; Hopkins & Rausher, 2012; Roda & Hopkins, 2019). The species flower simultaneously and are visited by the same pollinating species (Burgin et al., 2023; Hopkins & Rausher, 2012; Ruane & Donohue, 2007). Hybrids between the two *Phlox* species have strong or complete sterility compared to the two parental species indicating that hybridization is costly (Suni et al., 2018). Previous research demonstrates that reinforcing selection favored flower color divergence in sympatric populations of *P. drummondii*, thus decreasing hybridization with closely related *P. cuspidata* (Hopkins & Rausher, 2012; Hopkins & Rausher, 2014). Unlike *P. drummondii*, it is unclear whether *P. cuspidata* has evolved any mechanisms to reduce hybridization in sympatry. *Phlox cuspidata* is hypothesized to be self-compatible, indicating that variation in autonomous selfing could reduce hybridization and respond to reinforcing selection (Levin, 1985; Roda et al., 2017). In this study, we aim to determine whether selfing rate, timing of anther dehiscence, and key flower traits associated with mating system variation differ between allopatric and sympatric individuals across several *P. cuspidata* populations.

Materials and Methods

Study species

Phlox cuspidata (Polemoniaceae) is an annual wildflower that occurs throughout Eastern Texas (Fig. 1). Flowers are five-parted with sexual organs (pistil and anthers) fully inserted into a fused corolla tube. The stigma is positioned beneath two rows of anthers occurring at two heights in the corolla tube (Fig. 2). Flowers are light blue/purple, fragrant, and borne in clusters. Each flower produces a single, dehiscent fruit which contains up to three seeds. Based on its reduced floral size relative to other members of the *Phlox* genus, it is hypothesized that *P. cuspidata* is predominantly selfing (Levin, 1978; Levin, 1989). In a study using a single population and relying on a highly limited number of genetic markers, selfing rate in field conditions was inferred to be ~78% (Levin, 1978). To our knowledge, there have been no further quantifications of selfing rate in *P. cuspidata* in field or greenhouse conditions. *Phlox drummondii* has an active genetic self-incompatibility mechanism that prevents germination of self-pollen on the stigmatic surface (Roda & Hopkins, 2019). It is unknown whether *P. cuspidata* maintains any function of this genetic SI mechanism.

P. cuspidata grows in sympatry with a closely related species, *P. drummondii*, at the western edge of its range (Fig. 1). These *Phlox* species share pollinators and overlap temporally throughout their blooming season (Burgin et al., 2023; Hopkins & Rausher, 2012; Ruane & Donohue, 2007). As a result, hybridization can and does occur, resulting in the production of hybrids with significantly reduced fitness (Ferguson et al., 1999; Suni et al., 2018).

Genetic estimates of selfing rate using field-collected individuals

To infer rates of realized selfing in allopatric and sympatric populations, we grew seeds collected from natural populations and sequenced individuals to estimate genetic diversity. We collected seeds from naturally growing plants in 2019 and 2021 and grew one seed from 4-25 maternal plants from 12 populations (6 sympatric and 6 allopatric) for a total of 171 individuals. We incubated field-collected seeds in 500 ppm gibberellic acid in water for two days, vernalized planted seeds at 4 °C for ten days, and subsequently moved pots to growth chambers with controlled temperatures of 23 °C during the day and 20 °C at night to initiate germination.

We extracted DNA from fresh leaf tissue using the EZNA Plant DNA kit buffers (Omega Bio-Tek) combined with a chloroform extraction and two cold 96% ethanol washes as in Goulet-Scott et al. (2021). We prepared DNA libraries with double-digest restriction site-associated DNA sequencing (ddRAD-seq) protocol (Peterson et al., 2012). We used a Pippin Prep (Sage Biosciences) to select for 300-500bp fragments and cleaned pooled libraries with a Monarch PCR and DNA Cleanup kit (New England Biolabs). Samples were paired end sequenced (2x150 bp) at the Bauer Core of Harvard University on one Illumina NovaSeq 6000 lane. Illumina reads are accessible in NCBI's Sequence Read Archive (SRA; ##).

