Correlated evolution of self and interspecific incompatibility across the range of a Texas wildflower

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Summary

- Selection to prevent interspecific mating can cause an increase or a decrease in self-pollination in sympatric populations. Characterizing the geographical variation in self and interspecific incompatibilities within a species can reveal if and how the evolution of self and interspecific mate choice are linked.
- We used controlled pollinations to characterize the variation in self and interspecific incompatibility across 29 populations of *Phlox drummondii*. We evaluated seed set from these pollinations and described the developmental timing of variation in pollen–pistil compatibility.
- There is extensive quantitative variation in self-incompatibility and interspecific-incompatibility with its close congener *P. cuspidata*. *Phlox drummondii* populations that co-occur and hybridize with *P. cuspidata* have significantly higher interspecific incompatibility and self-incompatibility than geographically isolated *P. drummondii* populations. The strength of self and interspecific incompatibility is significantly correlated among individuals and the strength of both incompatibilities is explained by the success of pollen adhesion to the stigma.
- The correlated strength of self and interspecific incompatibility across the range of *P. drummondii* and the concurrent developmental timing of the pollen–pistil interaction, suggests these incompatibilities have an overlapping molecular mechanism. The geographical distribution of variation in incompatibilities indicates that this mechanistic link between incompatibilities may affect the evolution of mate choice in plants.

Introduction

Mechanisms of mate recognition are among the most rapidly evolving biological systems (Barrett, 2002), as illustrated by the diversity of floral displays in plants, and the miscellany of mating behaviors in animals. A major goal in evolutionary biology is to understand how these mate recognition systems evolve (Stebbins, 1974; Harder & Barrett, 1996; Igic *et al.*, 2008). In plants, two of the most critical components of mate choice are whether or not to reproduce with one's self and whether or not to reproduce with another species (Barrett, 2002; Barrett *et al.*, 2014). Pollen rejection through these recognition systems results in self-incompatibility (SI) and interspecific incompatibility (II). Both self and interspecific pollination have significant fitness consequences, and it is therefore important to understand if and how these two recognition systems interact to influence the evolution of mate choice.

Selection can favor either an increase or a decrease in self and interspecific fertilization. Offspring resulting from self-fertilization often suffer from reduced survival and fecundity due to the complementation of deleterious recessive alleles through increased homozygosity. This inbreeding depression can generate selection favoring self-incompatibility (Lande & Schemske,

1985; Schemske & Lande, 1985). Conversely, self-compatibility allowing for self-fertilization can be favored if pollinators or mates are limited because it provides reproductive assurance (Baker, 1955; Stebbins, 1974; Lloyd, 1992). The cost, or in some cases benefit, to hybridization between species generates selection to increase or decrease II. Fertilization by interspecific pollen often results in seed abortion, reduced hybrid survival and hybrid sterility (Lowry *et al.*, 2008; Baack *et al.*, 2015) and yet, hybridization between some taxa generates exceptionally fit or fecund offspring through heterosis (Lippman & Zamir, 2007; Goulet *et al.*, 2017).

Selection on self and interspecific pollination can vary among populations and across geography. For example, self-compatibility may be favored at the edge of a range where mates or pollinators are limited, but self-incompatibility may be favored at a range center where the cost of inbreeding depression outweighs the benefit of reproductive assurance from self-fertilization (Busch & Schoen, 2008; Busch & Delph, 2012; Layman *et al.*, 2017). Similarly, interspecific pollen is costly in sympatric regions where two species grow together and have an opportunity to hybridize, but not in allopatric regions (Fishman & Wyatt, 1999; Buide *et al.*, 2015; Rausher, 2017). Therefore spatial variation in the fitness consequences for both self and

interspecific pollination could result in spatial variation in self and interspecific incompatibility.

Selection on interspecific pollination can actually affect the evolution of self-pollination. This link between self and interspecific pollination can arise in one of two ways. First, increased self-fertilization can be favored by selection as a mechanism to decrease interspecific pollination and prevent costly hybridization. Specifically, plants with autogamous self-pollination can avoid interspecific pollen fertilization through self-pollen precedence (Fishman & Wyatt, 1999). Selection to decrease costly hybridization can shape the evolution of greater self-pollination in hybridizing populations, leading to a pattern of higher rates of self-fertilization in sympatry compared to allopatry. For example, this pattern has been found in *Arenaria uniflora* (Fishman & Wyatt, 1999)*, Leptosiphon jepsonii* (Goodwillie & Ness, 2005, 2013) and *Solanum habrochaites* (Broz *et al.*, 2017). In this way, selection to decrease hybridization can generate indirect selection to increase self-pollination through the evolution of increased self-compatibility (e.g. Fishman & Wyatt, 1999) or the evolution of flower morphologies that increase autogamous self-pollination (e.g. Briscoe Runquist & Moeller, 2014).

Second, selection to decrease interspecific matings can cause an increase in self-incompatibility in sympatric populations. It has long been hypothesized that the molecular and biochemical mechanisms causing SI can also cause pollen–pistil incompatibilities between species (Harrison & Darby, 1955; Abdalla, 1972; Pandey, 1981; Hancock *et al.*, 2003). If this mechanistic link exists, then selection to increase II could lead to a pleiotropic increase in SI (Bedinger *et al.*, 2017). There is evidence across a number of genera that overlapping pollen recognition and rejection systems cause SI and II. Species that have genetic SI tend to reject the pollen of closely related self-compatible species, whereas self-compatible species can be successfully fertilized by interspecific pollen (Harrison & Darby, 1955; Abdalla, 1972; de Nettancourt, 1977, 2001). This co-variation between SI and II has been termed the SI × SC rule, and results in an asymmetric barrier to hybridization called 'unilateral incompatibility'. In some systems, such as *Solanum* (de Nettancourt, 2001; Hancock *et al.*, 2003; Li & Chetelat, 2010, 2014, 2015) and *Nicotiana* (Murfett *et al.*, 1996), unilateral incompatibility arises because the genes causing SI also cause II. A pleiotropic link between SI and II implies that if selection acts on one incompatibility, the other type of incompatibility will show a correlated response to selection. Although the SI × SC rule has been documented across a variety of species, to our knowledge it has never been demonstrated that the link between incompatibilities can influence the strength of self-incompatibility mechanisms within a species.

