Reproductive isolation and life-history divergence between outcrossing and recently evolved selfing populations of *Arabidopsis lyrata*

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Summary

Mating system transitions from self-incompatibility to self-compatibility (i.e. being able to self-fertilize), represent a common evolutionary shift in plants and clearly play a role in speciation. Self-compatibility is also associated with an annual habit. However, many questions remain about the initial mechanistic basis of pre- and post-pollination reproductive isolation and life history divergence during the evolution of self-compatibility. I used *Arabidopsis lyrata*, a normally self-incompatible perennial that has recently experienced a shift to self-compatibility in several populations, to test whether the transition to self-compatibility has led to reproductive isolation and/or life history divergence. To test whether there was pre-pollination isolation between self-compatible (SC) and self-incompatible (SI) plants, I conducted a common garden experiment with plants from SC and SI populations and their between-population hybrids. First, I investigated whether there were differences in flowering phenology and/or pollinator visitation. Flowering largely overlapped between plants from SC and SI populations and I recovered no differences in pollinator visitation related to mating system. To test for post-pollination isolation between SC and SI populations, I compared seed sets resulting from crosses within populations, between populations of the same mating system, and between populations with different mating systems. Plants from SC and SI populations were not reproductively isolated via the investigated post-pollination mechanisms. Finally, I monitored survival of plants in the common garden over two years to test if the evolution of self-compatibility was accompanied by a shift towards the annual habit. Self-compatible plants exhibited a 39% decrease in survival after the first year compared to SI plants. While I found no strong evidence of pre- or post-pollination reproductive isolation between SC and SI populations, I did find unambiguous evidence for divergence in life history. Examinations of the dynamics of self-compatibility, reproductive isolation, and life history will continue to contribute to a more comprehensive understanding of mating system evolution.

Zusammenfassung

Der Übergang des Fortpflanzungssystems von Selbstinkompatibilität zu Selbstkompatibilität (d.h. der Fähigkeit, sich selbst zu befruchten) ist häufig bei Pflanzen und spielt zweifelsohne eine Rolle bei der Artenbildung. Auch steht die Selbstkompatibilität im Zusammenhang mit Einjährigkeit. Jedoch stehen noch viele Fragen offen, was die anfängliche mechanistische Basis der reproduktiven Isolation vor und nach der Bestäubung und die Änderungen des Lebenszyklus während der Evolution der Selbstkompatibilität betrifft. Ich verwendete *Arabidopsis lyrata*, eine normalerweise selbstinkompatible mehrjährige Pflanze, bei welcher vor kurzem in mehreren Populationen das Selbstinkompatibilitäts-System zusammengebrochen ist, um zu erforschen, ob die Transition zur Selbstkompatibilität zu reproduktiver Isolation und/oder Divergenz des Lebenszyklus der Pflanzen geführt hat. Um zu untersuchen, ob vor der Bestäubung bereits eine Isolation zwischen selbstkompatiblen (SC) und selbstinkompatiblen (SI) Pflanzen bestand, führte ich ein Common-Garden-Experiment mit Pflanzen aus SC- und SI-Populationen und den Hybriden zwischen beiden Populationen durch. Zuerst ermittelte ich, ob Unterschiede bei der Phänologie des Blühens und/oder der Bestäubervisitation bestanden. Beim Blühen der Pflanzen gab es zum großen Teil Überschneidungen zwischen Pflanzen aus SC- und SI-Populationen, und ich konnte keine Unterschiede bei der Bestäubervisitation feststellen, die mit dem Paarungssystem zusammenhingen. Um die Isolation zwischen SC- und SI-Populationen nach der Bestäubung zu testen, habe ich Gruppen von Samen verglichen, die jeweils aus Kreuzungen innerhalb von Populationen, aus Kreuzungen zwischen Populationen mit der gleichen Fortpflanzungsstrategie oder Kreuzungen zwischen Populationen mit unterschiedlichen Fortpflanzungsstrategien hervorgingen. Pflanzen aus SC- und SI-Populationen waren über die untersuchten Post-Bestäubungs-Mechanismen nicht reproduktiv isoliert. Schließlich verfolgte ich das Überleben von Pflanzen im Common Garden über einen Zeitraum von zwei Jahren, um zu erforschen, ob die Evolution der Selbstkompatibilität von einem Wechsel zur Einjährigkeit begleitet wurde. Das Überleben von selbstkompatiblen

Pflanzen zeigte im Vergleich zu SI-Pflanzen eine Abnahme von 39% nach dem ersten Jahr. Während ich keine stichhaltigen Beweise von reproduktiver Isolation zwischen SC- und SI-Populationen vor oder nach der Bestäubung finden konnte, so fand ich eindeutige Belege für die Divergenz beim Lebenszyklus. Untersuchungen der Dynamiken der Selbstkompatibilität, der reproduktiven Isolation und des Lebenszyklus werden weiterhin zu einem umfassenderen Verständnis der Evolution von Fortpflanzungsstrategien beitragen.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

List of Tables

Chapter 1

Chapter 2

Chapter 3

General Introduction

Plant breeding systems

Breeding systems, or systems that govern who mates with whom, are critical to the ecology and evolution of organisms. In plants, the breeding system encompasses both the variation in sex systems (i.e., whether plants are male, female, hermaphrodites, or a mixture) and the variation in mating systems (i.e., whether plants are inbreeding or outcrossing). Since sexual reproduction determines how genetic variation is transmitted through a population, breeding systems have profound effects on the ecology and evolutionary fates of plant populations (Charlesworth 2006, Barrett and Harder 2017). For instance, whether reproduction happens primarily by selfing or by outcrossing significantly influences gene flow, population genetic structure, and the effective population size of plant populations (Barrett and Harder 2017). Evolutionary shifts in plant breeding systems, such as transitions from being unable to produce viable offspring with oneself (i.e., self-incompatible) to being able to self-fertilize (i.e., self-compatible), are common in plants. These shifts can have substantial consequences for the evolution of plant populations. This dissertation examines the initial ecological and evolutionary dynamics of a recent shift from self-incompatibility to self-compatibility in several North American populations of *Arabidopsis lyrata.*

Mating system transitions from outcrossing to selfing

Mating-system transitions from self-incompatibility to self-compatibility are one of the most frequent evolutionary shifts in angiosperms (Barrett 2002). These transitions have likely played a substantial role in plant speciation as they have evolved independently and repeatedly across almost all major plant lineages (Barrett 2002). It is estimated that up to 15% of seed plants are predominantly selfing and many share a relatively recent common ancestor with outcrossing species (Goodwillie et al. 2005), suggesting that this mating system switch might provide a major axis promoting reproductive isolation. There are thought to be two main advantages of self-compatibility. First, plants that self-fertilize (or “self”) have an automatic transmission advantage of having self-compatible alleles because selfing individuals transmit 50% more genetic material to the next generation than individuals that do not self (Fisher 1941). This is because self-compatible plants can serve as a pollen parent to progeny of self-incompatible individuals and can also serve as a pollen and ovule parent to their own progeny (Fisher 1941). Self-compatibility is also advantageous for reproductive assurance in pollinator and/or mate limited situations (Darwin 1876, Baker 1955). Self-incompatible plants require conspecifics for reproduction and often require animal pollinators for pollen transfer. However, self-compatible plants can reproduce in the absence of conspecifics or pollinators.

There are also substantial costs associated with selfing. Inbreeding depression, or a reduction in fitness (i.e., fertility or survival) due to reproduction between related individuals (Darwin 1876), is a common result of self-fertilization (Charlesworth and Willis 2009). Plants that self-fertilize might also suffer negative consequences due to pollen discounting, or a reduction in success as a pollen parent (Nagylaki 1976, Holsinger et al. 1984). Self-fertilization can also result in a reduction in outcrossed seeds, or seed discounting (Lloyd and Schoen 1992, Busch and Delph 2012). Therefore, one would expect that lineages that have diverged in these two mating system types should face steep reproductive tradeoffs and could rapidly evolve reproductive isolating mechanisms.

The transition from self-incompatibility to self-compatibility is usually associated with a characteristic set of morphological and functional changes to a plant's flowers that promote self-fertilization, termed the "selfing syndrome" (Darwin 1876). This selfing syndrome is characterized by reductions in traits such as flower size, pollen/ovule ratios, decreased nectar and scent production, pollinator visitation, changes in flower number and altered reproductive timing, and has been observed in several plant genera containing species that have diverged for long periods of time (Wyatt 1984, Ritland and Ritland 1989, Foxe et al. 2009, Duncan and Rausher 2013, Tedder et al. 2015). Self-compatible plants are also typically annual (Stebbins 1970, Lloyd 1980, Barrett et al. 1996). The accumulation of these morphological and functional changes associated with reproduction and lifespan should lead to a reduction in gene flow between populations with different mating systems that could contribute to the development of reproductive barriers and ultimately speciation of selfing lineages (Rieseberg and Willis 2007). The importance of the selfing syndrome even at the earliest stages of divergence is supported by recently diverged sister groups (ca. 20,000 - 65,000 years) such as C*apsella rubella* and *C. grandiflora* (Foxe et al. 2009, Guo et al. 2009) and species in the genus *Clarkia* (Briscoe Runquist et al. 2014). However, which aspects of reproductive isolation might evolve in selfing lineages that have split even more recently remains unclear.