Raw reads were demultiplexed and filtered as described in Goulet-Scott et al., 2021. Reads were aligned to a complete assembly of the *P. drummondii* genome (Wu et al. 2023, unpublished), using BWA-MEM, and individuals were genotyped using STACKS v2.64 (Catchen et al., 2013; Li, 2013). Samples with a mean read depth under 10 were removed. Sites were filtered using BCFtools to include only biallelic SNPs with no more than 50% missingness across individuals (Danecek & Bonfield et al., 2021). Sites were further filtered using BCFtools to include only sites with a minor allele frequency greater than 5%. After these quality filtering steps, 70,118 sites remained. The final dataset included 86 sympatric and 68 allopatric individuals representing 6 sympatric and 6 allopatric populations (Table 1). Using the populations program in Stacks v2.64, we calculated the inbreeding coefficient F_{IS} averaged across loci for each population (Wright, 1949; Hartl & Clark, 1997; Danecek et al., 2011; Catchen et al., 2013). We inferred selfing rate (*s*) via its relationship to F_{IS} defined by the formula (see Hartl & Clark, 1997):

$$F_{IS} = \frac{s}{2-s}$$

Seed collection and plant growth for greenhouse experiments

To quantify traits involved in autonomous self-pollination, seeds from multiple sympatric and allopatric populations of *P. cuspidata* were collected in 2019 and 2021. These populations represent some but not all of those included in the genetic analyses (Fig. 1; Table 1). We generated maternal families by growing multiple seeds collected from the same plant in the field. Individuals within a maternal family range from full to half siblings sharing the same maternal parent. For a balanced design, we aimed to grow 20 maternal families from each of four sympatric and four allopatric populations. However, due to low germination rates, not all populations were included in subsequent analyses (Fig. 1; Table 1). In total, the experiment included 180 individuals representing 68 allopatric and 112 sympatric individuals (Table 1). Seeds were soaked in gibberellic acid and vernalized to induce germination as described above.

Flower and plant size

Germinated seeds were transplanted to 4.5-inch pots (using Pro-Mix HP Mycorrhizae potting media) and grown to flowering in a greenhouse with 16h of supplemented light and a temperature ranging from 23 °C to 26 °C. For each plant, we scanned six flowers (three face up and three on the side) and used ImageJ to calculate flower face diameter and flower length (Fig. A1). To estimate variation in plant height, the length between the second and third internode was recorded for each plant.

Anther-dehiscence timing

To determine timing of anther-dehiscence, four flowers were marked on one experimental plant per maternal family (n=79) at each of five bud developmental stages (e.g., Stage 0 = pigmented bud a day before opening, Stage 1 = day 1 of fully opened flower, Stage

2 = day 2 of fully opened flower, Stage 3 = day 3 of fully opened flower, Stage 4 = day 4 of fully opened flower; Fig. 2). For each flower included in the experiment, the anthers and attached corolla were removed at the assigned stage, leaving behind the pistil. We visually assessed anthers on the day they were removed from the calyx and recorded whether pollen dehiscence had occurred as a binary trait. Plants were grown in the greenhouse where all pollinators are excluded thereby preventing pollen transfer between flowers.

Timing of autonomous selfing

To determine timing of autonomous selfing, we recorded total fruit set from the experimental flowers that had anthers removed at each developmental stage from Stage 0 to Stage 4 (n=79) as described above. For a fruit to set, autonomously deposited self-pollen must have reached the receptive stigma prior to the stage at which the anthers were removed (Fig. 2). If dehisced pollen has not yet reached the receptive stigma, no fruit will form even at developmental stages when anthers have dehisced.

Anther-stigma distance

To quantify herkogamy (anther-stigma distance), two additional buds/flowers were marked at both developmental Stage 0 and 1 on the 79 plants included in the autonomous selfing experiment. The heights of the stamens and the pistil were measured from the bottom of the ovary. We calculated the distance from the stigma to the anthers by subtracting the height of the shortest stamen (closest to the stigma) from the height of the pistil.

Selfing rate and seed count

To estimate the autonomous selfing rate in the greenhouse, we bagged 10-15 unmanipulated flowers on one offspring per plant (n=79) and monitored for seed set. Selfing rate for each plant was calculated as the proportion of flowers that formed fruits. Seed count was estimated by counting the number of seeds developed from a known number of fruits.

Statistical analyses

We constructed a simple linear model that included zone (two categories: sympatry vs. allopatry) as a fixed factor to assess variation in F_{IS} and selfing rate inferred from genetic variation. Our greenhouse manipulative experiments aimed to test whether there were significant differences between sympatric and allopatric individuals of *P. cuspidata* in flower length and diameter, internode distance, timing of autonomous selfing, anther-stigma distance, selfing rate, and seed count. Specifically, we analyzed linear and generalized mixed models to test for differences in each of the seven traits independently. A linear mixed model was built for flower length, diameter and internode length separately with a normal distribution that included zone (two categories: allopatric and sympatric) as a fixed factor and random effects of maternal family to account for plants collected from the same mother and individual as multiple measurements were taken per plant.

A simple linear model was built for autonomous selfing rate in the greenhouse and seed count separately with zone (categorical) as a fixed factor and fruit number as an offset variable for seed count. To analyze variation in timing of selfing, a generalized linear model was built for fruit set with a binomial distribution that included zone as a categorical variable, stage as a numerical variable and their interaction, with individual as random effects as each plant included was observed for fruit set over the five developmental stages.