The two possible links between self and interspecific mate-choice lead to alternative hypotheses about how incompatibilities will evolve across populations. Determining the geographical distribution of variation in SI and the co-variation between SI and II can inform if selection to decrease interspecific matings decreases self-incompatibility to avoid hybrid matings, or increases self-incompatibility due to shared molecular mechanisms between II and SI. Here we investigate the link between self and interspecific mate choice in *Phlox drummondii*. *Phlox*

drummondii co-occurs and hybridizes with its congener, *Phlox cuspidata*, in a broad area of sympatry in eastern Texas (Levin, 1967, 1985; Ferguson *et al.*, 1999). The resulting hybrids are largely sterile (Ruane & Donohue, 2008; Suni & Hopkins, 2018) indicating that selection could favor mechanisms to decrease interspecific pollination in this region of sympatry (Levin, 1985; Hopkins & Rausher, 2012).

Phlox drummondii segregates quantitative genetic variation in self-incompatibility (Levin, 1985; Bixby & Levin, 1996). Most research investigating variation in SI and II focuses on qualitative variation across species that either have complete compatibility or incompatibility. The quantitative variation in degree of self-compatibility is called pseudo-self-compatibility (Nasrallah & Wallace, 1968; Levin, 1996). This partial compatibility is thought to arise from modifications of genes outside the S-locus involved in downstream pollen recognition and rejection (Leffel, 1963; Nasrallah & Wallace, 1968). It is not known if the genetic variation causing pseudo-self-compatibility could also cause intermediate levels of II.

Using this system we investigated the following questions: Is there variation in SI and II across the range of *P. drummondii*? If so, is SI weaker or stronger in populations sympatric with *P. cuspidata*? Do SI and II co-vary across individuals within a species as would be predicted by an overlapping genetic mechanism? Do SI and II occur at the same developmental stage? We answer these questions by quantifying variations in SI and II across the range of *P. drummondii* and evaluating when and where the pollen incompatibilities occur in the pistil. Finally, we test alternative hypotheses, unrelated to interspecific hybridization, as to why SI may vary across the range of *P. drummondii*. Specifically we test whether incompatibilities are associated with climate or population density – two environmental factors that could be proxies for mate availability. We also test whether selection for flower color in sympatry could have shaped the evolution of incompatibility through genetic hitchhiking.

Materials and Methods

Study species

Phlox drummondii Hook has a gametophytic SI system governed by a single major locus (Levin, 1993). However, the species has segregating genetic variation for the ability to reject self pollen, where seed set after self-pollinations varies quantitatively across plants (Bixby & Levin, 1996). *Phlox cuspidata*, is self-compatible and reproduces largely by selfing (Levin, 1978, 1989). *Phlox drummondii* pollen is completely compatible on *P. cuspidata* stigmas but the reciprocal cross is usually less successful (Suni & Hopkins, 2018). *Phlox drummondii* and *P. cuspidata* hybridize in areas where they grow sympatrically (Levin, 1985; Ferguson *et al.*, 1999; Roda *et al.*, 2017). Both species have similar light-blue flowers in allopatry, but in sympatry, *P. drummondii* has dark-red flowers (Levin, 1985). Flower color divergence in sympatry evolved due to selection to prevent maladaptive hybridization between species (Hopkins & Rausher, 2012). *Phlox drummondii* flower color varies across a sharp cline, with

populations that contain multiple flower-color types occurring at the edge between sympatry and allopatry (Hopkins *et al.*, 2014).

Plant collection and care

In May of 2014 and 2015 we collected seeds from nine *P. drummondii* natural populations that were in the area of the range sympatric with *P. cuspidata*, from nine allopatric populations, and from 11 populations with mixed flower colors at the edge of the area of sympatry and allopatry (Supporting Information Table S1). In 2014 we also collected seeds from five *P. cuspidata* populations. For each population we recorded their geographical location with a GPS receiver and collected fruits from *c.* 30 plants located at least 2 m apart.

We stored seeds at 4°C before growing them in Pro-Mix HP Mycorrizae potting media in the absence of pollinators at the Arnold Arboretum of Harvard University, Boston, MA, USA. Populations collected in 2014 were grown in a glasshouse with 16 h of supplemented light and a temperature ranging from 18°C to 27°C. Plants collected in 2015 were grown in growth chambers with 16 h of light, a daytime temperature of 23°C and a nighttime temperature of 18°C.

We grew an average of 12 plants per *P. drummondii* population, for a total of 343 plants (Table S1). We initially ran all statistical models described below as generalized linear mixed effect models (GLMs) including maternal population as a random effect. Maternal source population explained no variation and was therefore removed from the models. We also grew an average of eight individuals per population from the five *P. cuspidata* populations collected in 2014. These *P. cuspidata* plants were used as pollen sources in interspecific pollinations.

Controlled pollinations

We assessed self and interspecific incompatibility for each *P. drummondii* plant using seed set from three types of controlled pollinations: self-pollinations with pollen from the same plant, interspecific pollinations with pollen from a haphazardly selected *P. cuspidata* plant, and intraspecific pollinations with pollen from a haphazardly chosen *P. drummondii* plant (selected across all individuals growing independently of source population). Each *P. drummondii* plant was used once as a pollen source. We included the average number of intraspecific seeds set per cross per individual in every statistical model to control for plant maternal fertility.

Before performing pollinations, we emasculated the flower buds in each plant by plucking the corollas and the attached immature anthers. Three days after emasculation we used tweezers to deposit pollen collected from anthers of two to three mature flowers onto the fully developed stigmas of each emasculated flower. We crossed an average of seven flowers for each cross type on each maternal plant. Each crossed inflorescence was labeled with tape and bagged with tulle to prevent seed loss after explosive fruit dehiscence. In addition to the controlled crosses, we also bagged an average of 10 flowers on each plant and observed autogamous seed set. We collected and counted

seeds from all experimental inflorescences. *P. drummondii* has three ovules per flower and therefore seed set varies between zero and three per flower. We explored quantitative variation in this trait by crossing multiple flowers per cross type. Seed set from all pollinations was submitted to Dryad. Raw data for seed counts, pistil observations, and environmental variation across *P. drummondii* populations were deposited in Dryad (doi: 10.5061/dryad.dj3rs70). All analyses of these seed counts were preformed using the LME4 package in R (Bates *et al.*, 2015).