Reproductive isolation

The evolution of reproductive barriers is critical for the evolutionary independence of populations. Understanding how and why reproductive barriers arise during speciation has long been a central theme of evolutionary biology (Dobzhansky 1937, Stebbins 1950, Coyne and Orr 2004). There are several mechanisms that can lead to reproductive isolation, and they are usually characterized by the timing of their occurrence with respect to the formation of a zygote following the fusion of the maternal and paternal gametes. In animals, mechanisms that reduce the likelihood of viable zygote formation are typically characterized as prezygotic. Mechanisms that act after fertilization to reduce the viability and/or result in sterility of hybrids are classified as postzygotic. However, in plants some barriers can have both pre- and postzygotic components (Baack et al. 2015). Thus, in plants it is more precise to further distinguish reproductive barriers as pre- and post-pollination mechanisms. Pre-pollination mechanisms are entirely prezygotic and include differences in phenology and/or pollinator attraction. However, post-pollination mechanisms can have both pre- and postzygotic components and consist of differences in F_1 seed development and/or viability. Most plant species are isolated by a mixture of pre- and post-pollination mechanisms (Coyne and Orr 2004, Rieseberg and Willis 2007, Baack et al. 2015).

Pre-pollination mechanisms are generally expected to contribute more to the total reproductive isolation of species (Widmer et al. 2009, Baack et al. 2015), as they act earlier in the life history of plants and are generally individually stronger than post-pollination barriers (Ramsey et al. 2003, Coyne and Orr 2004, Lowry et al. 2008a). Accordingly, many studies have confirmed the importance of pre-pollination mechanisms to the reproductive isolation of self-compatible lineages from their self-incompatible ancestors (Martin and Willis 2007, Briscoe Runquist et al. 2014). However, some studies show that post-pollination mechanisms can also play an important role in reproductively isolating plant species (Fishman and Willis 2001, Yost and Kay 2009). It has also been suggested that post-pollination mechanisms are understudied due to their relatively cryptic nature compared to pre-pollination mechanisms (Yost and Kay 2009). Although the evolution of such reproductive barriers has been a central theme in evolutionary ecology, the importance of different pre- and/or post-pollination mechanisms as drivers of reproductive isolation after a recent transition from self-incompatibility to self-compatibility has rarely been studied. Furthermore, reproductive isolation between self-incompatible populations and newly evolved self-compatible populations may be accelerated if selection is driving populations to diverge in other ways, such as in life history traits.

Mating system influences on life history

The life histories of organisms are a collection of traits that govern reproduction and survival. These traits can have profound impacts on the fitness of organisms. In plants, life history traits include growth form, phenology, seed set, and lifespan. Mating systems may often influence the life histories of plants, and vice versa (Barrett et al. 1996, 2003). For example, comparative analyses have shown that self-compatibility is more common in herbaceous plants than in woody plants, and that annuals are self-compatible more often than perennials (Barrett et al. 1996). Mating system also has significant impacts on phenology across angiosperms, as outcrossing rate has been found to be strongly correlated with floral display and resource allocation to attractive floral structures (Goodwillie et al. 2010). Together, growth form, phenology, and lifespan encompass several aspects of life history that could influence the genetic structure and reproductive dynamics of plant populations. But, mating system transitions from self-incompatibility to self-compatibility may have especially important consequences for lifespan.

Shifts from self-incompatibility to self-compatibility can lead to reductions in lifespan, and ultimately the change from a perennial to an annual life history. The most commonly suggested mechanism for this is that the evolution of self-compatibility could increase the optimal level of total reproductive effort, thereby reducing lifespan and leading to an annual life history (Zhang 2000). For instance, self-fertilization in perennials could select for increased resource allocation to reproductive tissues instead of the vegetative structures needed for multi-year survival, which would favor the evolution of the annual habit (Friedman and Rubin 2015). It is also possible that the annual habit is favored in self-compatible plants because perennials suffer more negative consequences of self-fertilization than annuals. For instance, since perennials live for multiple years, the negative effects of inbreeding depression and/or seed discounting might accumulate over the years of a plant's lifespan (Morgan et al. 1997, Lloyd 2002, Lesaffre and Billiard 2019).

The transition from self-incompatibility to self-compatibility also has important genetic consequences for populations that can indirectly lead to reductions in lifespan. Self-fertilization inherently reduces effective population size (Heller and Smith 1978, Wright et al. 2013). Low effective population size makes self-compatible lineages more vulnerable to genetic degradation via inbreeding depression or genetic drift than self-incompatible lineages. This makes it more likely that self-compatible populations will experience a buildup of deleterious mutations that could lead to reduced lifespans. Ultimately, there are several ways that mating system can influence the life histories of plants. However, empirical tests of these associations remain rare.

Research gaps

Reproductive isolation between self-incompatible and self-compatible lineages

Few studies have attempted to quantify the relative importance of different mechanisms of reproductive isolation as potential ecological and demographic drivers of speciation following the recent evolution of selfing (Widmer et al. 2009). Ecologically relevant pre-pollination mechanisms such as changes in phenology and assortative pollinator visitation are thought to be especially important in maintaining/driving reproductive isolation between selfing species (Wendt et al. 2002, Lowe and Abbott 2004) and between selfing species and their outcrossing ancestors (Martin and Willis 2007). However, post-pollination mechanisms have likely been underappreciated as drivers of speciation (Yost and Kay 2009). Furthermore, most studies of reproductive isolation in selfing lineages have quantified the strength of these barriers in sets of highly divergent self-compatible and self-incompatible pairs/sets of (sub)species. Therefore, little is known about the dynamics of reproductive isolation in the earliest stages of the transition to selfing.

The association between self-compatibility and the annual life history

The association between self-compatibility and the annual life history has long been recognized (Stebbins 1950, Lloyd 1980, Barrett and Harder 2017). However, the directionality of this association remains unclear. For instance, it is unknown whether self-compatibility is more likely to evolve in annuals, or whether selection favors shorter lifespans in perennials that have evolved to be self-compatible. Most examinations of the evolutionary association between self-compatibility and the annual life history are based on analyses that treat mating system and habit as independent traits or assume that the annual or perennial habit is fixed and then investigate the circumstances that favor self-compatibility. Empirical studies that consider whether shifts to self-compatibility can select for changes in lifespan are nonexistent, likely due to the difficulty of testing this experimentally. To do so, one would need an ancestrally self-incompatible and perennial species that has evolved intraspecific variation in mating system (i.e., self-compatible populations that have not speciated from self-incompatible ancestors), such as *Arabidopsis lyrata*. By growing plants from ancestrally perennial and self-incompatible populations in a common environment with plants from recently evolved self-compatible populations, it is possible to compare reproductive investment and lifespans and determine if the shift to self-compatibility can lead to divergence in life history traits such as habit.

Arabidopsis lyrata as a study system

Arabidopsis lyrata ssp. *lyrata* (L.) O'Kane & Al-Shehbaz presents a unique opportunity to examine the role of different mechanisms of reproductive isolation in recently diverged self-compatible lineages. This species has experienced at least two independent and recent (<10,000 years ago) breakdowns in self-incompatibility across its range. In at least six of these populations, all plants are self-compatible, have low outcrossing rates, and therefore reproduce primarily through self-fertilization (Mable et al. 2005, Mable and Adam 2007, Foxe et al. 2010). The self-compatible and self-incompatible populations are geographically interspersed and do not exhibit consistent differences in population size or habitat type, providing a replicated framework that is ideal for studying the ecological drivers and evolutionary consequences of the evolution of self-compatibility.

Contributions of this thesis

The broad goal of this thesis is to investigate whether recently evolved self-compatible populations of *Arabidopsis lyrata* are reproductively isolated and/or have different life history traits than their self-incompatible ancestors.

In Chapter 1, I set up a common garden experiment in the native range of *A. lyrata* that simulated two phases of secondary contact between self-compatible and self-incompatible populations of *A. lyrata*. This allowed me to test whether the evolution of self-compatibility has led to prezygotic pre-pollination isolation through divergence in phenology and/or insect pollinator attraction. Specifically, I asked: 1) Does the timing and/or intensity of flowering differ between self-compatible and self-incompatible plants? 2) Is there a difference in pollinator visitation between self-compatible and self-incompatible plants? and 3) Is the visitation path of pollinators influenced by mating system? Then, using the empirical information on phenology and pollinator preferences, I simulated pollen transfer probabilities, (i.e., the opportunity for self- or outcross pollinations between plants from different populations), to determine how these vary between plants from different mating systems and how they combined might contribute to reproductive isolation.