For anther-stigma distances, a linear model of the minimum distance between the stigma and anther was built with zone and stage as categorical fixed factors along with their interaction and maternal family and individual as random effect because the minimum distance was measured on two flowers per stage per plant. Because there were more than twice the number of sympatric than allopatric individuals (Table 1), an equal number of sympatric individuals were randomly sampled 1,000 times and p-values were compared for all floral traits measured. All statistical analyses were performed in R (R Core Team, 2021) using package *lme4* (Bates et al., 2015).

Results

Genetic estimates of selfing from field-collected individuals

Genetic diversity measured from field collected individuals indicates some but not complete selfing in nature. After filtering for quality and coverage, we analyzed genetic diversity using 154 individuals (86 sympatric and 68 allopatric) from 12 populations (6 sympatric and 6 allopatric). Estimates of F_{IS} were similar across all populations with no differences between sympatric and allopatric populations (allopatric = 0.3377, sympatric = 0.3064) (Tables A1, A2). Inferred rates of selfing based on estimates of F_{IS} were intermediate and not significantly different between allopatric and sympatric populations (allopatric = 49.94%, sympatric = 45.29%) (Fig. 3a; Tables A1, A2).

Autonomous selfing in greenhouse-grown plants

At developmental Stage 0 (the day before bud opening) no marked flowers had anthers dehisced. By developmental Stage 1 (day 1 of open flower), all marked flowers had dehisced anthers. Fruit set increased over time across all populations, with 0% fruit set at developmental Stage 0 to nearly 100% fruit set by developmental Stage 4 (day 4 of open flower) (Fig. 3b). We detected no variation in anther dehiscence timing, autonomous-self fruit set across developmental stages, autonomous selfing rate or seed count between sympatric and allopatric populations (Table 2; Fig. 2; Fig. 3c, d, Fig. A2). On average, sympatric and allopatric *P. cuspidata* individuals had a 92% and 93% selfing rate respectively with 2.7 seeds per fruit (out of a maximum of 3) (Fig. 3c, d; Fig. A2).

Floral traits of greenhouse-grown plants

Flower length and diameter were significantly greater for sympatric populations of *P. cuspidata* compared to allopatric populations (Table 2; Fig. 3e, f; Fig. A2). In contrast, there were no significant differences between sympatric and allopatric individuals in internode length or anther-stigma distance. Although these differences did not pass significance, mean internode distance is larger among sympatric plants indicating overall larger plants in sympatry (Table 2; Fig. 3g, h; Fig. A2).

Discussion

The cost of producing low fitness hybrids can generate reinforcing selection favoring traits that decrease hybridization between sympatric species. We tested the hypothesis that reinforcement has favored the evolution of increased and earlier selfing in *P. cuspidata* individuals sympatric with *P. drummondii*. These two species share a broad region of geographic overlap, are known to hybridize in nature, and produce highly sterile offspring making the conditions seemingly ideal for reinforcement. However, our results indicate that the selfing syndrome is not more exaggerated in sympatry than allopatry in *P. cuspidata*. In fact, plants from both sympatric and allopatric populations are efficient selfers with high autonomous selfing rates in the greenhouse. Population genetic estimates of selfing rate demonstrate intermediate selfing in natural conditions, with no difference between sympatric and allopatric populations, consistent with the lack of difference observed in greenhouse grown plants. Our analysis of *P. cuspidata* populations spanning its Texas range revealed no significant differences in timing of selfing or other floral traits associated with selfing syndrome including anther dehiscence timing and anther-stigma distances between the allopatric and sympatric populations growing with *P. drummondii*. Contrary to the hypothesis of reinforcement favoring increased selfing in sympatry, we find that sympatric individuals have larger floral length and diameter than allopatric individuals.

Phlox cuspidata displays a highly efficient selfing syndrome throughout its geographic range. It has evolved small tubular corollas and anthers dehisce as soon as the flower opens. Under greenhouse conditions, autonomous selfing results in nearly 100% fruit set within four days after a flower opens. Despite this high potential for selfing, patterns of genetic variation indicate that the selfing rate in nature is only around 45-50%. The large difference in selfing rate estimated in the greenhouse versus via genetic data may be driven by inbreeding depression if selfed offspring have lower fitness compared to outcrossed offspring in nature. While there may be some contribution of inbreeding depression to this apparent difference, it is clear from the population genetic data that natural populations of *P. cuspidata* reproduce through both selfing and outcrossing. This finding is consistent with the complex relationship between the potential for selfing and realized outcrossing rates described across angiosperms. Although strongly self-incompatible species tend to have high outcrossing rates, strongly self-compatible species span a range of outcrossing rates (Raduski et al., 2011). Our data suggest that *P. cuspidata* is strongly self-compatible with an intermediate outcrossing rate in nature (~45-50%). Previous field observations demonstrate pollinator visitation to *P. cuspidata* flowers, supporting our finding of intermediate outcrossing rates in natural populations (Hopkins & Rausher, 2012; Briggs et al., 2018). Because *P. cuspidata* achieves reproduction through both outcrossing and selfing, reinforcing selection may act on traits that vary the timing and efficiency of self-fertilization to decrease costly hybridization with *P. drummondii*.