Regional variation in SI and II

We compared strength of SI and II across the range of *P. drummondii* to determine if these reproductive barriers are weaker or stronger in sympatry. We used GLMs with a negative-binomial error structure, total seed set from self-pollinations, interspecific pollinations, and autogamy as our dependent variables, and region (allopatry or sympatry with *P. cuspidata*) as our independent variable. We included the year of seed collection and average seeds per intraspecific cross as fixed effects. The number of self-pollinations performed was also included as an offset variable. Although we do not have specific predictions about the strength of SI in populations on the edge of allopatry and sympatry we did rerun our model including the 11 edge populations to evaluate if differences between allopatric and sympatric populations are due to their geographical position rather than the presence of *P. cuspidata* (i.e. edge and sympatric populations are geographically close but differ in *P. cuspidata* presence). Sample sizes between analyses vary due to technical errors resulting in seed loss. We tested significant fixed effects by comparing nested models using likelihood ratio tests. We also calculated the proportion of plants that were able to produce seeds by autogamy among the set of plants with the highest seed sets in self pollinations (i.e. 10% upper tail in the distribution, 24 plants).

Co-variation between SI and II

We further evaluated if seed set from self-pollinations was predicted by average seed set from interspecific pollinations. As above, we used a GLM with negative-binomial error structure and included year and average intraspecific seed set as a fixed effect and number of self-pollinations performed as an offset to account for any variation in the number of crosses performed. We also evaluated the strength of the linear correlation between self and interspecific incompatibility using a Spearman rank correlation as implemented by COR.TEST in R. For this analysis, we compared the ratio of the average seeds per cross from self-pollinations and intraspecific crosses to the average seeds per cross from interspecific and intraspecific pollinations.

Alternative hypotheses

We tested likely alternative hypotheses that could explain the distribution of variation in SI across the range of a species. First, we tested if SI variation is explained by mate availability as correlated with population density. In 2015, we estimated population

density at all *P. drummondii* seed-collection sites. Populations of *P. drummondii* usually occur as small, dense patches, likely due to their mechanism of seed dispersal through fruit explosion (Levin & Kerster, 1968; Watkins & Levin, 1990). Therefore we measured population density by placing a 1-m quadrat at three locations along the densest areas of each population and counting the number of contained plants (Table S1).

Second, we evaluated if climatic factors were associated with geographical variation in pollen-pistil incompatibilities. We determined the major axis of environmental variation across the range of *P. drummondii* using the location of 248 populations that have been identified by the authors over the past 10 yr. We used the geographical coordinates from these populations to extract climatic data from the WORLDCLIM v.1.4 database (Hijmans *et al.*, 2005) using the MAPTOOLS (Bivand, 2016), RASTER (Hijmans *et al.*, 2016), SP (Pebesma & Bivand, 2005) and RGDAL (Bivand *et al.*, 2016) packages in R. We retrieved monthly measurements of temperature and precipitation as well as altitude and 19 bio-climatic variables using grids with a 30-second resolution. We performed a principal component analysis (PCA) to separate populations along major axis of climatic variation (Table S1). We used the first three components to define the climate of each of the populations we investigated in this study.

Finally, we evaluated if self-incompatibility is predicted by flower color, because flower color is under divergent selection in allopatric and sympatric populations of *P. drummondii* (Hopkins & Rausher, 2012, 2014). For this analysis we used data from mixed-color populations on the edge of allopatry and sympatry, where flower color alleles and incompatibility alleles could naturally recombine if they are not physically linked to each other. In total, we scored 120 individuals belonging to 11 edge populations for flower color (light-blue, dark-blue, light-red or dark-red).

Ideally we would test all alternative hypotheses for the distribution of self-seed set in a single statistical model, but this was not possible due to technical and statistical issues. First, we analyzed our self-seed set data from the allopatric and sympatric populations sampled in 2015 using a GLM with negative-binomial error structure. Our model initially included region, population density, climate PC1-3, average intraspecific seed set, and number of self-pollinations as a covariate. This model suffered from multicollinearity as indicated by condition number ($\kappa = 141.83$) and Variance Inflation Factors (VIF for intraspecific seed set = 1.07, population density = 1.17, climate PC1 = 2.80, climate PC2 = 13.60, climate PC3 = 1.84, and region = 11.04). Because of the strong correlation between climate PC2 and region (allopatric/sympatric) we ran two models – one that included climate PC2 but not region and a second that included region but not climate PC2. We also ran a second model testing for the effect of PC2 that included the 11 edge populations. Finally, we used data from the edge population with mixed-colors to determine if seed set from self-pollinations was predicted by flower color. This GLM included flower color hue (red or blue), color intensity (light or dark) and average seed set from intraspecific pollinations as independent variables.

Pistil observations

The above analyses of seed set indicate that SI and II may share underlying molecular mechanisms. To further investigate this association we observed *P. drummondii* pistils after pollination with self, intraspecific and interspecific pollen. To best capture a relevant developmental variation associated with the strength of pollen pistil-incompatibilities we selected highly self-incompatible and highly self-compatible plants (eight SI plants and nine SC plants). From these 17 plants, we collected pistils 16 h after controlled pollination with self, conspecific or interspecific pollen. These pistils were fixed in a 63% ethanol, 5% formaldehyde, 5% acetic acid mix. For the observations, we washed samples three times with distilled water and then boiled them for 3 min in a 5% sodium sulfite solution. This step was standardized to ensure that only pollen that was not specifically adhered to the stigmas was washed away (Zinkl *et al.*, 1999). We then dyed the samples overnight at 4°C in a solution of 0.1% Aniline blue in 0.1 N potassium phosphate buffer. We 'squashed' the pistils on glass slides and observed the pollen using Zeiss Axioskop and Zeiss Axioimager fluorescence microscopes (Zeiss, New York, NY, USA). We observed an average of six pistils per cross-type for each maternal sample. For each pistil we counted the number of pollen grains adhered to the stigmas, the number of germinated pollen grains, and the number of pollen tubes reaching the base of the style.

We used GLMs with a negative-binomial error structure to test the effect of cross-type (i.e. self, interspecific, and interspecific), phenotype (i.e. SC or SI) and their interaction on each of the components of pollen development (i.e. pollen adhesion, germination and growth). Each component was modeled independently. Because counts at each step in the pollen development process depend on the previous step, each model included an offset term to control for the counts at the previous step. Specifically, number of pistils observed, number of pollen adhered, and number of germinated pollen grains were offsets for pollen adhesion, germination and pollen growth respectively. Pistils were processed in three batches so 'batch' was also included in the model as a term.

