

Limited phenological and pollinator-mediated isolation among selfing and outcrossing *Arabidopsis lyrata* populations

Courtney E. Gorman¹, Lindsay Bond², Mark van Kleunen^{1,3}, Marcel E. Dorken² and Marc Stift¹

¹Department of Biology, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany

²Department of Biology, Trent University, 1600 West Bank Drive, Peterborough, Ontario, Canada K9J 0G2

³Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation, Taizhou University, Taizhou 318000, People's Republic of China

 CEG, 0000-0001-7310-8803; MvK, 0000-0002-2861-3701; MED, 0000-0001-7400-5136; MS, 0000-0001-7801-9498

Keywords:

mating system, breeding system, pollinator visitation, phenology, pre-zygotic reproductive isolation, geitonogamy

Author for correspondence:

Courtney E. Gorman
e-mail: courtney.gorman@uni-konstanz.de

Transitions from outcrossing to selfing have been a frequent evolutionary shift in plants and clearly play a role in species divergence. However, many questions remain about the initial mechanistic basis of reproductive isolation during the evolution of selfing. For instance, how important are pre-zygotic pre-pollination mechanisms (e.g. changes in phenology and pollinator visitation) in maintaining reproductive isolation between newly arisen selfing populations and their outcrossing ancestors? To test whether changes in phenology and pollinator visitation isolate selfing populations of *Arabidopsis lyrata* from outcrossing populations, we conducted a common garden experiment with plants from selfing and outcrossing populations as well as their between-population hybrids. Specifically, we asked whether there was isolation between outcrossing and selfing plants and their between-population hybrids through differences in (1) the timing or intensity of flowering; and/or (2) pollinator visitation. We found that phenology largely overlapped between plants from outcrossing and selfing populations. There were also no differences in pollinator preference related to mating system. Additionally, pollinators preferred to visit flowers on the same plant rather than exploring nearby plants, creating a large opportunity for self-fertilization. Overall, this suggests that pre-zygotic pre-pollination mechanisms do not strongly reproductively isolate plants from selfing and outcrossing populations of *Arabidopsis lyrata*.

1. Background

Mating-system transitions from obligate outcrossing to predominantly selfing have arisen repeatedly across almost all major plant lineages [1]. Up to 15% of seed plants are predominantly selfing and many share a relatively recent common ancestor with outcrossing species [2]. The transition from outcrossing to selfing is usually associated with convergent evolution of a flower morphology that optimizes self-pollination and resource use (e.g. smaller flower size and/or a reduction in pollen, nectar and scent production), termed the 'selfing syndrome' [3–5]. These types of changes in flowering probably contribute to the reproductive isolation of selfing lineages [6], but it is unclear if they or their subsequent effects on pollinators are the main drivers of reproductive isolation in incipient selfing species.

Reproductive barriers are essential to the maintenance of evolutionary independence of parapatric populations (i.e. populations with slightly overlapping ranges). Reproductive isolation can be partial or complete, and most plant species are isolated by a combination of pre- and post-zygotic barriers [6–8], although pre-zygotic mechanisms tend to be more important [6,8–10]. In plants, pre-zygotic barriers include pre- or post-pollination mechanisms [8]. Post-pollination pre-zygotic mechanisms such as genetic incompatibilities can

cause differences in seed number, but pre-pollination mechanisms generally contribute more to the total reproductive isolation of plant species [6,9,10]. Although rarely addressed, this principle appears to hold for cases where a shift to self-fertilization has played a part in speciation. For example, in two closely related *Mimulus* species with a contrasting mating system, differences in mean flowering date and floral display contributed the most to reproductive isolation compared to other pre- and post-pollination mechanisms [11]. However, the importance of phenological shifts relative to shifts in pollinator preferences due to floral changes as drivers of rapid pre-zygotic reproductive isolation after the transition to self-fertilization [7] has rarely been studied.

Plant phenological traits and the behaviour of pollinators could readily interact. For example, pollinator behaviour and the number of flowers should both play a large role in determining the opportunity for the flowers of self-compatible plants to be fertilized by a different flower on the same plant (i.e. geitonogamy). Furthermore, geitonogamy could help to reproductively isolate self-compatible individuals. For instance, if pollinators commonly visit multiple flowers on the same individual, it could facilitate higher selfing rates of self-compatible individuals [12]. Different types of pollinators, such as flies versus bees, could also differ greatly in their pollination strategies [13]. Additionally, if pollinators more commonly visit plants in very close proximity, this could contribute to greater population viscosity (i.e. limited dispersal or gene flow among populations) and result in more matings among closely related individuals that share the same mating system [14–16]. Furthermore, due to potential differences in flower attractiveness between mating systems, pollinators might preferentially and repeatedly visit plants exhibiting a particular mating system type. Alternatively, at the earliest stages of divergence, pollinators might show limited or no ability to differentiate between plants with alternative mating types.

Here, we use *Arabidopsis lyrata* ssp. *lyrata* (L.) O’Kane & Al-Shehbaz to examine the role of differences in phenology and pollinator attraction as mechanisms of reproductive isolation in a recently diverged selfing lineage. This study system is ideally suited to address our questions as it has had multiple origins of selfing within the same species [17]. In several populations across the range of this normally outcrossing species (multi-locus outcrossing rates $t_m > 0.8$), all plants are self-compatible, have low outcrossing rates, and therefore reproduce primarily through selfing (t_m : 0.1–0.4) [17–19]. The selfing and outcrossing populations are geographically interspersed, therefore secondary contact following evolutionary divergence in parapatry is likely. Also, the transition to selfing in these populations is thought to have happened less than 10 000 years ago because (1) the range now occupied by outcrossing and selfing populations was mostly covered by ice during the last glacial maximum [17], and (2) the selfing populations have not developed a selfing syndrome [20]. Furthermore, these populations probably represent multiple transitions to selfing within different population genetic backgrounds (electronic supplementary material, table S1 and [17]). This raises the question of whether selfing populations have diverged from their outcrossing ancestors in traits conferring reproductive isolation. Similarly, given that outcrossing and selfing populations are at least partly interfertile and can regularly produce healthy offspring [21–23], between-population

hybrids may be a critical factor in determining whether secondary contact would lead to coalescence of the diverged populations or alternatively reinforce their evolved differences.