In Chapter 2, I used an existing seed collection from a controlled crossing design to examine whether self-compatible and self-incompatible *A. lyrata* populations are reproductively isolated via post-pollination prezygotic and/or postzygotic mechanisms. Specifically, I asked whether: 1) self-incompatible populations were reproductively isolated from one another; 2) self-compatible populations were reproductively isolated from one another; 3) there is more isolation between self-compatible populations than between self-incompatible populations; 4) there is more isolation between populations if they differ in mating system; and 5) whether there is asymmetric isolation between self-compatible and self-incompatible populations.

In Chapter 3, I used my common garden experiment in the native range of *A. lyrata* to examine if the evolution of self-compatibility in the normally perennial and self-incompatible *A. lyrata* was accompanied by a shift towards an annual habit. Specifically, I asked: 1) whether plants from self-compatible populations had reduced over-winter survival compared to plants from self-incompatible populations; 2) whether between-population cross-progeny had higher survival than within-population cross-progeny, and if this differed between self-compatible and self-incompatible populations; 3) whether the selfed progeny of plants from selfing populations suffered from inbreeding depression; and 4) whether plants that had a higher reproductive investment (i.e. flower and seed production) had lower survival.

Chapter 1: Limited phenological and pollinator-mediated isolation among selfing and outcrossing *Arabidopsis lyrata* populations

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Abstract

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 prezygotic 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 prezygotic pre-pollination mechanisms do not strongly reproductively isolate plants from selfing and outcrossing populations of *Arabidopsis lyrata*.

Introduction

Mating-system transitions from obligate outcrossing to predominantly selfing have arisen repeatedly across almost all major plant lineages (Barrett 2002). Up to 15% of seed plants are predominantly selfing and many share a relatively recent common ancestor with outcrossing species (Goodwillie et al. 2005). 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" (Darwin 1876, Jain 1976, Sicard and Lenhard 2011). These types of changes in flowering likely contribute to the reproductive isolation of selfing lineages (Rieseberg and Willis 2007), 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 postzygotic barriers (Coyne and Orr 2004, Rieseberg and Willis 2007, Baack et al. 2015), although prezygotic mechanisms tend to be more important (Rieseberg and Willis 2007, Lowry et al. 2008b, Widmer et al. 2009, Baack et al. 2015). In plants, prezygotic barriers include pre- or post-pollination mechanisms (Baack et al. 2015). Post-pollination prezygotic mechanisms such as genetic incompatibilities can cause differences in seed number and/or seed viability, but pre-pollination mechanisms generally contribute more to the total reproductive isolation of plant species (Rieseberg and Willis 2007, Lowry et al. 2008b, Widmer et al. 2009). 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 (Martin and Willis 2007). However, the importance of phenological shifts relative to shifts in pollinator preferences due to floral changes as drivers of rapid prezygotic reproductive isolation after the transition to self-fertilization (Coyne and Orr 2004) has rarely been studied.

Plant phenological traits and the behavior of pollinators could readily interact. For example, pollinator behavior 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 (Karron et al. 2009). Different types of pollinators, such as flies vs. bees, could also differ greatly in their pollination strategies (Gervasi and Schiestl 2017). 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 (Harder and Barrett 1996, Barrett et al. 2003, Mitchell et al. 2009). 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 (Foxe et al. 2010). 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) (Mable et al. 2005, Mable and Adam 2007, Foxe et al. 2010). 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 < 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 (Foxe et al. 2010), and 2) the selfing populations have not developed a selfing syndrome (Carleial et al. 2017a). Furthermore, these populations likely represent multiple transitions to selfing within different population genetic backgrounds (Appendix B and (Foxe et al. 2010)). 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 (Willi 2013a, 2013b), 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 behavior 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.

Methods

Study system

Arabidopsis lyrata spp. *lyrata* (L.) 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 (>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 (Mable et al. 2005). Additionally, many of these newly self-compatible populations have evolved high selfing rates (Foxe et al. 2010). Although selfing populations have on average slightly smaller corollas and reduced P:O ratios [20], population genetic background explains most variation in floral traits (Carleial et al. 2017a).

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 (Foxe et al. 2010) (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) (Fig. 1.1, Appendix B).

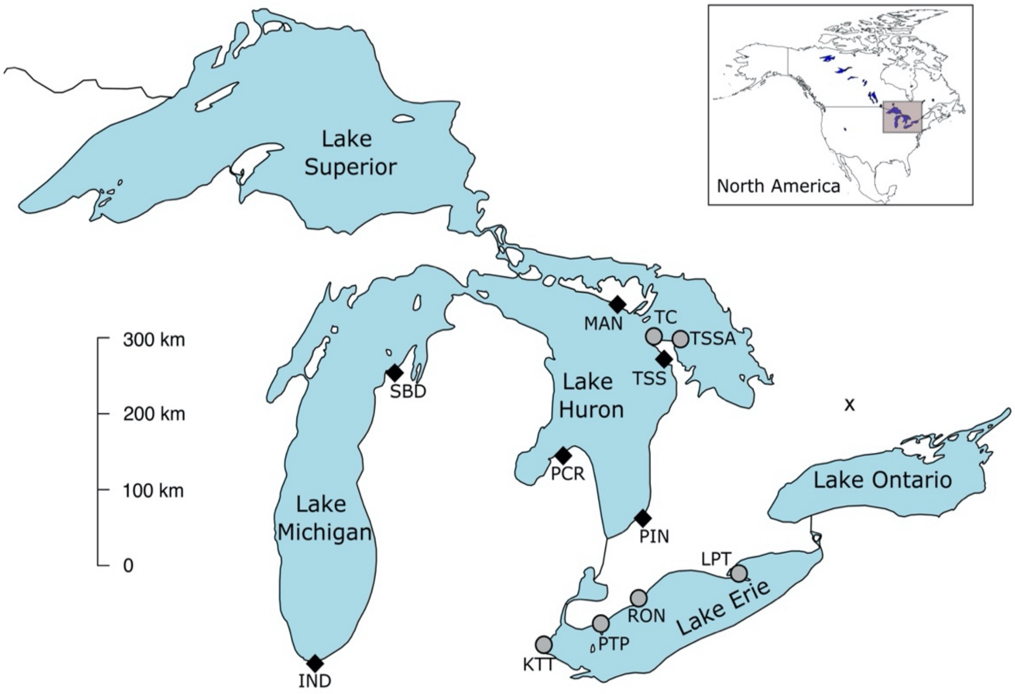

Figure 1.1 Map of the location of the common garden experiment in relation to the source populations. Gray circles represent selfing populations and black diamonds represent outcrossing populations. The black X represents the location of the common garden at Trent University, Peterborough, Ontario.

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 population ($SC{\times}SC_{between}$), and between SC and SI population reciprocally ($SI{\times}SC_{between}$ or $SC{\times}SI_{between}$) (see Appendix 3).

To increase the sample size for the $SI{\times}SI_{within}$ and $SC{\times}SC_{within}$ cross types, and to include material from the SC populations that was produced by selfing (SC×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 Appendix C for full details).

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

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 geographic range of the source populations (Fig. 1.1). From March 20 to 22, 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-hour days and a 21°C/18°C day/night cycle at 95% humidity. Between April 18 and May 1, 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 May 10, 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 3×6 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: $\mathrm{SI{\times}SI_{within}}$ (n=172), $\mathrm{SC{\times}SC_{within}}$ (n=175), SC×self (n=65), $\mathrm{SI{\times}SI_{between}}$ (n=203), $\mathrm{SC{\times}SC_{between}}$ (n=296), $\mathrm{SI{\times}SC_{between}}$ (n=314), $\mathrm{SC{\times}SI_{between}}$ (n=284).

Phenological data

To test for potential reproductive isolation between the cross types due to differences in phenology, we recorded daily for each plant whether it flowered and how many open flowers it had. 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.

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, California, 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 system. 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 interfering 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 neighbors 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-minute-long videos that were later trimmed to the central 10 minutes 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 hours of video) were analyzed 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*, *Syritta 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 (Fausto et al. 2001). 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 neighboring 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 (“$SI×SI_{within}$”, “$SC×SC_{within}$”, “SC×self”, “$SI×SI_{between}$”, “$SC×SC_{between}$”, “$SI×SC_{between}$”, “$SC×SI_{between}$”) – 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.