From these models we were specifically interested in testing if SI and SC plants differed in self and interspecific pollinations but not intraspecific pollinations. Such a finding would cause a significant interaction between plant phenotype and cross-type in our models. For models that showed a significant interaction we performed comparisons between self-compatible and self-incompatible plants for each of the three cross-types using the SUMMARY.GHLT function of R (Hothorn *et al.*, 2008).

We determined which component of pollen development best predicted seed set using a linear model that included the effects of cross-type, average pollen adhered per stigma, mean proportion of adhered pollen that germinated, mean proportion of germinated pollen that grew to the end of the style, and batch.

Our results indicate that pollen adhesion success on the stigma is an important developmental stage for the expression of incompatibility. We therefore tested if pollen adhesion success from

self-pollinations is predicted by pollen adhesion success from interspecific pollinations. This linear model also included 'batch'.

To confirm that differences in counts of adhered pollen are not the result of differences in pollen germination we counted adhered pollen from intraspecific, interspecific and self-pollinations between 1 and 2 h after pollination, which is before the onset of pollen tube germination. We used eight full sibling SI individuals and counted pollen on pistils from 62 intraspecific, 57 interspecific and 55 self-pollinations. Pistils were prepared and observed as described previously. We used a generalized linear model with Poisson error structure to test for an effect of cross type on number of pollen grains bound to stigma. The number of stigmas per pistil was included as an offset. We contrasted intraspecific pollination results from interspecific and self-pollination results using Wald tests.

Finally, we observed the interaction between intraspecific pollen and a stigma using a Scanning Electron Microscope (SEM). We used a cryo-SEM protocol to visualize stigmas from intraspecific pollinations collected 4 h after crossing. Stigmas were immediately dissected, introduced in liquid nitrogen for 2 min and then observed in a Jeol SEM 6010LV (Jeol Ltd, Tokyo, Japan).

Results

SI and II are greater in sympatric populations

We found widespread variation in seed set from self-pollinations and interspecific pollinations (Fig. 1a). Only 24% of individuals had complete SI (no seeds from self-pollinations). The average seed set for interspecific pollinations was greater than for self-pollinations, but less than for intraspecific pollinations (seed set per flower mean ± SE: intraspecific pollination = 1.99 ± 0.06; interspecific = 1.62 ± 0.05; self = 0.40 ± 0.03). There is variation in seed set from self and interspecific pollinations within all populations. For self-pollinations, the variation is greater in allopatric populations (mean standard deviation in seed set per population ± SD = 0.50 ± 0.14) and edge populations (SD = 0.46 ± 0.17) than in sympatric populations (SD = 0.26 ± 0.16). Autogamous seed set was low across all populations with only 40% of the most self-compatible plants producing any seeds by autogamy.

We evaluated if SI and II differed between allopatric regions across the range of *P. drummondii* and regions sympatric with *P. cuspidata*. We found that the presence or absence of *P. cuspidata* significantly predicted self, autogamous and interspecific seed set (Table 1). Sympatric populations contained more individuals with complete SI than allopatric regions (Fig. 1b). Individuals from the sympatric region had lower autogamy, as well as lower self and interspecific seed set than individuals from allopatric regions (Table 1; Fig. 1c). These findings support the hypothesis that there is a molecular or genetic link between SI and II. We re-ran a model including the 11 edge populations and found that region remained a significant factor in our model ($\chi^2(2, 286) = 6.01$, $P = 0.049$) with the edge populations having seed set from self-pollinations intermediate to

allopatric and sympatric populations (lsmean = 0.831, SE = 0.125).

II predicts SI

We evaluated if interspecific seed set predicts self-seed set success across *P. drummondii* individuals. We found that individuals that are more compatible with *P. cuspidata* are also more self-compatible (Table 1; Fig. 2). This finding also supports the hypothesis that SI and II share underlying molecular mechanisms.

Alternative hypotheses

We tested two alternative hypotheses as to what might explain the geographical variation in SI. First, we evaluated if SI levels are explained by changes in two indirect proxies for mate availability: population density and climate. Second, we evaluated if SI co-varies with flower color, a trait that is under divergent selection in sympatric and allopatric populations (Hopkins & Rausher, 2012, 2014).

We found no significant effect of population density on seed set from self pollinations (Table 1). This result was consistent across data sets that did and did not include the populations located at the edge of the area of sympatry.

We found limited support for the effect of climate on self-seed set (Table 1). The first three principal components of our climatic PCA explained 79% of the climatic variation (PC1 = 58%, PC2 = 15%, PC3 = 6%). PC2 was correlated with geographical longitude and with allopatric vs sympatric region, preventing us from including region and PC2 in the same statistical model. Our model that included the three climate variables, but not region, indicates a significant effect of PC2 on self-seed set. Alternative models with these two variables have indistinguishable AIC support. We added the edge populations to our data set and re-ran the model with the three climate variables. With this more complete data set we found no significant effect of PC2 on self-seed set ($\chi^2(1, 181) = 1.52$, $P = 0.676$).

Finally, we found no evidence that flower color hue (red or blue) or intensity (light or dark) affected self-seed set (Table 1).