Mating system variation is known to evolve to prevent costly hybridization in a variety of other systems across the angiosperm phylogeny. For example, case studies in *Arenaria uniflora* (Caryophyllaceae) (Fishman & Wyatt, 1999), *Collinsia rattanii* (Scrophulariaceae) (Randle et al., 2018) and *Clarkia xantiana* subsp. *parviflora* (Onagraceae) (Briscoe Runquist & Moeller, 2013) show evidence that reinforcement has favored the evolution of increased selfing in sympatric populations. Furthermore, a metanalysis in *Mimulus* found that sympatric sister species are more likely to have one species with characteristic selfing-syndrome floral traits than allopatric sister species, which may be caused by selection to avoid hybridization in sympatry (Grossenbacher & Whittall, 2011). Finally, theoretical work supports increased selfing as a viable and, under some conditions, favorable mechanism for decreasing hybridization between sympatric species (Castillo et al., 2016; Rausher, 2017). Collectively, previous work demonstrates how a shift in selfing rate can serve as a reproductive isolating mechanism favored by reinforcement. Despite this strong empirical and theoretical basis provided by other systems, we find no evidence of increased selfing or selfing syndrome traits in sympatric *P. cuspidata* individuals. There is no obvious difference between *Phlox* and other systems previously investigated that might explain why we do not find evidence of reinforcement in *P. cuspidata.* We discuss several hypotheses to explain this finding here.

First, it may be that the net selection favoring increased selfing is not as strong as we originally predicted. Hybrids of *P. cuspidata* and *P. drummondii* are nearly completely sterile, yet if hybridization is exceedingly rare, the overall strength of reinforcing selection will be low. It has already been demonstrated that flower color variation in the sympatric species *P. drummondii* can decrease hybridization by 50% (Hopkins & Rausher, 2012). As a result, there may be very little current hybridization to generate selection favoring increased selfing in sympatric *P. cuspidata*. Theoretical analyses demonstrate that it is difficult for reinforcement to complete speciation (i.e., stop hybridization completely) because the strength of selection diminishes as reproductive isolation increases (Bank et al., 2011).

Additionally, it could be that realized selfing is higher than estimated by the population genetic measures presented here. If selfing in nature is as high as selfing in the greenhouse, selection would have little room to act to increase selfing rate in sympatry.

Selfing estimates based on population-level heterozygote deficiency (F_{IS}) are imperfect because they rely on assumptions rarely met in nature. Previous studies demonstrate that F_{IS}-based calculations tend to overestimate the selfing rate relative to progeny arrays when the selfing rate is < 50% (Jarne & Auld, 2006). Therefore, we expect the true selfing rate in natural *P. cuspidata* populations to be lower, or at least not much higher, than the estimates presented here. More precise estimates of selfing based on progeny arrays would provide additional clarity (Colicchio & Herman, 2020).

Furthermore, the cost of hybridization favoring increased selfing could be partially countered by selection against selfing due to inbreeding depression and its associated costs. If selection towards maintaining genetic diversity with higher outcrossing rates is high, then net selection on the timing of selfing and selfing rate will be weak or counter to the expectations of reinforcement (Epinat & Lenormand, 2009; Pickup et al., 2019). This hypothesis is particularly intriguing for two reasons. First, we found that flowers from sympatric individuals were larger. Floral display size is an important signal for pollinators, and these findings may reflect selection to increase pollinator attraction. Consistent with increased pollinator attraction, we inferred that selfing rates in nature were somewhat, although not significantly, lower in sympatry than allopatry. Second, we found that autonomous selfing rate in the greenhouse is low on the first day a flower is open (~10%) and remains low through the second day (~25%). As a result, there is high potential for outcrossing during the first days of a flower's lifespan. These data are consistent with the delayed selfing hypothesis which suggests that selfing occurring after outcrossing opportunities have passed should be selected for under a wide range of ecological conditions (Goodwillie & Weber, 2018). If *P. cuspidata* experiences selection for delayed selfing as a mechanism to maintain opportunities for outcrossing, it may explain why we fail to find evidence for the evolution of earlier selfing in sympatric populations. Further investigations of pollinator visitation, timing of selfing in natural populations, and the relative fitness of selfed versus outcrossed offspring could address these hypotheses.