Statistical analyses

All statistical analyses were done in R 3.5.1 (R Core Team 2019). To test if there were differences in the time of peak flowering and duration of flowering between SI and SC cross types ($SI×SI_{within}$, $SC×SC_{within}$, SC×self) and between within-population cross types and between-population cross types ($SI×SI_{within}$, $SC×SC_{within}$, SC×self versus $SI×SI_{between}$, $SC×SC_{between}$, $SI×SC_{between}$, $SC×SI_{between}$), we used Gaussian linear mixed-effects models implemented in lme4 (Bates et al. 2014) using *cross type* as a fixed effect, and *maternal ID* (nested within *maternal population*) and *paternal 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, 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’ (Gelman and Hill 2006)). 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 (Korner-Nievergelt et al. 2015). Specifically, $p(\beta) \propto 1$ was implemented for the model coefficients and $p(\sigma^2) \propto 1/\sigma^2$ for the variance parameters. 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 $\mathrm{SI{\times}SI_{within}}$ and $\mathrm{SC{\times}SC_{within}}$ cross types (and to create the SC×self cross type, see Appendix C), we also ran all analyses without the extra material but this did not change the findings (see Appendix E).

Pollinator visitation rate (per plant) was analyzed separately for the two main visitor classes hoverflies and solitary bees. The cross type ‘SC×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 (Bates et al. 2014). In other words this results in a faster but less precise parameter estimation for generalized mixed effect models (Bates et al. 2014). These models used a log-link function. Improper prior distributions were used, as in the analyses of time of peak flowering and flowering duration.

Pollinator visitation paths were analyzed in two ways. The probability that a pollinator would make a certain choice after landing on a flower was analyzed with a multinomial logistic regression as implemented in the function multinom in the package 'nnet' (Venables and Ripley 2002). *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'; (Therneau and Grambsch 2000)) 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 '$\mathrm{SI}\times\mathrm{SI}_{\mathrm{within}}$' 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.

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 behavior to model opportunities for outcrossing between ‘SI×SI$_{within}$’ and ‘SC×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 $\boldsymbol{K_{ij}}$ - the “pollen transfer probabilities” outlined in (Brunet and Charlesworth 2006) (see Appendix D for full details).

Results

Phenology

Of the 1509 plants in the common garden, 938 flowered (62%). Although six plants died before flowering, the non-flowering plants were largely due to plants that survived the whole season but never produced a flowering stem and therefore never flowered. The main flowering period lasted six weeks from June 1 to July 14, although a few individuals flowered later (nine individuals flowered a second time and 10 individuals flowered for the first time as late as September) (Appendix A). The probability of an individual flowering did not strongly differ among the SI×SI$_{within}$ and SC×SC$_{within}$ cross types (60% and 40%, respectively; CrI overlapping; Fig. 1.2a), but the flowering probability of the SC×self cross type (15%) was lower (Fig. 1.2a). 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; Fig. 1.2a). Additionally, the direction of the cross for the SI×SC$_{between}$ and SC×SI$_{between}$ crosses did not have an obvious effect on flowering probability, as both cross types had a 65% probability of flowering (Fig. 1.2a).

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×SI$_{\text{within}}$ and SC×SC$_{\text{within}}$ cross types, and the SC×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×SI$_{\text{between}}$; Fig. 1.2b). The day of peak flowering did not differ between SI×SC$_{\text{between}}$ and SC×SI$_{\text{between}}$ plants, indicating that cross direction did not influence the time of peak flowering (CrIs overlapping; Fig. 1.2b). 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×SI$_{\text{between}}$ cross type tended to reach peak flowering one to two days later (Fig. 1.2b). Similarly, flowering duration did not strongly differ among the cross types (Fig. 1.2c), but the SC×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) (Fig. 1.2c).

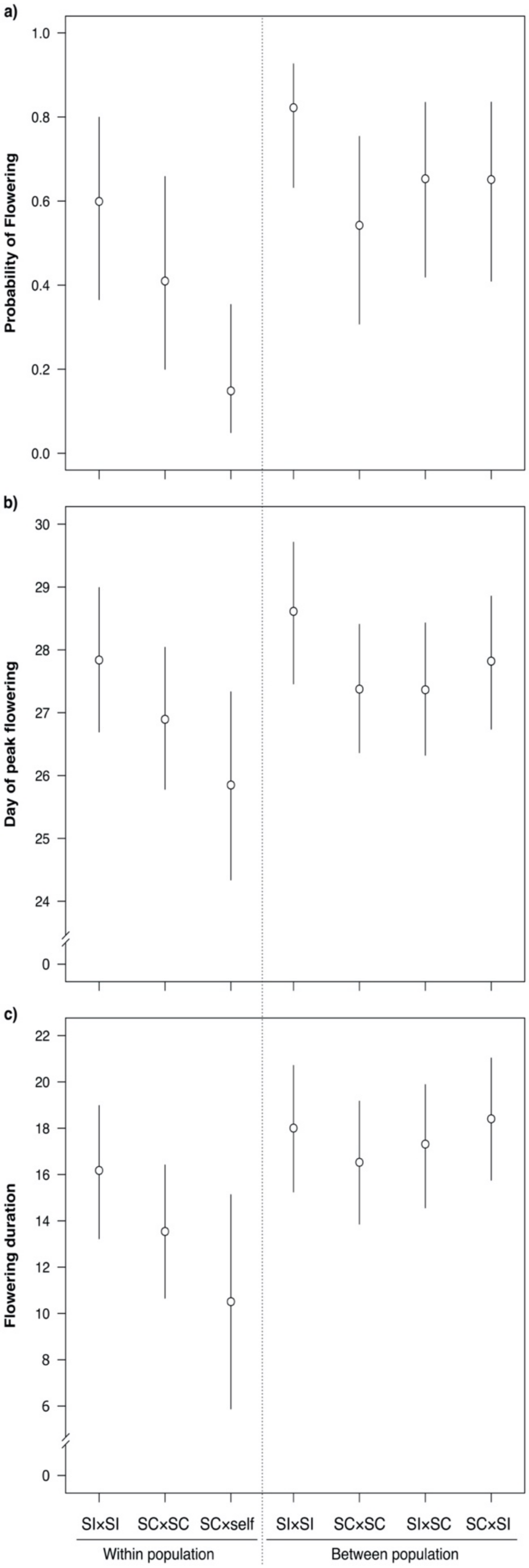

Figure 1.2 Panel of effect plots for the differences in phenological traits among the cross types (n=1509). Fitted values and credible intervals were obtained through simulation of the linear mixed model output. a) The probability of flowering per cross type. b) The day of peak flowering per cross type (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×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.

Pollinator visitation and opportunities for pollen-transfer

Solitary bees and hoverflies were the predominant visitors, and they had similar visitation frequencies and no clear pattern of preference for any of the cross types (compare Fig. 1.3a and 1.3b, all CrIs overlapping). The behavior of both types of pollinators appeared to increase the opportunity for geitonogamous self-pollination, as ~50% of the movements between flowers were to a different flower on the same plant (Fig. 1.4). 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 1.1). In other words, pollinators were more prone to visit the nearest plant, regardless of the cross type or the number of flowers on the neighboring plant.