Pollen adhesion success predicts SI and II

We examined the developmental basis of self and interspecific incompatibility by observing pollen adhesion, germination and tube growth in incompatible and compatible individuals. There was a large variation in the number of pollen grains adhered to the stigma after pollinations with different types of pollen (Fig. 3a,b). The variation in number of pollen grains adhered to the stigma is explained by a significant interaction between cross type and phenotype (Table 2). Pairwise contrasts indicated that self-compatible individuals had more pollen adhered to the stigma than self-incompatible individuals from both self ($Z = 7.17$, $P < 0.001$) and interspecific pollination ($Z = 4.78$, $P < 0.001$), but had similar pollen adhered after intraspecific pollinations ($Z = 1.85$, $P = 0.431$) (Fig. 3c). We did not find a
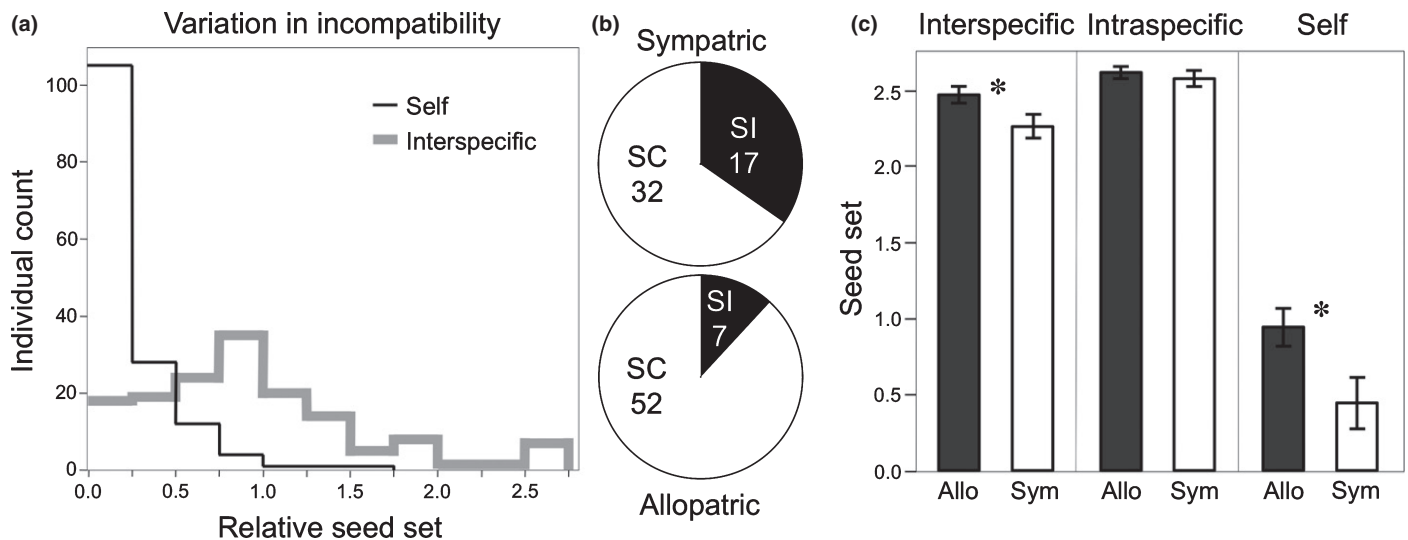
Fig. 1 Incompatibilities are stronger in sympatric *Phlox drummondii* populations. (a) Distribution of relative seed set (with respect to intraspecific pollinations) in self-pollinations and interspecific pollinations. (b) Number and proportion of plants that produce some seeds in self-pollinations (SC) and no seeds in self-pollinations (SI) in allopatric and sympatric populations. (c) Seed set from interspecific, intraspecific, and self-pollinations in allopatric (Allo) and sympatric (Sym) populations. Least squared means ± SE are displayed. Asterisks indicate significant differences ($P < 0.05$) between allopatric and sympatric populations.

significant effect of phenotype or an interaction between cross type and phenotype on pollen germination or pollen tube growth (Table 2). Self-pollinations had reduced proportions of pollen germination and pollen tube growth but these proportions did not differ between self-compatible and self-incompatible plants. Interspecific pollinations did not show a reduced success for these later components of pollen development.

When controlling for all other stages of pollination, pollen adhesion is the only developmental stage that significantly predicted seed set across treatments (Table 3). Furthermore the number of interspecific pollen grains adhered to a plant's stigma significantly predicts the number of self pollen grains adhered to the plant's stigma ($F(1, 35) = 4.8$, $P = 0.035$).

We observed pollinated SI pistils before the onset of pollen germination to confirm that differences in counts of adhered pollen grains do not result from differences in pollen germination. We found that pollen adhesion to the stigma is significantly greater in intraspecific pollinations than in interspecific ($Z = 9.17$, $P < 0.001$) and self-pollinations ($Z = 15.57$, $P < 0.001$; mean ± SE of adhered pollen: intraspecific = 17.77 ± 2.66, interspecific = 11.49 ± 1.60, self = 7.14 ± 1.42).

We used the SEM to visualize the interaction between the pollen and the stigma during adhesion. We found impressions on the papillae surface at the point of contact with compatible pollen suggesting alterations in the extracellular protein of the pellicle or the underlying cell wall (Fig. 3d) (Zinkl *et al.*, 1999).

Discussion

The evolution of self-recognition systems within a species can be shaped by selection against hybridization with a sympatric species. Our investigations of the link between self and interspecific mate-choice in *P. drummondii* resulted in three major

findings: both SI and II are stronger in sympatric populations with *P. cuspidata* than in allopatric populations; the strength of II predicts the strength of SI among *P. drummondii* individuals; and the strength of SI and II are both predicted by pollen adhesion to the stigma. These three findings suggest that SI and II share developmental or genetic mechanisms, and that this mechanistic link influences the evolution of mate choice across the range of *P. drummondii*. Our study is the first to find that quantitative variation in the strength of self-incompatibility is correlated with the strength of interspecific incompatibility across a species range.

Stronger SI and II in sympatry

We found that *P. drummondii* has stronger self and interspecific incompatibility in regions sympatric with *P. cuspidata*. *P. drummondii* and *P. cuspidata* flower simultaneously, share pollinators, and produce hybrids (Erbe & Turner, 1962; Levin, 1967, 1975, 1985). Hybrids are vigorous but have low fertility (Levin, 1967, 1975; Suni & Hopkins, 2018), and thus interspecific reproduction is costly relative to conspecific reproduction. The cost of hybridization causes selection to favor traits that decrease reproduction between *Phlox* species (Hopkins & Rausher, 2012). Greater II in sympatry relative to allopatry is consistent with pollen–pistil incompatibility evolving in response to selection to decrease hybridization. Furthermore, stronger SI in sympatry suggests that overlapping molecular mechanisms between SI and II affect the evolution of self-recognition in *P. drummondii*.