In a common-garden experiment set within the native range of *A. lyrata*, we simulated two phases of secondary contact between selfing and outcrossing populations. The first phase corresponds to initial contact between parental plants from selfing and outcrossing populations. The second phase corresponds to secondary contact between admixed plants (hybrids between populations) and parental plants. This allowed us to test whether the evolution of selfing has led to pre-pollination isolation through divergence in phenology and/or insect pollinator attraction. Specifically, we asked whether there was reproductive isolation between outcrossing and selfing plants and their between-population hybrids through differences in (1) the timing or intensity of flowering, and (2) pollinator visitation rates and paths. Based on this, we tested whether phenological differences and pollinator behaviour reduced the opportunities for pollen exchange between mating systems. Moreover, as geitonogamy can also contribute to reproductive isolation via effects on rates of selfing versus outcrossing, we quantified the relative frequency of within-plant pollinator movements.

2. Methods

(a) Study system

Arabidopsis lyrata spp. *lyrata* is a small, short-lived perennial that is native to North America. It occurs in dry-mesic habitats with shallow soils, such as rock outcrops and sand dunes. Individual plants can produce several stems that terminate in racemes of numerous (greater than 20) small white flowers. The primary pollinators of *A. lyrata* are small solitary bees and hoverflies, which are attracted to the nectar and pollen of the flowers. The ancestral condition in *Arabidopsis lyrata* is self-incompatibility; however, the barrier to self-fertilization has broken down in several North American populations [18]. Additionally, many of these newly self-compatible populations have evolved high selfing rates [17]. Although selfing populations have on average slightly smaller corollas and reduced pollen-ovule (P:O) ratios [20], population genetic background explains most variation in floral traits [20].

(b) Crossing designs

To generate the material needed to simulate secondary contact between diverged selfing and outcrossing populations, we sowed field-collected seeds from 12 North American *A. lyrata* populations with known breeding and mating systems [17] (seeds were kindly provided by Barbara Mable, University of Glasgow). These included six populations characterized as outcrossing (high outcrossing rates, high frequency of self-incompatible individuals, hereafter referred to as SI populations) and six populations characterized as selfing (low outcrossing rates, high frequency of self-compatible individuals, hereafter referred to as SC populations) (figure 1; electronic supplementary material, table S1).

To generate the material needed to simulate admixture between the parental populations and their between-population hybrids, we performed a full diallel cross in 2014 and 2015 with six plants of each of the six SI and six SC populations. This cross produced progeny with the following cross types: within SI population ($SI \times SI_{within}$), within SC population ($SC \times SC_{within}$), between SI population ($SI \times SI_{between}$), between SC

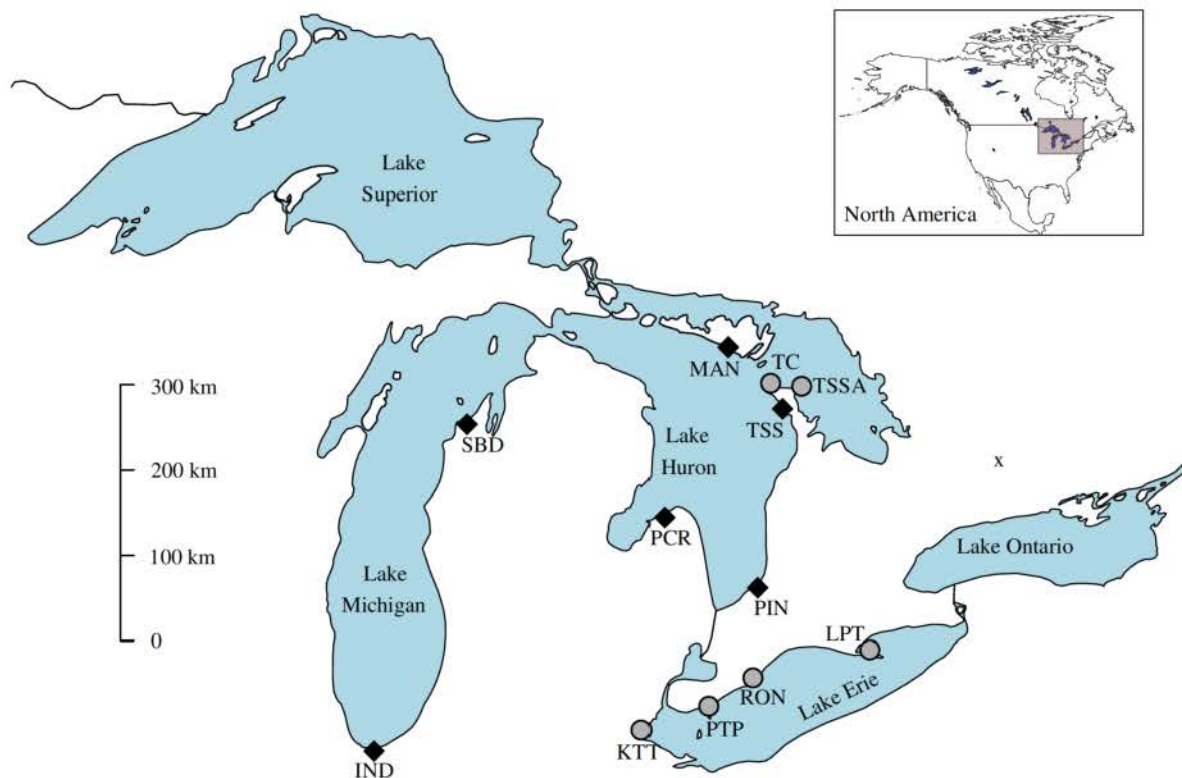


Figure 1. Map of the location of the common garden experiment in relation to the source populations. Grey circles represent selfing populations and black diamonds represent outcrossing populations. The black X represents the location of the common garden at Trent University, Peterborough, Ontario. (Online version in colour.)

population ($SC \times SC_{\text{between}}$), and between SC and SI population reciprocally ($SI \times SC_{\text{between}}$ or $SC \times SI_{\text{between}}$) (see electronic supplementary material, S2).

To increase the sample size for the $SI \times SI_{\text{within}}$ and $SC \times SC_{\text{within}}$ cross types, and to include material from the SC populations that was produced by selfing ($SC \times \text{self}$ cross type), we also used seeds that had been produced in 2012 and 2013 by manually cross- and self-pollinating between 5 and 12 additional plants per population (see the electronic supplementary material, S2 for full details).

All pollinations were done by emasculating recipient flowers prior to anther dehiscence and rubbing a freshly dehiscid anther from the pollen donor (the same individual in self-pollinations) over the recipient's stigma.

(c) Experimental design of common garden experiment

To test whether differences in phenology and flower-visitor attraction can reproductively isolate plants from selfing populations, we set up an outdoor common garden experiment at Trent University in Peterborough, Ontario, Canada. This location is at an intermediate latitude within the geographical range of the source populations (figure 1). From 20 to 22 March 2018, for each seed family, up to 50 seeds were sown on a moistened peat-based substrate in one pot. Plants were grown in climate chambers with 11 h days and a 21°C/18°C day/night cycle at 95% humidity. Between 18 April and 1 May, when seedlings had developed at least two true leaves, we transplanted three haphazardly chosen seedlings from each germinated seed family to individual Stuewe and Sons Ray Leach 'Cone-tainers' (Tangent, Oregon, USA) with the same peat-based substrate. On 10 May, plants were moved outside to the common garden, prior to any flowering.