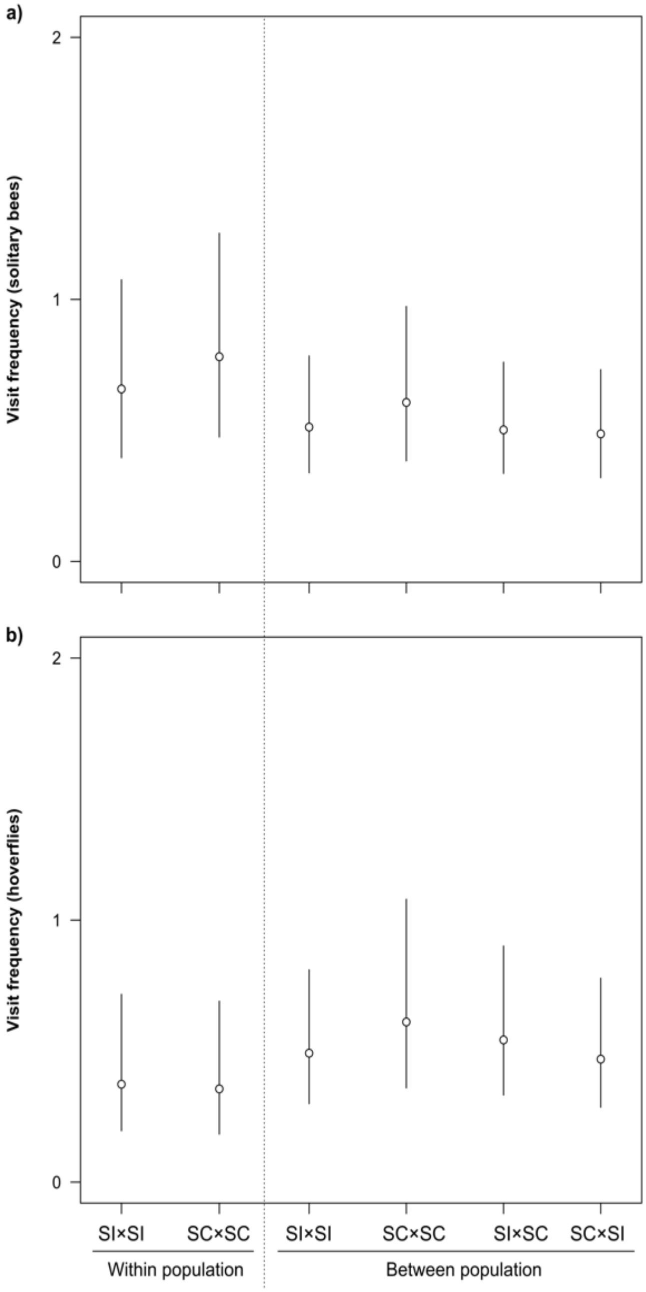

Figure 1.3 Differences in pollinator visitation (number of visits) by a) solitary bees and b) hoverflies among the cross types (n=502). Fitted values and credible intervals were obtained through simulation of the negative binomial generalized linear mixed model output. Vertical bars represent the 95% credible intervals. Cross types are SI×SI$_{\text{within}}$ and SC×SC$_{\text{within}}$ (crosses within SI and SC populations, respectively), SI×SI$_{\text{between}}$ and SC×SC$_{\text{between}}$ (crosses between SI and between SC populations, respectively),

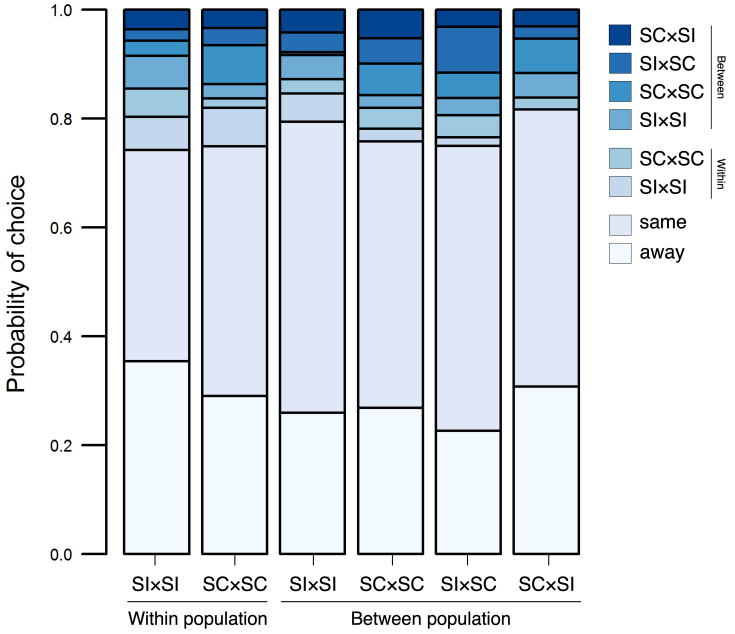

Figure 1.4 Stacked bar plot representing the probability of pollinators making a choice to visit an individual of any cross type after visiting an individual of a certain cross type. Probabilities were obtained from the multinomial model (and thus corrected for the number of available flowers on each plant in the array). Pollinators could also choose to visit a flower on the same plant ("same") or to leave the experimental setup ("away"). The cross types on the x-axis represent the cross type of the initial visit. The stacked blue bars represent the probability of that cross type being selected after a visit to the cross type on the x-axis.

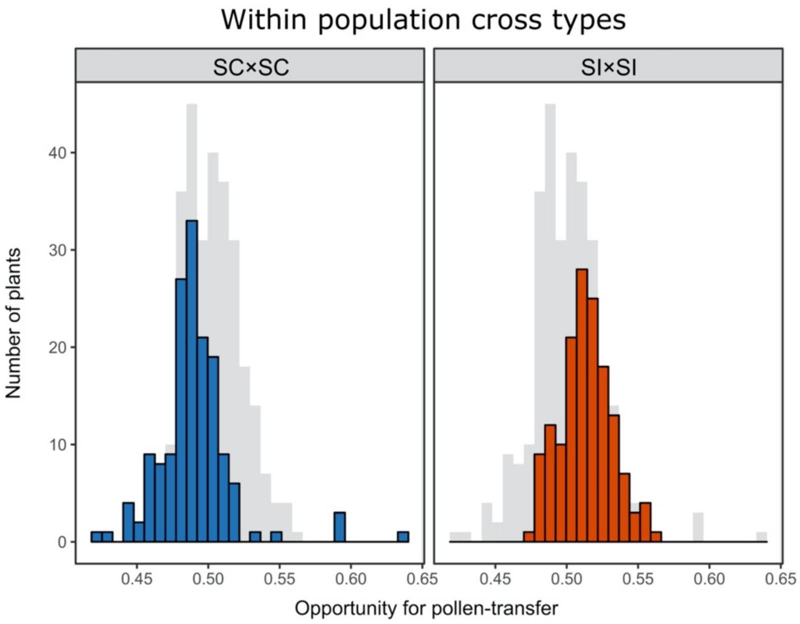

Figure 1.5 Representative bootstrapped run of the distribution of the opportunity for between-cross type pollen-transfer for the SC×SC$_{\text{within}}$ (blue) and SI×SI$_{\text{within}}$ (red) cross types. The parameter estimate for the difference in transfer-probability for the shown run was 0.024, 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.

Table 1.1 Summary of results from the conditional logistic regression model that analyzed whether *relative flower number*, *relative distance*, or *cross type* influenced the pollinator path. Cross type $\text{SI}\times\text{SI}_{\text{within}}$ was used as the baseline. Hoverflies: n=546, number of events = 142, Likelihood ratio test= 62.16 on 7 df, p=<.001*. Solitary Bees: n=541, number of events=140, Likelihood ratio test= 45.24 on 7 df, p=<.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. Significant effects are highlighted in **bold**. 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	SE	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	**0.11**	**0.38**	**-5.73**	**<.001***	**0.05**	**0.24**
	$\text{SC}\times\text{SC}_{\text{within}}$	2.01	0.39	1.80	0.07	0.94	4.30
	$\text{SI}\times\text{SI}_{\text{between}}$	1.32	0.39	0.71	0.48	0.62	2.82
	$\text{SC}\times\text{SC}_{\text{between}}$	0.96	0.39	-0.12	0.91	0.44	2.07
	$\text{SI}\times\text{SC}_{\text{between}}$	0.80	0.36	-0.64	0.52	0.40	1.60
	$\text{SC}\times\text{SI}_{\text{between}}$	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	**0.06**	**0.43**	**-6.56**	**<.001***	**0.03**	**0.14**
	$\text{SC}\times\text{SC}_{\text{within}}$	1.06	0.45	0.14	0.89	0.44	2.55
	$\text{SI}\times\text{SI}_{\text{between}}$	1.09	0.41	0.21	0.83	0.49	2.44
	$\text{SC}\times\text{SC}_{\text{between}}$	1.70	0.40	1.34	0.18	0.78	3.71
	$\text{SI}\times\text{SC}_{\text{between}}$	1.03	0.40	0.07	0.95	0.47	2.27
	$\text{SC}\times\text{SI}_{\text{between}}$	0.83	0.41	-0.44	0.66	0.37	1.86

The opportunity for between versus within cross type pollen-transfer was nearly equal both for the $\text{SI}\times\text{SI}_{\text{within}}$ and the $\text{SC}\times\text{SC}_{\text{within}}$ cross types (Fig. 5). In other words, slight shifts in phenology and flowering intensity (Appendix A) were unlikely to lead to reproductive isolation.

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 prezygotic 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.

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 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 (Gervasi and Schiestl 2017). 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 behavior of the pollinators could favor 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 (Darwin 1876, Harder and Barrett 1995), 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, neighboring plants could be highly related to each other (Willi and Maattanen 2011). As a consequence, the observed behavior 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 (Foxe et al. 2010, Mable et al. 2017), geitonogamy may help overcome this mate limitation and provide reproductive assurance (Baker 1955, Hesse and Pannell 2011). 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(Fisher 1941, Porcher and Lande 2005), but in the absence of pollen limitation, geitonogamy should result in strong pollen discounting (Harder and Wilson 1998). 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.