Another possible explanation for the observed absence of reinforcing selection is a lack of genetic variation underlying selfing related traits available for selection to act upon. Across all individuals, from all populations, we found that all anthers dehisced on the first day of the open flower. Although we only monitored anther dehiscence once per day, we found no evidence that anthers could release pollen earlier than flower opening in this species or later than the morning of flower opening. Alternatively, there could be negative pleiotropic effects of adjusting anther dehiscence timing due to correlated developmental traits or fertility. If strong purifying selection results in highly coordinated timing of floral development, we would expect to find little to no variation in anther dehiscence timing consistent with our findings in *P. cuspidata*. Future work should focus on investigating the extent of adaptive divergence in other floral traits across populations of *P. cuspidata* to clarify whether anther dehiscence timing is a uniquely invariant trait.

In summary, we found no systematic difference between sympatric and allopatric individuals of *P. cuspidata* in the timing of selfing, anther dehiscence timing, anther-stigma distance, or self-seed set between the sympatric and allopatric populations. Despite the high potential for selfing in *P. cuspidata*, population genetic estimates of selfing rate indicate intermediate selfing rates in nature, with no difference in sympatric versus allopatric populations. Although reinforcement underlies mating system divergence in other systems, we find no evidence of reinforcing selection acting on the timing of selfing in *P. cuspidata*. Understanding why reinforcement occurs in some cases but not others can help clarify its overall importance to the speciation process and the dynamics under which reinforcement evolves.

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Author Contribution

BMS, GAB and RH designed the study. BMS and GAB collected the data. BMS and GAB analyzed the data. BMS, GAB and RH wrote the manuscript.

Data Availability Statement

The data and statistical code that support the findings of this study are available on Dryad.

Conflict of Interest Statement

The authors declare no conflict of interest.

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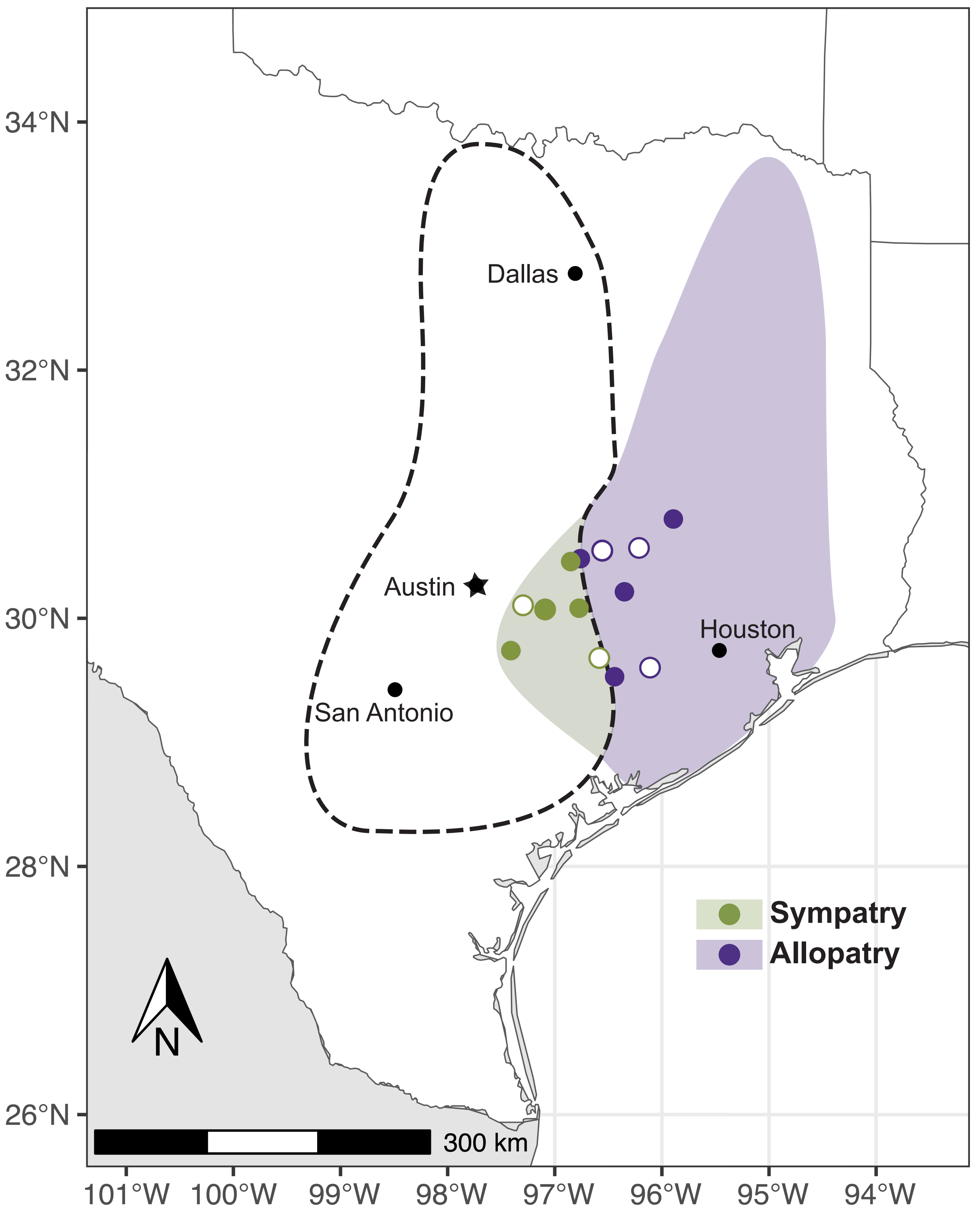
Figure captions