This is the first study that we know of to find greater SI and lower autogamy in sympatric populations relative to allopatric populations. In other systems rates of self-fertilization are higher in sympatric populations relative to allopatric populations

Table 1 Factors affecting variation in self and interspecific seed set in *Phlox drummondii*

Model	Response[a]	n	AIC	Factor[b]	Estimate	SE	χ^2	P
Regional variation in SI and II	Self SS	172	716.5	Intercept	−2.1	0.31		
				Region	−4.99	0.2	5.83	**0.016**
				Intraspecific AvSS	0.27	0.13	3.51	0.061
				Year	1.08	0.21	21.8	**< 0.001**
	Interspecific SS	167	1079.7	Intercept	−0.09	0.13		
				Region	−0.21	0.09	4.92	**0.027**
				Intraspecific AvSS	0.24	0.06	14.97	**< 0.001**
				Year	0.32	0.1	10.89	**< 0.001**
	Autogamy	152	456.7	Intercept	−1.11	0.3859		
				Region	−0.87	0.32	6.29	**0.012**
				Intraspecific AvSS	−0.11	0.18	0.35	0.553
				Year	−1.12	0.29	13.35	**< 0.001**
Co-variation between SI and II	Self SS	160	675.75	Intercept	−2.59	0.33		
				Interspecific AvSS	0.37	0.12	8.6	**0.003**
				Intraspecific AvSS	0.19	0.13	1.63	0.202
				Year	0.8	0.22	11.35	**< 0.001**
Alternative hypotheses	Self SS	103	519.95	Intercept	−1.61	0.39		
				Density	0.01	0.02	0.44	0.505
				PC Climate 1	0.01	0.01	0.43	0.513
				PC Climate 2	−0.4	0.02	4.37	**0.036**
				PC Climate 3	−0.02	0.04	0.25	0.616
				Intraspecific AvSS	0.3	0.14	3.81	0.051
	Self SS	103	519.87	Intercept	−1.18	0.38		
				Region	−0.43	0.2	4.38	**0.036**
				Density	0.01	0.02	2.23	0.634
				PC Climate 1	−0.001	0.01	0.004	0.948
				PC Climate 3	−0.002	0.04	0.002	0.958
				Intraspecific AvSS	1.94	0.05	3.36	0.067
	Self SS	120	546.08	Intercept	−1.36	0.29		
				Flower Hue	−0.14	0.24	0.3	0.581
				Flower Intensity	−0.21	0.25	0.71	0.399
				Intraspecific pollination AvSS	0.37	0.29	8.01	**0.004**

[a]SS, seed set.
[b]AvSS, average seed set.
Statistically significant values ($P < 0.05$) are indicated in bold text.

(Rausher, 2017). This latter pattern is consistent with a selection which favors increased self-pollination as a mechanism to avoid hybridization (Antonovics & Bradshaw, 1968; Allard, 1975). In some of these species, such as *Arenaria uniflora* (Fishman & Wyatt, 1999), *Leptosiphon jepsonii* (Goodwillie & Ness, 2005, 2013) and *Solanum habrochaites* (Baek *et al.*, 2016; Broz *et al.*, 2017) there is an intraspecific variation in SI, and sympatric populations are more compatible. Other species lack SI systems but display intraspecific variation for flower features associated with autogamy (Smith & Rausher, 2008; Briscoe Runquist & Moeller, 2014; Buide *et al.*, 2015).

An increase in self-compatibility does not necessarily lead to increased rates of self-fertilization in the field (Sicard & Lenhard, 2011; Duncan & Rausher, 2013). This depends on selfing pre-empting ovules that would otherwise be fertilized with pollen from outcross and interspecific pollen. Species lacking morphological and developmental features associated with autogamous self-pollination (e.g. small anther–pistil distance and synchrony between pistil receptivity and pollen dehiscence) may show low rates of self-fertilization despite being self-compatible. In these species, decreasing SI will not

necessarily cause an avoidance of hybridization. In *P. drummondii*, we found that most individuals, even if they were self-compatible, did not set autogamous seeds. Given the floral morphology of the species, selfing is expected to be rare and likely to be pollinator mediated (Levin, 1975; Watkins & Levin, 1990; Bixby & Levin, 1996). Therefore, in this species, it is unlikely that increased self-compatibility would result in higher rates of self-pollination and therefore lower rates of hybridization in the wild.

A previous study found that sympatric *P. drummondii* populations are more self-compatible than allopatric populations (Levin, 1985). This study used similar methods as ours and also sampled populations across the range of the species. Given that our findings are consistent across two flowering seasons and 29 populations, it is unlikely that the discrepancies between the two studies are the result of fluctuations in SI levels across time or geographical region. Levin used fewer crosses per plant (two instead of seven) and did not control for intraspecific crossing success when evaluating differences in self-pollination success. These differences could explain the discrepancies in our results.
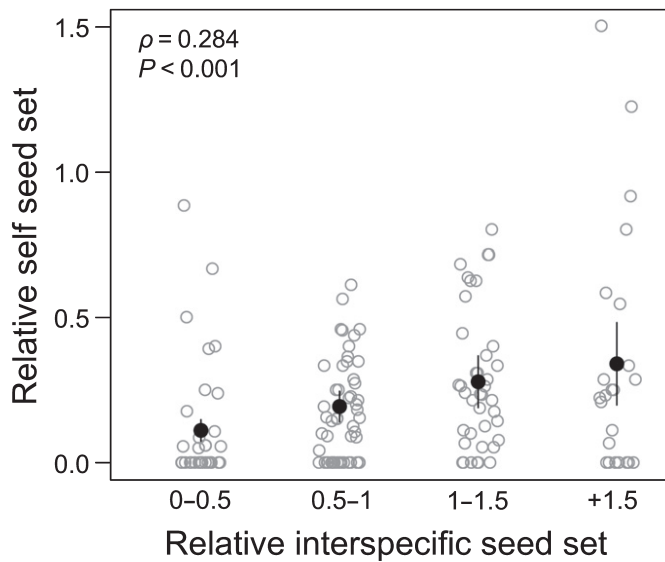
Fig. 2 Co-variation in seed set from self-pollinations and interspecific pollinations in *Phlox drummondii*. Relationship between relative seed set (with respect to intraspecific pollinations) in self-pollinations and interspecific pollinations. Interspecific pollination data were binned. Individual plants are represented by grey dots, the mean ± SE for each bin is displayed in black. Spearman rank correlation coefficients (ρ) and significance (P) are provided. Un-binned data were used for analyses.

Co-variation in incompatibilities

The concurrent geographical pattern of variation in self and interspecific incompatibility in *P. drummondii* arises from among-individual co-variation in the strength of SI and II. Variation in both incompatibilities segregate within populations and yet the strength of these two incompatibilities remains significantly correlated among individuals, suggesting that self and interspecific pollen recognition share molecular mechanisms.