Within the common garden, plants were organized in a randomized block design. There were three replicates of three $3 \times$

6 m blocks. Each of the nine resulting blocks contained between 150–180 individual plants distributed randomly over 180 positions within 9 cone-tainer trays with plants from each seed family and cross-type evenly distributed among the blocks. In total, 1509 plants were raised in the common garden. Sample sizes for the cross types were: $SI \times SI_{\text{within}}$ ($n = 172$), $SC \times SC_{\text{within}}$ ($n = 175$), $SC \times \text{self}$ ($n = 65$), $SI \times SI_{\text{between}}$ ($n = 203$), $SC \times SC_{\text{between}}$ ($n = 296$), $SI \times SC_{\text{between}}$ ($n = 314$), $SC \times SI_{\text{between}}$ ($n = 284$).

(d) Phenological data

To test for potential reproductive isolation between the cross types due to differences in phenology, for each plant we recorded flowering dates and the number of open flowers per day. Open flowers were defined as flowers with visible reproductive organs (stigma and anthers) and that still had petals attached to the flower. Besides calculating opportunities for pollen-transfer between outcrossing and selfing populations, this allowed us to compare the time to onset of flowering, flowering duration, and time of peak flowering (i.e. the day when each individual had the greatest number of open flowers) for each individual for each of the cross-types.

(e) Flower visitor observations

To test for differences in insect attraction and flower visitor movements within and between plants, we recorded flowers with GoPro Hero Session (San Mateo, CA, USA) cameras. Specifically, we tested whether there were differences in the potential for geitonogamous selfing (visitor movement within the same plant), and for outcrossing (i.e. visitor movement between plants) within and between mating systems. Prior to recording we counted the number of open flowers on each plant. To standardize the recording procedure, 4–6 flowering plants (depending on their size) were taken from their blocks and placed in a tray located at the

front of their respective blocks. This method ensured clear video footage of multiple focal plants simultaneously, while minimizing interference with the visual context of the pollinators provided by the configuration of plants in the block design. To make sure that focal plants had a different set of neighbours for each set of observations, we combined flowering individuals systematically according to their position in the block, going through the block in three different ways: (1) taking consecutive plants in a vertical direction, (2) taking consecutive plants in a horizontal direction and (3) taking plants from the same position but in different trays. Due to this approach, the cross type of the focal plants combined in the video-frames was random.

We recorded 12–15 min videos that were later trimmed to the central 10 min to exclude potential effects of disturbance during starting and stopping the cameras. In total, 500 videos were taken throughout the flowering period, of which a random subset of 140 videos (23.3 h of video) were analysed by the first author in a random order. In total, these videos included 379 unique individuals (41% of all flowering individuals in the common garden), and 123 plants were observed in multiple videos. For each visitor, we recorded whether it was a solitary bee or hoverfly, the duration of the visit and the path it took (see below). Finer taxonomic identification was not possible due to the video resolution, but we took high-quality photographs to identify the most common visitors: hoverflies (Syrphidae) *Eristalis arbustorum*, *Syriffa pipiens*, *Sphaerophoria* sp. and *Toxomerus marginatus*, and solitary bees from the family Halictidae (kindly identified by Bill Crins, Toronto, Canada).

The entire path that each visitor took after its initial visit to a flower in the frame was recorded to test whether plants from SC populations received fewer visits than those from SI populations as is expected in selfing plants [24]. Moreover, because pollinators will often focus on exploiting one type of flower and/or floral scent, we tested whether visitors were more likely to move to neighbouring plants with the same mating system than to plants with a different mating system, and whether progeny from crosses between mating system received fewer visits than progeny from crosses within the same mating system. We classified visitor paths as: ‘away’—the visitor left the video frame after an initial visit; ‘same’—the visitor visited a second flower on the same individual; or to one of the cross types as defined above ($SI \times SI_{within}$, $SC \times SC_{within}$, $SC \times self$, $SI \times SI_{between}$, $SC \times SC_{between}$, $SI \times SC_{between}$, $SC \times SI_{between}$)—the visitor went to a flower on a different individual. This allowed us to classify the flight paths of the visitors and compare visitation rates among destinations.

(f) Statistical analyses

All statistical analyses were done in R 3.5.1 [25]. To test if there were differences in the time of peak flowering and duration of flowering between SI and SC cross types ($SI \times SI_{within}$, $SC \times SC_{within}$, $SC \times self$) and between within-population cross types and between-population cross types ($SI \times SI_{within}$, $SC \times SC_{within}$, $SC \times self$ versus $SI \times SI_{between}$, $SC \times SC_{between}$, $SI \times SC_{between}$, $SC \times SI_{between}$), we used Gaussian linear mixed-effects models implemented in lme4 [26] that included *cross type* as the only fixed effect and *maternal plant ID* nested within *maternal population* and *paternal plant ID* nested within *paternal population* as random effects. To test if there were differences in the probability of flowering between the cross types, we used a binomial mixed-effects model with the same random effects structure as the Gaussian models. To make comparisons among cross types for both the Gaussian and binomial models, we obtained posterior distributions of model parameter estimates through simulations (5000 values were directly simulated from the joint posterior distribution of the model parameters using the function `sim` of the R package ‘arm’ [27]). Because we had no prior information, we used ‘improper’ prior distributions (i.e. distributions with density

functions that do not integrate to 1 and are therefore not ‘proper’ probability distributions [28]). Specifically, $p(\beta) \propto 1$ was implemented for the model coefficients and $p(\sigma^2) \propto 1/\sigma^2$ for the variance parameters. Therefore, for both the Gaussian and binomial models, all information in the results is derived directly from the data. The medians of the simulated values from the joint posterior distributions of the model parameters were then used as estimates, and the 2.5% and 97.5% quantiles were used as the lower and upper limits of the 95% credible intervals. To rule out that our results were affected by the additional within-population crosses that we had added to increase the sample sizes for the $SI \times SI_{within}$ and $SC \times SC_{within}$ cross types (and to create the $SC \times self$ cross type; see electronic supplementary material, S2), we also ran all analyses without the extra material, but this did not change the findings (see electronic supplementary material, S4).