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 (Moore and Lewis 1965). 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 likely function as a bridge to further gene exchange between selfing and outcrossing plants, which could potentially lead to the parental populations merging (Rhymer and Simberloff 1996). However, this further depends on post-pollination mechanisms that determine the relative success of crosses between non-admixed SI or SC plants compared to crosses between non-admixed and admixed plants. Therefore, whether the resulting admixed populations will maintain a mixed mating system (Goodwillie et al. 2005), or evolve to become predominantly selfing or outcrossing remains to be tested. Initially, as inbreeding depression tends to be low (Carleial et al. 2017b, Li et al. 2019), selfing may be favored due to the associated inherent transmission advantage. However, on longer timescales, expression of drift load may select against selfing as shown in selfing populations of *A. lyrata* (Willi 2013a)(but see(Joschinski et al. 2015)). It would therefore be of interest to monitor the performance and mating system of admixed populations over multiple years.

Conclusions

Our common garden experiment showed that although pollinator behavior 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.,(Ramsey et al. 2003, Briscoe Runquist et al. 2014)). The weak isolation between SC and SI populations of *A. lyrata* is likely because its transition to selfing is even more recent, and has not led to evolution of a selfing syndrome (Carleial et al. 2017a). Future studies could investigate if reproductive isolation due to prezygotic 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.

Chapter 2: Post-pollination prezygotic and postzygotic mechanisms play little role in the reproductive isolation of selfing and outcrossing *Arabidopsis lyrata* populations

Courtney E. Gorman, Yan Li, Marcel Dorken, and Marc Stift

Abstract

Selfing restricts gene flow. Therefore, mating system transitions from outcrossing to selfing could result in reproductive isolation between selfing and outcrossing lineages and frequently provide a starting point for speciation. In plants that have diverged along a selfing axis, reproductive isolation can be caused by a variety of pre- and post-pollination prezygotic and postzygotic mechanisms. In animals, prezygotic barriers tend to evolve faster than postzygotic ones. But, in plants this is not necessarily the case. The significance of post-mating, post-fertilization, and early acting post-zygotic barriers has been investigated far less in plants than in animals. To test whether post-pollination isolation exists between North American *Arabidopsis lyrata* plants from self-compatible (SC) and self-incompatible (SI) populations we compared seed sets resulting from crosses within populations, between populations of the same mating system, and between populations with different mating system. We found that plants from SC and SI populations of *A. lyrata* are neither reproductively isolated via post-pollination prezygotic mechanisms nor by early acting postzygotic mechanisms. Despite the differences in mating system, we conclude that the newly evolved selfing populations in North American *A. lyrata* show little evidence of post-pollination reproductive isolation.

Introduction

Mating system transitions from obligately outcrossing to predominantly selfing are one of the most frequent evolutionary shifts in angiosperms and have occurred repeatedly across almost all major plant lineages (Barrett 2002). Approximately 15% of seed plants are predominantly selfing and many share a relatively recent common ancestor with an outcrossing species (Goodwillie et al. 2005), suggesting that the mating system transition to selfing is also likely a major axis of speciation. In line with this, several studies have demonstrated that transitions from outcrossing to selfing can lead to rapid speciation in plants (Foxe et al. 2009, Guo et al. 2009, Briscoe Runquist et al. 2014). The transition to selfing is itself an isolating mechanism because selfing by its nature reduces gene flow. Once gene flow is restricted, other isolating mechanisms that enforce restricted gene flow are likely to arise (Bateson 1909, Dobzhansky 1937, Muller 1942), leading to further divergence between lineages. Here, we test whether the recent transition to selfing in populations of *A. lyrata* has been accompanied by the evolution of post-pollination reproductive isolation.

Selfing limits gene flow via reduction in pollen transfer (Wright et al. 2013, Brys et al. 2014). Selfing can also indirectly contribute to reproductive isolation (Coyne and Orr 2004) because the evolution of selfing is often associated with characteristic changes in floral morphology and reproductive allocation that optimize self-pollination, termed the “selfing syndrome” (Sicard and Lenhard 2011). Such changes could promote reproductive isolation between newly evolved selfing lineages and their outcrossing ancestors by reducing pollinator visitation and/or the ability of conspecific pollen to reach the stigma. Furthermore, selfing could facilitate speciation via genetic drift, as self-fertilization substantially reduces effective population size (Heller and Smith 1978, Wright et al. 2013), and could therefore reduce gene flow between selfing and outcrossing lineages. Additionally, once there is genetic isolation of selfing populations, genetic incompatibilities such as Bateson–Dobzhansky–Muller incompatibilities between selfing and outcrossing lineages can evolve that reduce fitness if there is secondary contact between the diverged populations (Bateson

1909, Dobzhansky 1937, Muller 1942, Fishman and Willis 2001). Yet, how and if these consequences of selfing contribute to the evolution of reproductive barriers in newly evolved selfing lineages remains unclear.

There are many types of reproductive barriers, which are typically characterized as prezygotic (i.e. mechanisms that reduce the likelihood of viable zygote formation) or postzygotic (i.e. mechanisms that act after fertilization to reduce the viability and/or sterility of hybrids) in animal systems. However, in plants some barriers such as reduced hybrid seed set can have both pre- and postzygotic components (Baack et al. 2015). Therefore, in plants it is more precise to distinguish between pre- and post-pollination mechanisms. Pre-pollination mechanisms are exclusively prezygotic and include differences in geography, phenology and/or pollinator attraction. However, post-pollination mechanisms can have both pre- and postzygotic components and consist of differences in F_1 seed formation and/or viability. Most plant species are isolated by a combination of pre- and post-pollination mechanisms (Coyne and Orr 2004, Rieseberg and Willis 2007, Baack et al. 2015). Pre-pollination mechanisms are generally expected to contribute more to total reproductive isolation after the transition to selfing (Widmer et al. 2009, Baack et al. 2015), as they act earlier in the life history of plants and are often individually stronger than post-pollination barriers (Ramsey et al. 2003, Coyne and Orr 2004, Lowry et al. 2008a). However, post-pollination mechanisms have likely been underappreciated as drivers of speciation due to their relatively cryptic nature (Yost and Kay 2009).

Post-pollination mechanisms can also act asymmetrically to isolate selfing lineages. There are several examples of self-compatible species that can hybridize with related self-incompatible species as females, but not as males due to inhibition of pollen tube growth in the style (reviewed in (Levin 1971)). Additionally, cyto-nuclear incompatibilities (i.e. incompatibilities between organellar and nuclear genes) between the parental genomes can result in asymmetric post-zygotic reproductive isolation (Tiffin et al. 2001, Caruso et al. 2012). Furthermore, conflicts between female and male parents over resource allocation to offspring can result in asymmetric post-pollination reproductive isolation between self-compatible and self-incompatible lineages (Brandvain and Haig 2005, Willi 2013b). However, surprisingly few studies have attempted to quantify the relative importance of different post-pollination mechanisms of reproductive isolation, and the strength of possible asymmetries as potential drivers of speciation after the evolution of selfing (Widmer et al. 2009). Here we use within- and between mating system crosses of *Arabidopsis lyrata* ssp. *lyrata* (L.) O'Kane & Al-Shehbaz to examine the role of differences in seed number and weight as mechanisms of post-pollination reproductive isolation in a recently diverged selfing lineage.

Arabidopsis lyrata has become a popular system for studying the early dynamics of the evolutionary shift to selfing, because it displays intraspecific breeding- and mating system variation in its North American populations (Mable et al. 2005). Most North American populations are self-incompatible and outcrossing (multi-locus outcrossing rates t_m>0.8), but in several populations all plants are self-compatible, and reproduce primarily through selfing (t_m: 0.1-0.4) (Mable et al. 2005, Mable and Adam 2007, Foxe et al. 2010). The self-incompatible and self-compatible populations are geographically interspersed and do not exhibit consistent differences in population size or habitat type. The transition to selfing in these populations is thought to have happened recently (< 10,000 years ago) as the range now occupied by the self-incompatible and self-compatible populations was mostly covered by ice during the last glacial maximum and the selfing populations have not developed clear phenotypes associated with a selfing syndrome (Foxe et al. 2010, Carleial et al. 2017a). Furthermore, these populations likely represent multiple transitions to selfing within different population genetic backgrounds ((Foxe et al. 2010, Mable et al. 2017) Appendix B). Due to the multiple intraspecific origins of selfing, *A. lyrata* is ideally suited for examining the role of post-pollination mechanisms of reproductive isolation in recently diverged selfing lineages.

In this study, we examined post-pollination reproductive isolation in *A. lyrata* by quantifying patterns of seed set of crosses involving self-compatible (SC) plants from populations with high selfing rates (hereafter: SC populations) and self-incompatible (SI) plants from populations with high outcrossing rates (hereafter: SI populations). Specifically, we compared crosses within populations and between populations with the same mating system and between populations with a different mating system. As reproductive barriers can act asymmetrically in plants (Tiffin et al. 2001, Turelli and Moyle 2007), we also tested whether there was an effect of cross-direction. Specifically, we compared seed number and average seed weight to assess reproductive isolation due to post-pollination mechanisms, and asked whether: (1) SI populations were isolated from one another; (2) SC populations were isolated from one another; (3) there is more isolation between SC populations than between SI populations; (4) there is more isolation between populations if they differ in mating system; and (5) whether there is asymmetric isolation between SI and SC populations.