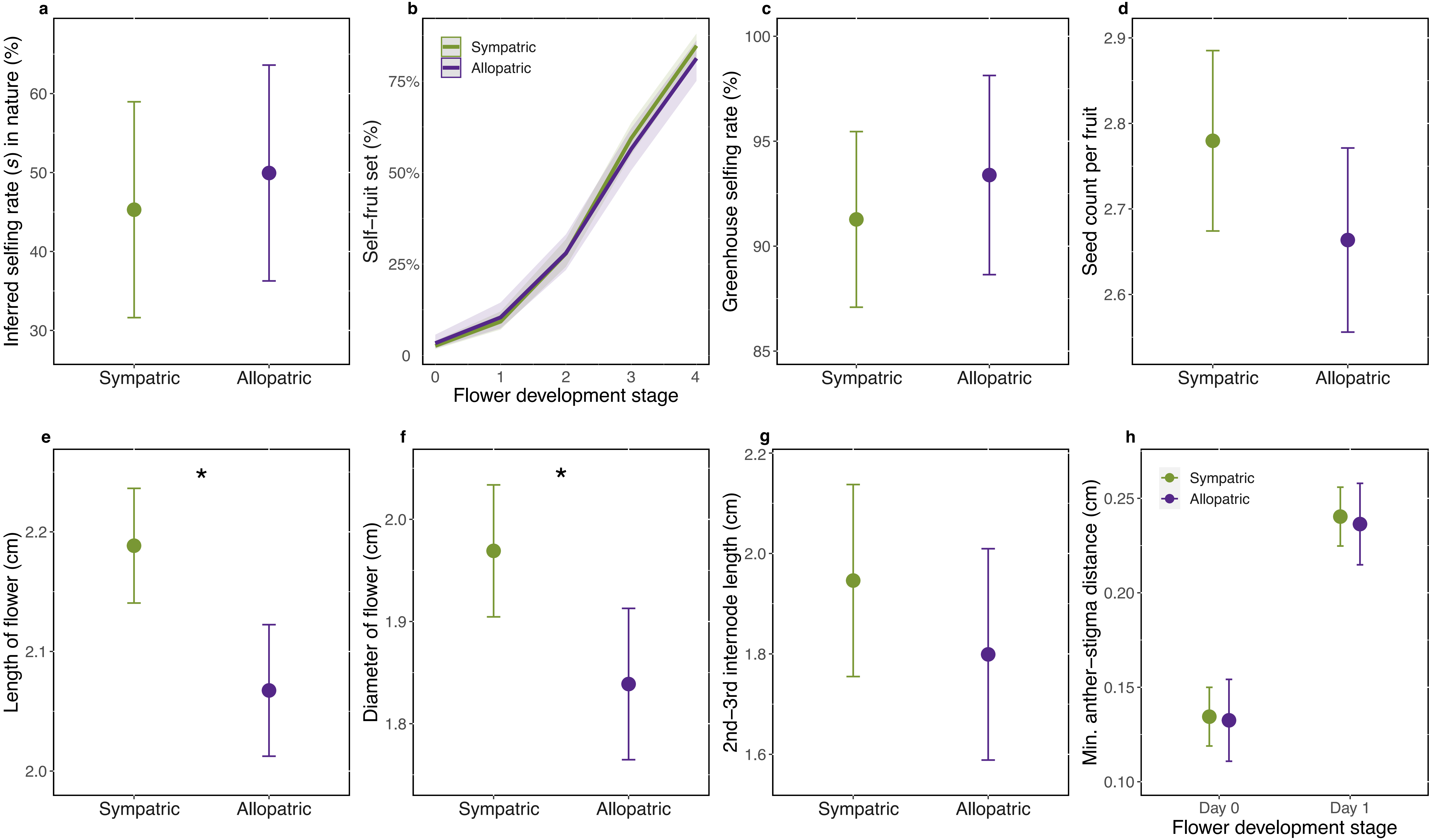
Figure 1: The range of *Phlox cuspidata* extends throughout Eastern Texas. The range of *P. drummondii* is indicated with a dashed black line. The allopatric portion of *P. cuspidata*'s range is shaded purple while the sympatric range is shaded green. All marked points represent locations of sampled populations used in this experiment (allopatric populations are shown in purple while sympatric populations are shown in green) with solid circles represent those included in the greenhouse experiment.