Pollinator visitation rate (per plant) was analysed separately for the two main visitor classes hoverflies and solitary bees. The cross type ‘ $SC \times self$ ’ was excluded from the analyses of pollinator visitation due to low sample size. To test if there were differences in the frequency of pollinator visits among the cross types, two identical generalized linear mixed-effects models with negative binomial distributions with *number of visits* as the response variable (one model for visits made by hoverflies and another one for solitary bees) were used. The explanatory variables were *cross type* and *flower number* as fixed effects, and *maternal ID* nested within *maternal population* and *paternal ID* nested within *paternal population* as random effects. In these models, the number of adaptive Gauss-Hermite quadrature points (nAGQ) was set to zero, which optimizes the random effects and the fixed-effects coefficients in the penalized iteratively reweighted least-squares step [26]. In other words, this results in a faster but less precise parameter estimation for generalized mixed effect models [26]. These models used a log-link function and improper prior distributions.

Pollinator visitation paths were analysed in two ways. The probability that a pollinator would make a certain choice after landing on a flower was analysed with a multinomial logistic regression as implemented in the function `multinom` in the package ‘nnnet’ [29]. *Path* in the multinomial model included all cross types and the same plant (opportunity for geitonogamy) as path options, along with the option of leaving the observation frame. This model included both *cross type* and *flower number* as fixed effects and *path* as the response variable using a logit link function. To further parse the pollinator preference and the effect of flower number and distance between plants in the frame, a conditional logistic regression (function `clogit`, package ‘survival’; [30]) was performed. The conditional logistic regression was performed separately for hoverflies and solitary bees and included the insect’s selection for any of the cross types in the same video frame as the response variable, as well as *relative flower number*, *relative distance* and *cross type* as fixed effects, and finally *switch ID* as the strata. The strata command specifies the group of observations inherent to our video recordings. The strata in this case specifies the group of choice options for each pollinator in each video. Switch ID was defined as: what the insect selected (1) and everything the insect did not select (0) and incorporated information about the distance to the other individuals and the flower number relative to the other individuals. The cross type ‘ $SI \times SI_{within}$ ’ was used as the baseline as this cross type represents the ancestral condition in *A. lyrata*. *Relative flower number* and *relative ranked distance* were obtained by dividing by the maximum value within the same video-frame.

(g) Pollen-transfer probabilities

To examine whether there were differences in the opportunities for outcross pollination between selfing and outcrossing plants, we used the empirical information on phenology and pollinator

behaviour to model opportunities for outcrossing between 'SI $\times$ SI_{within}' and 'SC $\times$ SC_{within}' plants. Between-population cross types were excluded from these analyses. In other words, we were interested in whether plants from SC populations of *A. lyrata* had more opportunity to mate with each other than with plants from SI populations as a potential mechanism of reproductive isolation. To do this, we used calculations of K_{ij} —the 'pollen transfer probabilities' outlined in [31] (see electronic supplementary material, S3 for full details).

3. Results

(a) Phenology

Of the 1509 plants in the common garden, 938 flowered (62%). Although six plants died before flowering, the non-flowering plants were largely represented by plants that survived the whole season but never produced a flowering stem and therefore never flowered. The main flowering period lasted six weeks from 1 June to 14 July, although a few individuals flowered later (nine individuals flowered a second time and 10 individuals flowered for the first time as late as September; electronic supplementary material, figure S1). The probability of an individual flowering did not strongly differ among the SI $\times$ SI_{within} and SC $\times$ SC_{within} cross types (60% and 40%, respectively; CrI overlapping; figure 2a), but the flowering probability of the SC $\times$ self cross type (15%) was lower (figure 2a). So, while progeny formed by selfing flowered less, merely having the ability to self did not substantially decrease the probability of flowering when compared to individuals from outcrossing populations. The between-cross types did not differ from the within-population cross types in the probability of flowering (54%–82%; CrIs overlapping; figure 2a). Additionally, the direction of the cross for the SI $\times$ SC_{between} and SC $\times$ SI_{between} crosses did not have an obvious effect on flowering probability, as both cross types had a 65% probability of flowering (figure 2a).

The time of peak flowering showed a very similar pattern as flowering probability. Again, there were no strong differences in the time of peak flowering between the SI $\times$ SI_{within} and SC $\times$ SC_{within} cross types, and the SC $\times$ self cross type peaked in flowering about one to two days earlier (average = 27.4 days; median SC-self = 25.8) than the other within or between cross types (however, CrIs overlapped among all cross types except SI $\times$ SI_{between}; figure 2b). The day of peak flowering did not differ between SI $\times$ SC_{between} and SC $\times$ SI_{between} plants, indicating that cross direction did not influence the time of peak flowering (CrIs overlapping; figure 2b). There were also no strong differences in peak flowering between the within-population and between-population cross types (CrIs overlapping), with the exception that the SI $\times$ SI_{between} cross type tended to reach peak flowering one to two days later (figure 2b). Similarly, flowering duration did not strongly differ among the cross types (figure 2c), but the SC $\times$ self cross type tended to have a shorter duration (6–15 days) than the other within-population cross types (11–19 days) or between-population cross types (14–21 days) (figure 2c).

(b) Pollinator visitation and opportunities for pollen-transfer

Solitary bees and hoverflies were the predominant visitors, and they had similar visitation frequencies and no clear