Co-variation between self and interspecific incompatibility has been observed across many closely related species of plants giving rise to the SI × SC rule (Lewis & Crowe, 1958). In some systems, SI and II are known to result from overlapping developmental and genetic mechanisms (Covey *et al.*, 2010; Bedinger *et al.*, 2011; Baek *et al.*, 2016). For example, loci contributing to unilateral incompatibility in *Solanum* co-localize with the S-locus (Chetelat & DeVerna, 1991; Bernacchi & Tanksley, 1997) and other loci known to mediate self-incompatibility (Covey *et al.*, 2010). Investigations of the molecular basis of the SI × SC rule focus predominantly on qualitative variation in incompatibility across species and not quantitative variation within species. In previously studied systems transitions from SI to SC are unidirectional and complete, usually involving mutations in the S-locus (Nasrallah *et al.*, 2002; Tovar-Méndez *et al.*, 2014). Once self-compatibility is complete and fixed in a species it can no longer respond to selection to increase incompatibility. Therefore, these systems may be good models for understanding the molecular basis of the SI × SC rule, but they cannot necessarily tell us if and how the shared molecular basis of self and interspecific incompatibility affect the evolution of these traits. By examining patterns of genetic variation in SI within a species, whether it is quantitative variation as in *P. drummondii* or qualitative as in most *Solanum* species (Baek *et al.*, 2015, 2016), we can gain a
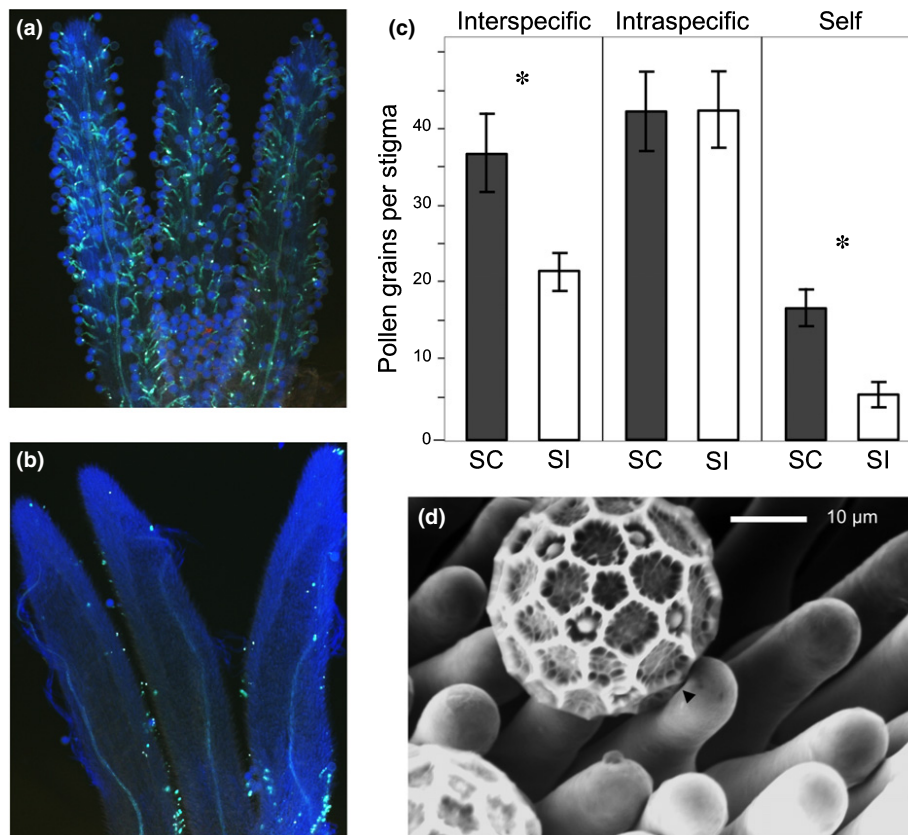

Fig. 3 Pollen adhesion determines reproductive incompatibilities in *Phlox drummondii*. (a, b) Aniline blue staining of stigmas after (a) compatible and (b) incompatible pollinations. (c) Pollen adhesion in self-compatible (SC) and self-incompatible (SI) plants for three types of pollinations 16 h after pollination. Means ± SE are displayed. Asterisks indicate significant differences (P < 0.05) between SI and SC plants. (d) Scanning electron micrographs of pollinated stigmas after intraspecific pollination. Compatible pollen creates impressions in the stigma surface (arrow).

Table 2 Effect of cross type and self-incompatibility phenotype on three pollen development components in *Phlox drummondii*

Response	n	Factor	df	χ^2	P
Number adhered pollen	289	Phenotype	1	48.57	**< 0.001**
		Cross type	2	112.27	**< 0.001**
		Batch	2	40.89	**< 0.001**
		Phenotype × Cross type	2	12.98	**0.002**
Number pollen germinated	252	Phenotype	1	2.94	0.086
		Cross type	2	23.05	**< 0.001**
		Batch	2	15.51	**< 0.001**
		Phenotype × Cross type	2	3.93	0.140
Number pollen tube growth	202	Phenotype	1	2.75	0.100
		Cross type	2	6.67	**0.036**
		Batch	2	57.63	**< 0.001**
		Phenotype × Cross type	2	0.40	0.817

Statistically significant values ($P < 0.05$) are indicated in bold text.

Table 3 Effect of pollen development components on seed set in *Phlox drummondii*

Factor	Sum of squares	df	F	P
Mean pollen adhered	2.375	1	4.341	**0.039**
Proportion of germinated pollen	1.094	1	1.999	0.160
Proportion of pollen grown	0.839	1	1.534	0.218
Cross type	9.736	2	8.899	**< 0.001**
Batch	3.232	2	2.958	0.056
Residuals	66.187	121		

Statistically significant values ($P < 0.05$) are indicated in bold text.

better understanding of the factors influencing the evolution of incompatibilities.

Quantitative variation in the strength of SI – pseudo-self-compatibility – occurs in a diversity of plants species (Levin, 1996). Some of these plants are in genera that show patterns of incompatibility across species consistent with the SI × SC rule (Baek *et al.*, 2015, 2016), and yet, to our knowledge, this is the first time a correlation between SI and II has been documented within a species showing pseudo-self-compatibility. Although little is known about the genetic basis of pseudo-self-compatibility, most evidence suggests that it results from mutations outside the S-locus in genes downstream from the initial recognition reaction (Tantikanjana *et al.*, 1993; Stone *et al.*, 2003; Murase *et al.*, 2004; Baldwin & Schoen, 2017). Previous studies have shown that the quantitative variation in SI in *P. drummondii* is genetically controlled and highly heritable (Bixby & Levin, 1996). If, as in other plants, genes outside the S-locus cause pseudo-self-compatibility in *P. drummondii*, then the correlation between SI and II also likely arises due to shared molecular mechanisms outside the S-locus dependent recognition. However, the correlation between SI and II can have alternative causes, including developmental constraints and maternal environmental effects. Further

research is therefore needed to confirm the genetic overlap between SI and II in this system.