Figure 2: Floral development of *Phlox cuspidata* including cross sections of the floral tube (top and bottom panels). Five stages of floral and anther-stigma development (left to right) where Stage 0 = Pigmented bud a day before opening with no anther dehiscence (slight damage occurred to anthers during dissection), Stage 1 = Day 1 of a fully open flower with dehisced anthers, Stage 2 = Day 2 of open flower with some pollen on stigma, Stage 3 = Day 3 of open flower starting to wilt with most pollen around and on stigma, and Stage 4 = Day 4 showing flower completely wilted with extensive pollen having reached stigma.

Figure 3: Variation in traits between sympatric (green) and allopatric (purple) *Phlox cuspidata* individuals including (A) selfing rate inferred from genetic data (B) autonomous self-fruit set across flower developmental stage (C) total autonomous selfing rate in greenhouse conditions (D) seed count per fruit from autonomous self-set seeds in the greenhouse (E) flower length (F) flower diameter (G) internode length between 2nd and 3rd leaf pair (H) minimum distance between anther and stigma. The mean of the seven traits measured are plotted as points. 95% confidence intervals are indicated as bars. Asterisks (*) indicate significant differences in flower length and diameter between sympatric and allopatric populations.


34°N
32°N
30°N
28°N
26°N
101°W
100°W
99°W
98°W
97°W
96°W
95°W
94°W
Dallas
Austin
San Antonio
Houston
Sympatry
Allopatry
N
300 km


Flower development
Cross section of floral tube
corolla tube
anther
filament
stamen
stigma
style
ovary
pistil
Stage 0
Stage 1
Stage 2
Stage 3
Stage 4


a
Inferred selfing rate (s) in nature (%)
30
40
50
60
Sympatric
Allopatric
b
Sympatric
Allopatric
Self−fruit set (%)
0
25%
50%
75%
0
1
2
3
4
Flower development stage
c
Greenhouse selfing rate (%)
85
90
95
100
Sympatric
Allopatric
d
Seed count per fruit
2.6
2.7
2.8
2.9
Sympatric
Allopatric
e
*
Length of flower (cm)
2.0
2.1
2.2
Sympatric
Allopatric
f
*
Diameter of flower (cm)
1.8
1.9
2.0
Sympatric
Allopatric
g
2nd−3rd internode length (cm)
1.6
1.8
2.0
2.2
Sympatric
Allopatric
h
Sympatric
Allopatric
Min. anther−stigma distance (cm)
0.10
0.15
0.20
0.25
Day 0
Day 1
Flower development stage

Appendices for manuscript: Experimental and genetic analysis of selfing reveal no reinforcement in *Phlox cuspidata*