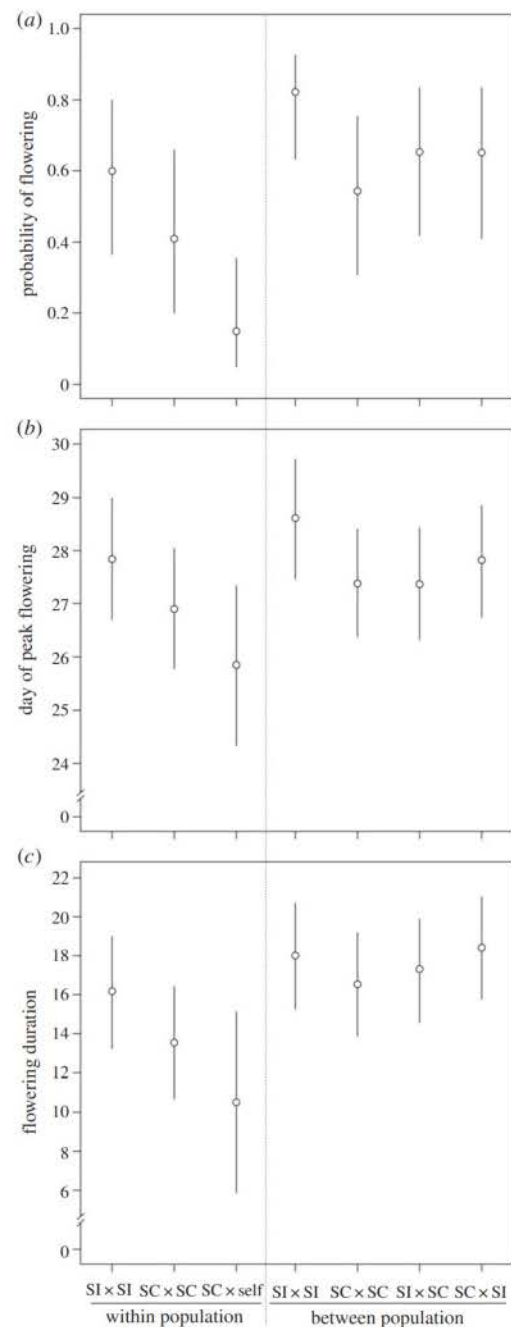


Figure 2. Panel of effect plots for the differences in phenological traits among the cross types ($n = 1509$). Fitted values and credible intervals were obtained through simulation of the linear mixed model output. (a) The probability of flowering per cross type. (b) The day of peak flowering per cross type (calculated as the day where each individual had the highest number of flowers during the total flowering period). (c) The flowering duration (days) per cross type. Vertical bars in all figures represent the 95% credible intervals. Cross types are SI $\times$ SI_{within} and SC $\times$ SC_{within} (crosses within SI and SC populations, respectively), SC $\times$ self (self-fertilizations within SC populations), SI $\times$ SI_{between} and SC $\times$ SC_{between} (crosses between SI and between SC populations, respectively), and SI $\times$ SC_{between} and SC $\times$ SI_{between} (reciprocal crosses between SI and SC populations). SI, self-incompatible, SC, self-compatible.

pattern of preference for any of the cross types (compare figure 3a and b, all CrIs overlapping). The behaviour of both types of pollinators appeared to increase the opportunity

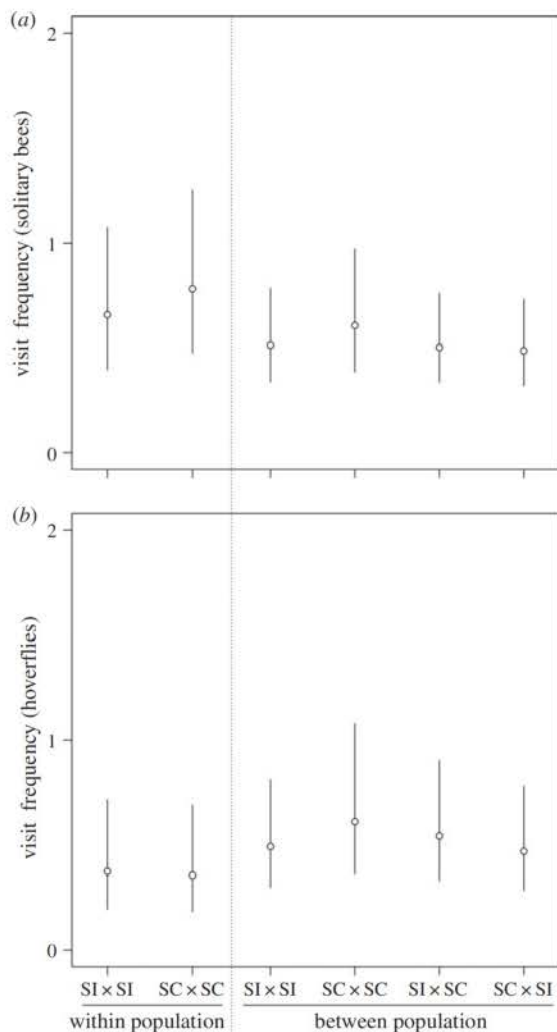


Figure 3. Differences in pollinator visitation (number of visits) by (a) solitary bees and (b) hoverflies among the cross types ($n = 502$). Fitted values and credible intervals were obtained through simulation of the negative binomial generalized linear mixed model output. Vertical bars represent the 95% credible intervals. Cross types are $SI \times SI_{within}$ and $SC \times SC_{within}$ (crosses within SI and SC populations, respectively), $SI \times SI_{between}$ and $SC \times SC_{between}$ (crosses between SI and between SC populations, respectively), and $SI \times SC_{between}$ and $SC \times SI_{between}$ (reciprocal crosses between SI and SC populations). SI, self-incompatible, SC, self-compatible.

for geitonogamous self-pollination, as approximately 50% of the movements between flowers were to a different flower on the same plant (figure 4). When cases of a pollinator visiting another flower on the same plant were not considered, the odds of an initial visitor moving to a plant in the frame decreased by 89% (solitary bees) and 94% (hoverflies) for each unit increasing relative distance (significantly negative odds-ratios for relative distance; table 1). In other words, pollinators were more prone to visit the nearest plant, regardless of the cross type or the number of flowers on the neighbouring plant.

The opportunity for between versus within cross type pollen-transfer was nearly equal both for the $SI \times SI_{within}$ and the $SC \times SC_{within}$ cross types (figure 5). In other words, slight shifts in phenology and flowering intensity (electronic supplementary material, figure S1) were unlikely to lead to reproductive isolation.

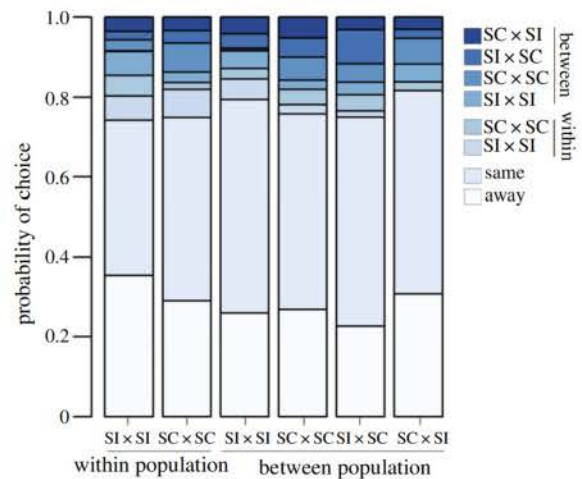


Figure 4. Stacked bar plot representing the probability of pollinators making a choice to visit an individual of any cross type after visiting an individual of a certain cross type. Probabilities were obtained from the multinomial model (and thus corrected for the number of available flowers on each plant in the array). Pollinators could also choose to visit a flower on the same plant (same) or to leave the experimental set-up (away). The cross types on the x-axis represent the cross type of the initial visit. The stacked bars represent the probability of that cross type being selected after a visit to the cross type on the x-axis. Cross types are $SI \times SI_{within}$ and $SC \times SC_{within}$ (crosses within SI and SC populations, respectively), $SI \times SI_{between}$ and $SC \times SC_{between}$ (crosses between SI and between SC populations, respectively), and $SI \times SC_{between}$ and $SC \times SI_{between}$ (reciprocal crosses between SI and SC populations). SI, self-incompatible, SC, self-compatible. (Online version in colour.)