Reduced stigma–pollen adhesion characterizes incompatible interactions

Pollen–pistil incompatibilities can arise at a variety of developmental stages (Edlund *et al.*, 2004; Swanson *et al.*, 2004). Successful pollen must adhere to the stigmatic surface, germinate, grow a pollen tube through the style, find the ovule, and fertilize the egg. Pollen adhesion to the stigmatic surface was the greatest barrier for both self and interspecific pollen in *P. drummondii*. The coincident timing of pollen–pistil interactions further suggests an overlapping molecular basis of SI and II. Our results also suggest that SI in *P. drummondii* has a distinct developmental mechanism from other described systems.

Compared to intraspecific pollinations, self-pollinations showed fewer pollen grains adhered to the stigma, lower pollen germination and lower rates of pollen tube growth. However the number of self pollen grains that adhered to the stigma was the only trait that differed between SI and SC *P. drummondii* individuals. Although we used a protocol to remove unbound pollen from the stigmas (Zinkl *et al.*, 1999) it is possible that only germinated pollen grains remained adhered. Under this scenario our measurements of pollen adhesion would actually reflect germination. This seems unlikely given that we found pollen specifically bound to the stigmas before the onset of germination. The physical interaction between pollen and stigma observed with the SEM also points to active mechanism of pollen binding. Our results indicate that SI in *Phlox* is a complex phenotype involving multiple stages of pollen–pistil interaction, but variation in SI within the species is due to variation in the pollen adhesion mechanism.

Phlox stigmas are dry and papillate (Heslop-Harrison & Shivanna, 1977) and likely to require active pollen hydration during adhesion. In other plants, such as *Arabidopsis thaliana*, the adhesion of pollen to a dry stigma is a complex multi-step process requiring inter-cellular recognition and signaling (Swanson *et al.*, 2004). Pollen must first be captured on the stigma, then crosslinking between the pollen coat and stigma occurs to allow for hydration of pollen, and finally pollen tubes tether to the papilla by penetrating the stigma cuticle (Swanson *et al.*, 2004). Pollen adhesion is known to mediate discrimination against self and interspecific pollen in species with dry stigmas (Zinkl *et al.*, 1999; Edlund *et al.*, 2004; Swanson *et al.*, 2004), however these species have sporophytic SI mechanisms (Clarke & Newbigin, 1993; Golz *et al.*, 1995; Franklin-Tong & Franklin, 2003), and not gametophytic SI as is seen in *Phlox* (Levin, 1993). Most gametophytic SI systems involve the rejection of self-pollen during pollen tube growth in the style and not on the stigma surface (Edlund *et al.*, 2004; Swanson *et al.*, 2004; Bedinger *et al.*, 2017). An exception is gametophytic SI in *Papaver*, which involves stigmatic protein secretions that trigger self-specific termination of pollen tube growth shortly after germination (Franklin-Tong, 2008; Wilkins *et al.*, 2014, 2015). Although SI in *Phlox* also appears to involve pollen interacting with stigmatic papillae, this

occurs before pollen tube germination. Further research is needed to understand how the biochemical processes of pollen adhesion mediates incompatibilities in *Phlox*.

Alternative hypotheses do not explain the geographical variation in SI

We explored three alternative explanations for a geographical variation in the strength of SI. First, self-fertilization can be advantageous when pollinators or mates are unreliable by providing reproductive assurance (Nasrallah & Wallace, 1968; Barrett, 2002; Good-Avila & Stephenson, 2002; Vallejo-Marín & Uyenoyama, 2004; Brennan *et al.*, 2005; Goodwillie *et al.*, 2005; Mable *et al.*, 2005). We found that population density, a proxy for mate availability, does not predict variation in self-compatibility across the range of *P. drummondii*. This is consistent with previous findings, that outbreeding rates are independent of population density in this species (Watkins & Levin, 1990). More studies are necessary to thoroughly test the reproductive assurance hypothesis, including measurements of pollen limitation across populations as well as empirical evaluations of the fitness effects of different SI levels under low pollen availability (Busch & Schoen, 2008; Busch & Delph, 2012; Layman *et al.*, 2017). Second, the ability to attract or reward pollinators as well as pollinator abundance may vary across geography due to changes in climatic factors such as temperature or water availability (Eckert *et al.*, 2010). We find little evidence that climatic variation shapes the evolution of self-compatibility in *Phlox*. Finally, our analyses from mixed color populations indicate that variations in SI and flower color segregate independently and thus the evolution of incompatibility is not a correlated response to selection on flower color in sympatric populations of *P. drummondii*. Most *Phlox* taxa are self-incompatible (Wherry, 1955; Grant & Grant, 1965), suggesting that SI mechanisms evolved in the ancestors of Phlox species and ancestral polymorphism in SI has been maintained to varying degrees across the range of *P. drummondii*. Although there are likely other possible hypotheses as to why SI varies across the range of *P. drummondii*, our investigations indicate that the evolution of SI is, at least in part, shaped by selection of II in sympatric populations.

Concluding remarks

Rejection of self and interspecific pollen takes place in a common arena – the pistil. Therefore evolution in this arena is likely to affect recognition of both types of pollen. We have demonstrated a significant correlation between strength of self and interspecific incompatibility within *P. drummondii*. This variation is geographically structured such that individuals in sympatric populations have stronger incompatibilities than in allopatric populations. Our results suggest self and interspecific pollen-pistil recognition have overlapping molecular mechanisms causing a correlated evolution of incompatibility across the range of *P. drummondii*. Although a correlation across species in self and interspecific incompatibility is common, we were able to show that this correlation persists within a species.

Author contributions

F.R. and R.H. designed the experiments. F.R. and R.H. conducted plant collections. F.R. conducted lab and glasshouse work. F.R. and R.H. analyzed data. F.R. and R.H. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Natural *Phlox drummondii* and *Phlox cuspidata* populations sampled

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