Methods

Study System

Arabidopsis lyrata ssp. 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 (>20) small white flowers. Each fruit (silique) can produce up to 40 seeds. The ancestral condition in *A. lyrata* is self-incompatibility (multi-locus outcrossing rates t_m>0.8, Appendix B), however the barrier to self-fertilization has broken down in several North American populations (Mable et al. 2005). Additionally, these newly self-compatible populations have evolved high selfing rates (t_m: 0.1-0.4; Appendix B) (Foxe et al. 2010).

Crossing Design

To generate the material needed to test whether diverged SI and SC populations are reproductively isolated via post-pollination mechanisms, we first sowed field-collected seeds from 12 North American *A. lyrata* populations with known breeding (SC versus SI) and mating systems (Foxe et al. 2010) (seeds were kindly provided by Barbara Mable, University of

Glasgow). These included six populations with high outcrossing rates, high frequency of SI individuals, hereafter referred to as SI populations) and six populations with low outcrossing rates, high frequency of SC individuals; hereafter, SC populations) (Fig. 1, Appendix B). We then performed controlled crosses in 2014 and 2015 with six plants of each of the six SI and six SC populations. This crossing design 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 population ($SC \times SC_{between}$), and between SC and SI population reciprocally (SI×SC or SC×SI) (see Appendix C for full details).

Seed yield and weight

To obtain the average number of zygotes per fruit for each cross combination, for each of the 1770 possible fruits, we determined whether the fruits produced seeds, and then counted all seeds (including seeds of which the development that appeared to have been aborted prematurely). To obtain the average seed mass per fruit, we pooled all seeds per fruit and excluded those that did not produce seeds. We then weighed each fruit's seeds to the nearest µg using a microbalance (Mettler Toledo XP2U).

Statistical analyses

All statistical analyses were done in R 3.5.1 (R Core Team 2019). To test if seed number per fruit differed among the cross types, we used a generalized linear mixed-effects model with a Poisson distribution implemented in *lme4* with *cross type* as a fixed effect, and *maternal ID* (nested within *maternal population*) and *paternal ID* (nested within *paternal population*) as random effects. To test if the average seed weight of the crosses that produced seeds (n=1667 out of 1770) differed among the cross types, we used a Gaussian linear mixed-effects model with *cross type* as a fixed effect and the same random-effects structure described above.

To make post-hoc comparisons between specific cross type combinations, we used the *glht* function in the *multcomp* package (see Table 2.1) (Hothorn 2008) with a custom contrast matrix to test whether: (C1) SI populations are isolated from each other via post-pollination mechanisms; (C2) SC populations are isolated from each other via post-pollination mechanisms; (C3) SC populations are more isolated from each other than SI populations; (C4) there is isolation due to breeding system differences; and (C5) there is asymmetric isolation between SI and SC populations.

Results

Out of the 1770 possible crosses, 1667 produced seeds. The frequency of cross failure was evenly distributed among cross types (percent of failed crosses by cross type: $SI \times SI_{within}$: 7.80%, $SC \times SC_{within}$: 7.32%, $SI \times SI_{between}$: 8.77%, $SC \times SC_{between}$: 3.13%, SI×SC: 3.99%, SC×SI: 7.72%). Plants from different SI populations appear to show little isolation from each other via post-pollination mechanisms, because progeny resulting from crosses between SI populations did not have a lower seed set than progeny from crosses within SI populations. In fact, the opposite was true, with crosses between SI populations yielding a higher seed number (20.5% increase) and average seed weight (4.8% increase) than crosses within SI populations. In other words, there was heterosis rather than outbreeding depression for all traits (significant effects of C1, Table 2.1, Fig. 2.1). Similarly, plants from SC populations were not isolated from each other via post-pollination mechanisms and showed heterosis rather than outbreeding depression for all traits (significant effects of C2 for seed number and seed weight; Table 2.1, Fig. 2.1). We found a higher seed number (39.2 % increase) and average seed weight (11.4% increase) for progeny from between SC population relative to progeny from crosses within SC populations. There was no significant difference in the magnitude of the effects of C1 and C2 (no significant effect of C3, Table 2.1, Fig. 2.1). Breeding system did not have a significant effect on seed number but did on average seed weight (significant effect of C4, Table 2.1, Fig. 2.1), as seeds produced by within mating system crosses were larger than seeds produced by between mating system crosses. Cross direction in the reciprocal crosses (SI×SC and SC×SI cross types) did not have a significant effect on seed number (SI×SC: 13.89 and SC×SI: 15.03) or average seed weight (SI×SC: 110.47 µg and SC×SI: 131.58 µg) (no significant effect of C5, Table 2.1, Fig. 2.1).

Figure 2.1 a) Effect plot of seed number per fruit by cross type (n = 1770). b) Effect plot of average seed weight by cross type (n = 1667). Predictions from the generalized linear mixed-effects models were obtained with the *ggpredict* function in the *ggeffects* R package (Lüdecke 2018). Colored points are predicted values and error bars represent 95% confidence intervals.

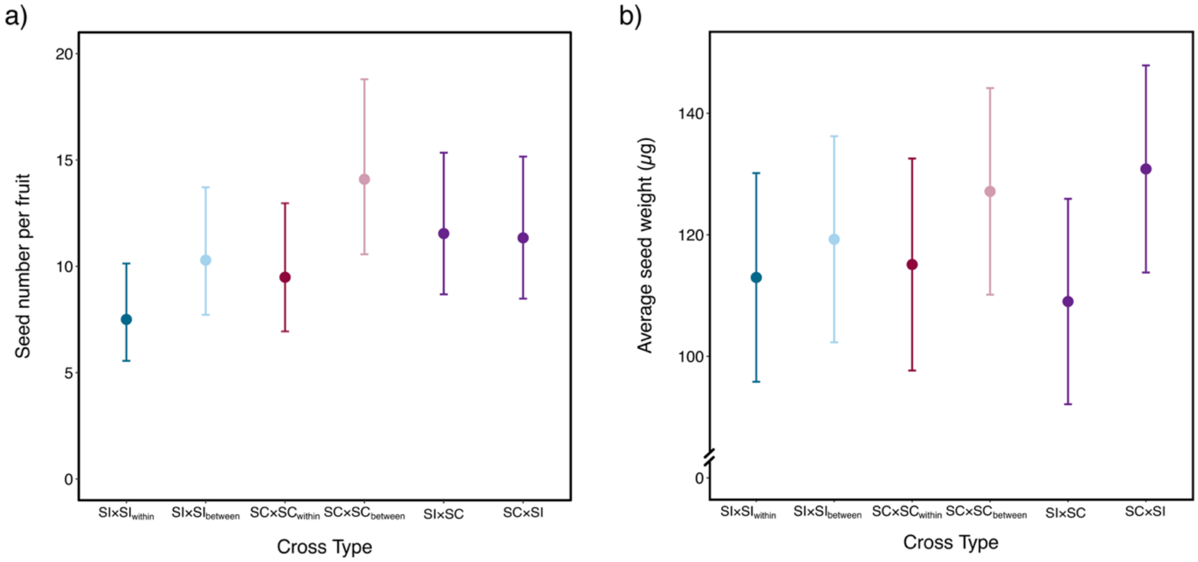

Table 2.1 Post-hoc hypothesis testing for seed traits using a contrast matrix (*glht* function in the multcomp package(Hothorn et al. 2008)). Abbreviations: SE= standard error; z= Wald statistic, SI = self-incompatible, and SC = self-compatible. Significant effects are highlighted in **bold**.

	Seed number				**Seed weight**				**Contrast definitions**					
Post hoc comparison	**Estimate**	**SE**	**z**	**Pr(>|z|)**	**Estimate**	**SE**	**z**	**Pr(>|z|)**	**SI×SI within**	**SI×SI between**	**SC×SC within**	**SC×SC between**	**SI×SC**	**SC×SI**
C1: Isolation between SI populations?[a]	**0.316**	**0.065**	**4.852**	**<0.001**	**6.284**	**2.092**	**3.004**	**0.012**	-1	1	0	0	0	0
C2: Isolation between SC populations?[a]	**0.396**	**0.078**	**5.081**	**<0.001**	**12.033**	**2.557**	**4.706**	**<0.001**	0	0	-1	1	0	0
C3: Difference in magnitude of effect C1 and C2?[b]	-0.040	0.051	-0.788	0.898	-2.874	1.648	-1.744	0.302	-0.5	0.5	0.5	-0.5	0	0
C4: Isolation due to breeding systems differences?[c]	-0.051	0.033	-1.542	0.424	**-3.274**	**1.111**	**-2.948**	**0.015**	0	-0.5	0	-0.5	0.5	0.5
C5: Asymmetric isolation between SI and SC?[d]	0.018	0.207	0.086	1.000	-21.814	12.249	-1.781	0.282	0	0	0	0	1	-1

[a] A negative estimate would indicate reproductive isolation (i.e., outbreeding depression) between populations. A positive estimate would indicate the opposite (i.e., heterosis).