4. Discussion

Our common garden experiment simulating secondary contact between SI and SC populations showed that phenology largely overlapped between plants from SI and SC populations. There were also no differences in pollinator preference related to mating system. Regardless of mating system, pollinators tended to move between flowers on the same plant, thus facilitating opportunities for geitonogamy. Our models of pollen-transfer probabilities, which integrated differences in phenology (timing and intensity of flowering), revealed equal opportunities for pollen-transfer within- and among mating systems. Together, this suggests that pre-zygotic pre-pollination mechanisms do not strongly reproductively isolate plants from SC and SI populations of *Arabidopsis lyrata* when grown in the same environment. However, because plants simultaneously open multiple flowers, and pollinators predominantly move from flower to flower on the same plant, our data suggest that there is a large opportunity for geitonogamy in this system.

(a) Pollinator visitation

Our results show that differences in pollinator preference do not play a large role in the reproductive isolation of the newly diverged SC populations. Neither of the two main visitor types (hoverflies and solitary bees) showed any preferences for particular cross types. Additionally, when given a choice, pollinators preferred to stay on the same individual versus exploring nearby plants regardless of cross type. When they did choose to visit another plant, pollinators

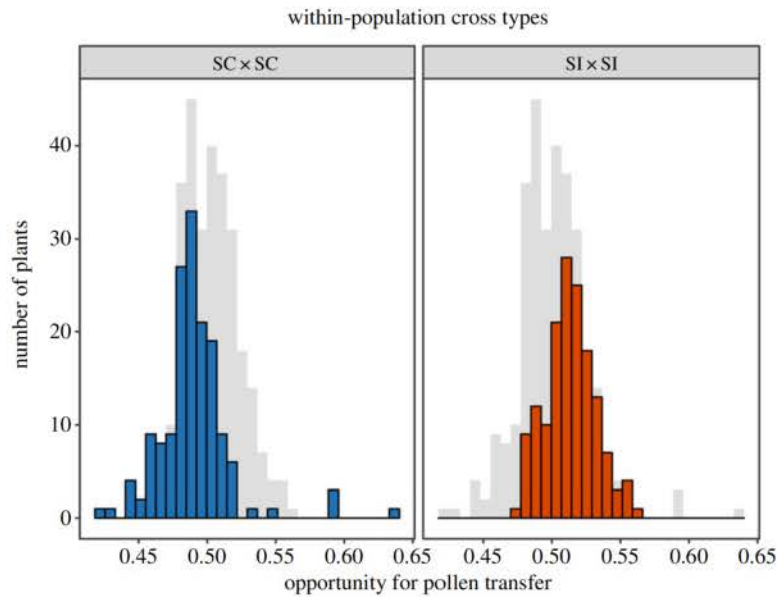


Figure 5. Representative bootstrapped run of the distribution of the opportunity for between-cross-type pollen-transfer for the $SC \times SC_{within}$ (blue, left panel) and $SI \times SI_{within}$ (red, right panel) cross types. The grey bars represent the sum of the distribution. The parameter estimate for the difference in transfer probability for the run shown was 0.024, and thus close to the mean value of 0.020 across all the bootstrapped samples. There were no significant differences in the opportunities for pollen transfer between the cross types. (Online version in colour.)

Table 1. Summary of results from the conditional logistic regression model that analysed whether *relative flower number*, *relative distance* or *cross type* influenced the pollinator path. Cross type $SI \times SI_{within}$ was used as the baseline. Hoverflies: $n = 546$, number of events = 142, likelihood ratio test = 62.16 on 7 d.f., $p \leq 0.001^*$. Solitary bees: $n = 541$, number of events = 140, likelihood ratio test = 45.24 on 7 d.f., $p \leq 0.001^*$. Symbols and abbreviations used in the column headings: SE, standard error; z, Wald statistic. For both hoverflies and solitary bees, relative plant distance had the greatest influence on pollinator path. There was no strong preference for any of the cross types or individuals with more flowers. Significant effects are highlighted in *italics*. The 95% confidence interval is the confidence interval of the odds ratio. *Relative flower number* and *relative distance* were estimated relative to the other individuals in the observation. These variables were transformed to range between 0 and 1.

visitor	fixed effect	odds ratio	s.e.	z	Pr(> z)	95% confidence interval	
solitary bees	relative flower number	1.10	0.32	0.29	0.77	0.59	2.04
	relative distance	<i>0.11</i>	<i>0.38</i>	<i>-5.73</i>	<i><0.001*</i>	<i>0.05</i>	<i>0.24</i>
	$SC \times SC_{within}$	2.01	0.39	1.80	0.07	0.94	4.30
	$SI \times SI_{between}$	1.32	0.39	0.71	0.48	0.62	2.82
	$SC \times SC_{between}$	0.96	0.39	-0.12	0.91	0.44	2.07
	$SI \times SC_{between}$	0.80	0.36	-0.64	0.52	0.40	1.60
	$SC \times SI_{between}$	0.91	0.38	-0.25	0.81	0.43	1.91
hoverflies	relative flower number	0.90	0.34	-0.32	0.75	0.46	1.75
	relative distance	<i>0.06</i>	<i>0.43</i>	<i>-6.56</i>	<i><0.001*</i>	<i>0.03</i>	<i>0.14</i>
	$SC \times SC_{within}$	1.06	0.45	0.14	0.89	0.44	2.55
	$SI \times SI_{between}$	1.09	0.41	0.21	0.83	0.49	2.44
	$SC \times SC_{between}$	1.70	0.40	1.34	0.18	0.78	3.71
	$SI \times SC_{between}$	1.03	0.40	0.07	0.95	0.47	2.27
	$SC \times SI_{between}$	0.83	0.41	-0.44	0.66	0.37	1.86

preferred to go to the closest plant regardless of how many flowers it had or what cross type it was. This lack of pollinator preference could be due to the pollinators being generalists, as previous studies have shown that hoverflies, for instance, are not very choosy with respect to the plants

they visit [13]. Additionally, pollinators may not be able to distinguish between cross types, as there are only subtle floral differences between SC and SI populations: shape and size are very similar, although selfers do have reduced pollen production [20]. Either way, pollinator preference

seems to be playing little role in differentiating the selfing from outcrossing populations of *A. lyrata*.