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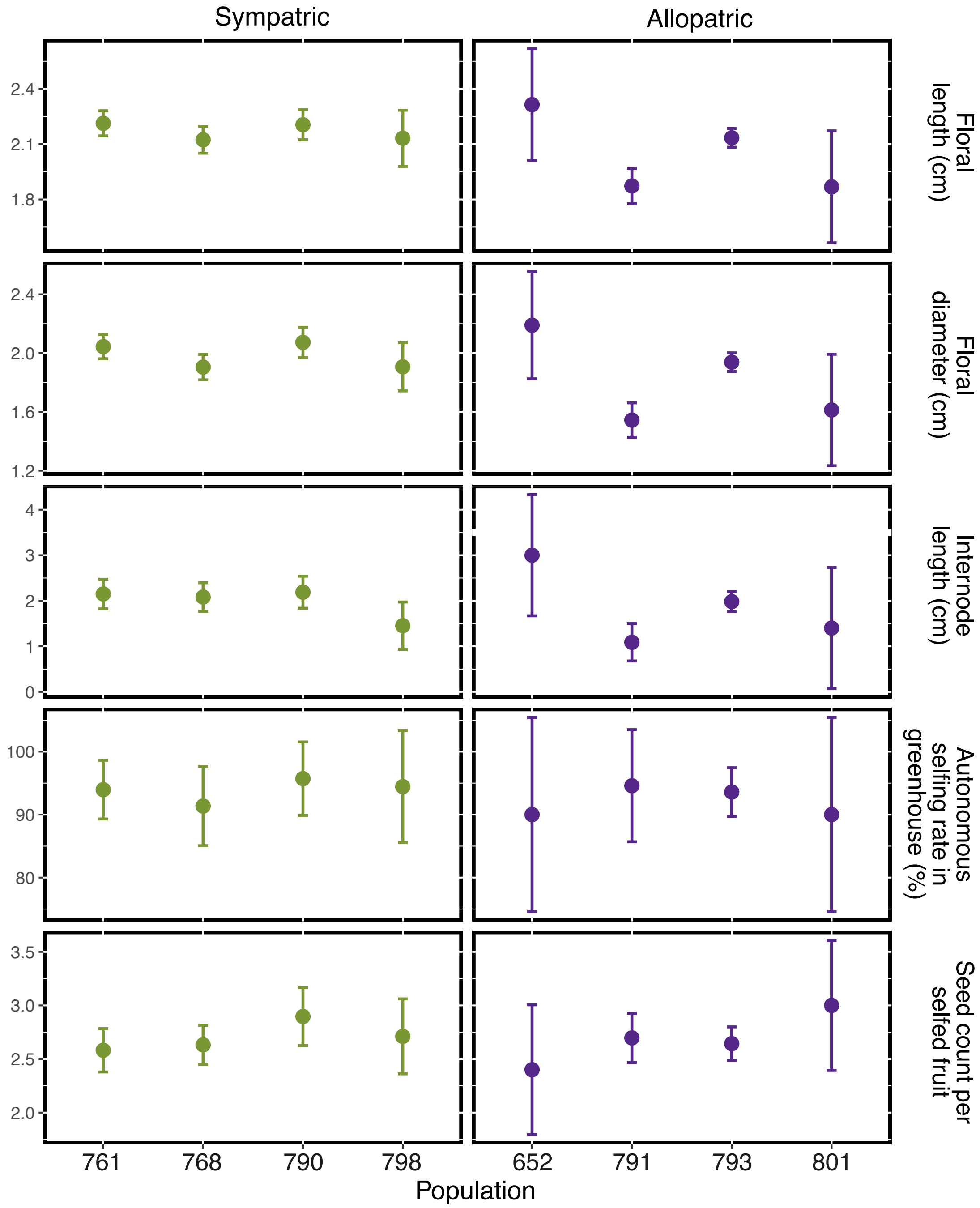
*authors contributed equally

Appendix Figure 1. Floral size was measured by scanning six flowers per individual plant. **(A)** Scans included three face-down flowers to measure floral diameter as indicated by the dotted black line. The diameter was calcuated by summing both dotted lines. **(B)** An additional three flowers were scanned on their side to measure flower height as indicated by the dotted black line. Flower height was then calculated by summing both dotted lines. All measurements were calculated using ImageJ.

Appendix Figure 2. Floral trait variation by population in sympatry versus allopatry. Sympatric populations are shown in the left panel in green. Allopatric populations are shown in the right panel in purple. Predicted population means are plotted as points and 95% confidence intervals are shown as lines. Floral traits plotted from top to bottom include floral length, flower diameter, internode length (2nd-3rd internode), autonomous selfing rate under greenhouse conditions and seed count per selfed fruit.



Appendix Table 1. Summary of linear models to detect the effect of zone (sympatry vs. allopatry) on F_{IS} and selfing rate (*s*) inferred from genetic variation.

	F-statistic	**df**	**p-value**
F_{IS}	0.2276	10	0.644
Selfing rate (*s*) in nature	0.2869	10	0.604

Appendix Table 2. Per population estimate of F_{IS} and selfing rate (*s*) inferred from genetic variation.

Population	**Zone**	**F_{IS}**	***s***	**No. of individuals**
659	sympatry	0.331	0.4973704	6
668	sympatry	0.0376	0.07247494	8
761	sympatry	0.3349	0.50176043	20
768	sympatry	0.3992	0.57061178	21
790	sympatry	0.3637	0.53340177	17
798	sympatry	0.3717	0.54195524	14
	Mean	**0.30635**	**0.45292909**	**86**
651	allopatry	0.2643	0.41809697	8
652	allopatry	0.4216	0.5931345	25
653	allopatry	0.3681	0.53811856	9
666	allopatry	0.2789	0.43615607	4
791	allopatry	0.2408	0.38813669	4
793	allopatry	0.4522	0.62277923	18
	Mean	**0.33765**	**0.49940367**	**68**
Total				**154**