Nevertheless, the behaviour of the pollinators could favour selfing for several other reasons. For instance, we found that pollinators often visit different flowers on the same individual, irrespective of mating system. This should provide ample opportunity for within-individual pollen transfer [3,32], and thus for self-compatible individuals to self-fertilize through geitonogamy. Moreover, when pollinators moved between plants, they mainly moved between nearby individuals. Given that *A. lyrata* seeds have no mechanisms to promote seed dispersal, and plants can produce over 1000 seeds per season, neighbouring plants could be highly related to each other [33]. As a consequence, the observed behaviour of pollinators could cause mate limitation in self-incompatible plants, making the transfer of cross-pollen rare and/or mainly from incompatible partners (e.g. from relatives that share S-alleles). In self-compatible plants, on the other hand, which occur at low frequency in outcrossing populations [17,34], geitonogamy may help overcome this mate limitation and provide reproductive assurance [35,36]. Theoretically, selfing populations could also evolve in the absence of mate limitation, but only if self-compatible plants have a transmission advantage over self-incompatible plants. Such a transmission advantage can arise when pollen discounting is weak [37,38], but in the absence of pollen limitation, geitonogamy should result in strong pollen discounting [39]. Therefore, it is most likely that the evolution of high selfing rates in six of the North American *A. lyrata* populations was driven by conditions with persistent mate or pollinator limitation, for example during colonization.

(b) Potential consequences of admixture

We found that between-population hybrid cross types had a similar phenology and pollinator visitation as the parental cross types. Earlier studies have shown that hybrids between outcrossing and selfing plants can be intermediate for phenological traits. For instance, in the genus *Clarkia*, hybridization between SC and SI populations resulted in floral characteristics and flowering times that were intermediate between the parental populations [40]. Our results show similar relationships among between-population hybrids and the parental populations in regard to flowering probability and time of peak flowering. This suggests that in a scenario of secondary contact, between-population hybrids would probably function as a bridge to further gene exchange between selfing and outcrossing plants, which could potentially lead to the parental populations merging [41]. However, this further depends on post-pollination mechanisms that determine the

relative success of crosses between non-admixed SI or SC plants compared with crosses between non-admixed and admixed plants. Therefore, whether the resulting admixed populations will maintain a mixed mating system [2], or evolve to become predominantly selfing or outcrossing remains to be tested. Initially, as inbreeding depression tends to be low [42,43], selfing may be favoured due to the associated inherent transmission advantage. However, on longer time scales, expression of drift load may select against selfing as shown in selfing populations of *A. lyrata* [22] (but see [44]). It would therefore be of interest to monitor the performance and mating system of admixed populations over multiple years.

5. Conclusion

Our common garden experiment showed that although pollinator behaviour may isolate selfers by promoting geitonogamy, SI and SC *A. lyrata* populations are only weakly reproductively isolated via pre-pollination mechanisms. These findings differ from findings in other systems with a recent transition to selfing (e.g. [11,45]). The weak isolation between SC and SI populations of *A. lyrata* is probably because its transition to selfing is even more recent, and has not led to evolution of a selfing syndrome [20]. Future studies could investigate if reproductive isolation due to pre-zygotic pre-pollination mechanisms are larger in natural populations, giving specific attention to parapatric SC and SI populations. Moreover, to what extent other mechanisms such as niche differentiation and genetic incompatibilities contribute to reproductive isolation remains to be investigated.

Authors' contributions. M.S. conceived the project. C.E.G., M.E.D., M.v.K. and M.S. designed the experiment. C.E.G. and L.B. collected the data. C.E.G. and M.E.D. analysed the data with input from M.v.K. and M.S., C.E.G. and M.S. wrote the paper with input from all authors. All authors have approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests. We declare we have no competing interests.

Funding. This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project 388824194 to M.S.

Acknowledgements. Christina Steinecke and Ryan Holt helped with data collection. Colleen White helped with transplanting. Bill Crins taxonomically identified pollinators. Fränzi Korner-Nievergelt and Pius Korner-Nievergelt (oikostat GmbH) helped with parts of the statistical analyses. Barbara Mable provided the source material (seeds) from wild populations. Yan Li, Ekaterina Mamonova and Sina Konitzer-Glückner helped with the crosses to produce the seeds used in the experiment.

References

- Barrett SCH. 2002 The evolution of plant sexual diversity. *Nat. Rev. Genet.* **3**, 274–284. (doi:10.1038/nrg776)
- Goodwillie C, Kalisz S, Eckert CG. 2005 The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Syst.* **36**, 47–79. (doi:10.1146/annurev.ecolsys.36.091704.175539)
- Darwin C. 1876 *The effects of cross and self fertilisation in the vegetable kingdom*. London, UK: J. Murray.
- Jain SK. 1976 The evolution of inbreeding in plants. *Annu. Rev. Ecol. Syst.* **7**, 469–495. (doi:10.1146/annurev.es.07.110176.002345)
- Sicard A, Lenhard M. 2011 The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. *Ann. Bot.* **107**, 1433–1443. (doi:10.1093/aob/mcr023)
- Rieseberg LH, Willis JH. 2007 Plant speciation. *Science* **317**, 910–914. (doi:10.1126/science.1137729)
- Coyne J, Orr H. 2004 *Speciation*. Sunderland, MA: Sinauer Associates.
- Baack E, Melo MC, Rieseberg LH, Ortiz-Barrientos D. 2015 The origins of reproductive isolation in plants.

- New Phytol.* **207**, 968–984. (doi:10.1111/nph.13424)
9. Lowry DB, Rockwood RC, Willis JH. 2008 Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. *Evolution* **62**, 2196–2214. (doi:10.1111/j.1558-5646.2008.00457.x)
 10. Widmer A, Lexer C, Cozzolino S. 2009 Evolution of reproductive isolation in plants. *Heredity* **102**, 31–38. (doi:10.1038/hdy.2008.69)
 11. Martin NH, Willis JH. 2007 Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* **61**, 68–82. (doi:10.1111/j.1558-5646.2007.00006.x)
 12. Karron JD, Holmquist KG, Flanagan RJ, Mitchell RJ. 2009 Pollinator visitation patterns strongly influence among-flower variation in selfing rate. *Ann. Bot.* **103**, 1379–1383. (doi:10.1093/aob/mcp030)
 13. Gervasi DDL, Schiestl FP. 2017 Real-time divergent evolution in plants driven by pollinators. *Nat. Commun.* **8**, 14691. (doi:10.1038/ncomms14691)
 14. Harder LD, Barrett SCH. 1996 Pollen dispersal and mating patterns in animal-pollinated plants. In *Floral biology*, pp. 140–190. Boston, MA: Springer.
 15. Barrett SCH, Richards AJ, Bayliss MW, Charlesworth D, Abbott RJ. 2003 Mating strategies in flowering plants: the outcrossing–selfing paradigm and beyond. *Phil. Trans. R. Soc. B* **358**, 991–1004. (doi:10.1098/rstb.2003.1301)
 16. Mitchell RJ, Irwin RE, Flanagan RJ, Karron JD. 2009 Ecology and evolution of plant–pollinator interactions. *Ann. Bot.* **103**, 1355–1363. (doi:10.1093/aob/mcp122)
 17. Foxe JP, Stift M, Tedder A, Haudry A, Wright SI, Mable BK. 2010 Reconstructing origins of loss of self-incompatibility and selfing in North American *Arabidopsis lyrata*: a population genetic context. *Evolution* **64**, 3495–3510. (doi:10.1111/j.1558-5646.2010.01094.x)
 18. Mable BK, Robertson AV, Dart S, Di BC, Witham L. 2005 Breakdown of self-incompatibility in the perennial *Arabidopsis lyrata* (Brassicaceae) and its genetic consequences. *Evolution* **59**, 1437–1448. (doi:10.1111/j.0014-3820.2005.tb01794.x)
 19. Mable BK, Adam A. 2007 Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. *Mol. Ecol.* **16**, 3565–3580. (doi:10.1111/j.1365-294X.2007.03416.x)
 20. Carleial S, van Kleunen M, Stift M. 2017 Small reductions in corolla size and pollen: ovule ratio, but no changes in flower shape in selfing populations of the North American *Arabidopsis lyrata*. *Oecologia* **183**, 401–413. (doi:10.1007/s00442-016-3773-4)
 21. Willi Y. 2013 The battle of the sexes over seed size: Support for both kinship genomic imprinting and interlocus contest evolution. *Am. Nat.* **181**, 787–798. (doi:10.1086/670196)
 22. Willi Y. 2013 Mutational meltdown in selfing *Arabidopsis lyrata*. *Evolution* **67**, 806–815. (doi:10.1111/j.1558-5646.2012.01818.x)
 23. Gorman CE, Steinecke C, van Kleunen M, Dorken ME, Stift M. 2020 A shift towards the annual habit in selfing *Arabidopsis lyrata*. *Biol. Lett.* **16**, 20200402. (doi:10.1098/rsbl.2020.0402)
 24. Fausto JA, Eckhart VM, Geber MA. 2001 Reproductive assurance and the evolutionary ecology of self-pollination in *Clarkia xantiana* (Onagraceae). *Am. J. Bot.* **88**, 1794–1800. (doi:10.2307/3558355)
 25. R Core Team. 2019 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
 26. Bates D, Mächler M, Bolker BM, Walker SC. 2015 Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48. (doi:10.18637/jss.v067.i01)
 27. Gelman A, Hill J. 2006 *Data analysis using regression and multilevel/hierarchical models*. Cambridge, UK: Cambridge University Press.
 28. Komer-Nievergelt F, Roth T, von Felten S, Guélat J, Almasi B, Komer-Nievergelt P. 2015 *Bayesian data analysis in ecology using linear models with R, BUGS, and Stan*. London, UK: Academic Press.
 29. Venables WN, Ripley BD. 2002 *Modern applied statistics with S*. Berlin, Germany: Springer.
 30. Therneau TM, Grambsch PM. 2000 *Modeling survival data: extending the Cox model*. Berlin, Germany: Springer.
 31. Brunet J, Charlesworth D. 1995 Floral sex allocation in sequentially blooming plants. *Evolution* **49**, 70–79. (doi:10.1111/j.1558-5646.1995.tb05959.x)
 32. Harder LD, Barrett SCH. 1995 Mating cost of large floral displays in hermaphrodite plants. *Nature* **373**, 512–515. (doi:10.1038/373512a0)
 33. Willi Y, Määttäen K. 2011 The relative importance of factors determining genetic drift: mating system, spatial genetic structure, habitat and census size in *Arabidopsis lyrata*. *New Phytol.* **189**, 1200–1209. (doi:10.1111/j.1469-8137.2010.03569.x)
 34. Mable BK, Hagmann J, Kim ST, Adam A, Kilbride E, Weigel D, Stift M. 2017 What causes mating system shifts in plants? *Arabidopsis lyrata* as a case study. *Heredity* **118**, 52–63. (doi:10.1038/hdy.2016.99)
 35. Baker HG. 1955 Self-compatibility and establishment after 'long-distance' dispersal. *Evolution* **9**, 347–349. (doi:10.1111/j.1558-5646.1955.tb01544.x)
 36. Hesse E, Pannell JR. 2011 Density-dependent pollen limitation and reproductive assurance in a wind-pollinated herb with contrasting sexual systems. *J. Ecol.* **99**, 1531–1539. (doi:10.1111/j.1365-2745.2011.01880.x)
 37. Fisher RA. 1941 Average excess and average effect of a gene substitution. *Ann. Eugen.* **11**, 53–63. (doi:10.1111/j.1469-1809.1941.tb02272.x)
 38. Porcher E, Lande R. 2005 The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *J. Evol. Biol.* **18**, 497–508. (doi:10.1111/j.1420-9101.2005.00905.x)
 39. Harder LD, Wilson WG. 1998 A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. *Am. Nat.* **152**, 684–695. (doi:10.1086/286199)
 40. Moore DM, Lewis H. 1965 The evolution of self-pollination in *Clarkia xantiana*. *Evolution* **19**, 104. (doi:10.2307/2406299)
 41. Rhymer JM, Simberloff D. 1996 Extinction by hybridization and introgression. *Annu. Rev. Ecol. Syst.* **27**, 83–109. (doi:10.1146/annurev.ecolsys.27.1.83)
 42. Carleial S, van Kleunen M, Stift M. 2017 Relatively weak inbreeding depression in selfing but also in outcrossing populations of North American *Arabidopsis lyrata*. *J. Evol. Biol.* **30**, 1994–2004. (doi:10.1111/jeb.13169)
 43. Li Y, van Kleunen M, Stift M. 2019 Sibling competition does not magnify inbreeding depression in North American *Arabidopsis lyrata*. *Heredity* **123**, 723–732. (doi:10.1038/s41437-019-0268-1)
 44. Joschinski J, van Kleunen M, Stift M. 2015 Costs associated with the evolution of selfing in North American populations of *Arabidopsis lyrata*? *Evol. Ecol.* **29**, 749–764. (doi:10.1007/s10682-015-9786-3)
 45. Briscoe Runquist RD, Chu E, Iverson JL, Kopp JC, Moeller DA. 2014 Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* **68**, 2885–2900. (doi:10.1111/evo.12488)
 46. Gorman CE, Bond L, van Kleunen M, Dorken ME, Stift M. 2020 Data from: Limited phenological and pollinator-mediated isolation among selfing and outcrossing *Arabidopsis lyrata* populations, v3. Dryad, Dataset. (doi:10.5061/dryad.x0k6djh7)