[b] A negative estimate would indicate that the magnitude of C1 is smaller than the magnitude of C2. A positive estimate would indicate the opposite (that the magnitude of C1 is larger than the magnitude of C2)

[c] A negative estimate would indicate reproductive isolation between breeding systems. A positive estimate would indicate the opposite (i.e., heterosis).

[d] A significant estimate would indicate asymmetry in reproductive isolation between breeding systems. A positive estimate would indicate that crosses with SI mothers have higher trait values than crosses with SC mothers (i.e., SI×SC > SC×SI). A negative estimate would indicate the opposite, i.e. that crosses with SI mothers have lower trait values than crosses with SC mothers (i.e., SI×SC < SC×SI)

Discussion

Our main finding is that SI and SC populations of North American *A. lyrata* ssp. *lyrata* are not reproductively isolated via post-pollination mechanisms from other populations within the same mating system, or from each other. To the contrary, both for the set of SI and the set of SC populations, we found that seed number and seed weight were higher when plants from different populations were crossed. In other words, there was heterosis for these traits. Also, the magnitude of this heterosis was not significantly different between the breeding systems. Critical to our understanding of reproductive isolation, heterosis tends to accelerate gene flow between populations and thereby limits population divergence and speciation (Ingvarsson and Whitlock 2000, Bierne et al. 2002). Thus, our results effectively show the opposite of what we would expect if SI populations were reproductively isolated from each other or SC populations were isolated from each other. Moreover, comparing the reciprocal SI×SC and SC×SI cross types provided no evidence of asymmetric pre- or postzygotic post-pollination barriers between SI and SC populations, as cross direction did not significantly influence seed number or seed weight.

No evidence for speciation by selfing

Our results, taken together with the results of previous work, indicate that speciation by selfing has not occurred in *A. lyrata*. This is in contrast to other plant systems, where the transition to self-compatibility has been found to be associated with rapid speciation of highly selfing lineages (Foxe et al. 2009, Guo et al. 2009, Briscoe Runquist et al. 2014). *Arabidopsis lyrata* differs from these systems in that SC populations of *A. lyrata* have not yet developed a selfing syndrome (Carleial et al. 2017a) and that the SI and SC populations are not strongly reproductively isolated via pre-pollination mechanisms (Gorman et al. 2020a). Since the transition to selfing in SC populations of *A. lyrata* likely occurred in the last 10,000 years (Foxe et al. 2010), it is possible that there has not been enough time for the evolution of reproductive barriers between these populations. However, there is some evidence that the

SC populations have diverged from the SI populations in lifespan (Gorman et al. 2020b). Therefore, reproductive barriers, especially if they are influenced by lifespan, could become stronger as SC populations continue to diverge from SI populations.

No asymmetric reproductive isolation between breeding systems

Our results show no evidence that post-pollination barriers are acting asymmetrically to reproductively isolate SI and SC *A. lyrata* populations. This contrasts with previous work that found that seed size in *A. lyrata* depended on the mating system of the pollen donor (Willi 2013b). However, our study differed in that we used the same plants as the sire and the dam in the reciprocal crosses, while the previous study used haphazardly chosen pollen donors (Willi 2013b). It is also possible that we did not detect an effect of cross direction because we used average seed weight as a proxy for seed size, while the previous study used surface area (Willi 2013b). For instance, if a cross produced large seeds without endosperm, this would give seeds with a higher surface area but low weight. Nevertheless, we found no significant effect of cross direction for the SI×SC and SC×SI cross types on seed number or seed size. Therefore, asymmetrically acting post-pollination barriers are not likely playing a large role in the reproductive isolation of SI and SC *A. lyrata* populations.

Potential consequences of secondary contact

We found that seeds produced by the between mating system crosses (i.e., SI×SC and SC×SI) had similar seed number and seed weight to plants produced by the between-population within mating system crosses (i.e., $SI \times SI_{between}$ and $SC \times SC_{between}$). Previous work on pre-pollination mechanisms of reproductive isolation in *A. lyrata* found that plants produced by between mating system crosses had similar phenological traits to plants produced by within mating system crosses (Gorman et al. 2020a). Taken together, this indicates that in a scenario of secondary contact (i.e. SI and SC populations coming into contact with one another), SI and SC populations would likely merge. The two mating system types substantially overlap in phenology and lack pollinator specificity. This ample opportunity for cross-pollination means that there are likely no post-pollination barriers to interbreeding as well as no differences in seed numbers. Therefore, it is likely that in a scenario of secondary contact *A. lyrata* would initially maintain a mixed-breeding system containing both SI and SC plants (Goodwillie et al. 2005). Whether such admixed populations would evolve to become predominantly selfing or outcrossing on longer timescale remains to be tested. To elucidate this, it would be important to compare the performance of SI versus SC plants over multiple years in a common environment.

Conclusions

Our experiment showed that SI and SC populations of *A. lyrata* are not strongly reproductively isolated via pre- or postzygotic post-pollination mechanisms that affect seed set. It should be noted that we only examined seed number and seed weight of F1 progeny and might therefore have missed genetic incompatibilities that only become detectible in later generations (Dobzhansky 1937, Muller 1942, Fishman and Willis 2001). However, a follow-up experiment showed that seeds formed by F1 plants in a common garden were as viable as seeds formed by the parental plants (Gorman, unpublished data). Thus, combined with previous findings that SI and SC *A. lyrata* populations are not strongly reproductively isolated by pre-pollination mechanisms (Gorman et al. 2020a), we conclude that the selfing populations in North American *A. lyrata* show little to no detectable signs of incipient speciation.

Chapter 3: A shift towards the annual habit in selfing *Arabidopsis lyrata*

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Abstract

An annual life history is often associated with the ability to self-fertilize. However, it is unknown whether the evolution of selfing commonly precedes the evolution of annuality, or vice versa. Using a two-year common garden, we asked if the evolution of selfing in the normally perennial *Arabidopsis lyrata* was accompanied by a shift towards the annual habit. Despite their very recent divergence from obligately outcrossing populations, selfing plants exhibited a 39% decrease in over-winter survival after the first year compared to outcrossing plants. Our data ruled out the most obvious underlying mechanism: differences in reproductive investment in the first year did not explain differences in survival. We conclude that transitions to selfing in perennial *A. lyrata* may be accompanied by a shift towards annuality, but drivers of the process require further investigation.

Introduction

Mating system transitions from obligate outcrossing to predominant selfing are frequent evolutionary shifts in angiosperms (Barrett 2002). Established selfing species display a characteristic flower morphology known as the ‘selfing syndrome’ (Darwin 1876, Sicard and Lenhard 2011) and are typically annual (Stebbins 1970, Lloyd 1980, Barrett et al. 1996). However, it is unclear whether this association exists because the transition to selfing is more likely in annuals, or because selection favors shorter lifespans in perennials that have evolved selfing. Most of our understanding about the evolutionary association between selfing and the annual life history is based on analyses that treat these as independent traits or assume that an annual or perennial life history is fixed and then explore the conditions favoring selfing. Empirical studies that consider the alternative, that shifts to selfing can select for changes in lifespan, are conspicuously lacking. To test if the transition to selfing in a perennial species can lead to a shift to annuality, we used the intraspecific mating system variation in North American populations of the ancestrally self-incompatible and perennial *Arabidopsis lyrata*, which has evolved several selfing populations. Specifically, we determined if survivorship in a native-range common garden is consistent with a shift towards a more annual life history in plants from recently evolved perennial selfing populations.

Several hypotheses could explain the link between lifespan and mating system, and the order in which these traits evolve. Selection could favor the evolution of selfing in annuals, as annuals have only one opportunity to reproduce and would incur a lower risk of reproductive failure if selfing evolved (Stebbins 1950, 1957). Alternatively, selection could favor the evolution of the annual habit in initially perennial selfing populations. The evolution of self-fertilization may increase the optimal level of total reproductive effort thereby reducing survival and leading to an annual life history (Zhang 2000). In particular, selfing in perennials could select for increased resource allocation to reproductive tissues rather than to vegetative structures needed for perennial survival, favoring the evolution of the annual habit (Friedman and Rubin 2015). The high costs of selfing in perennials due to inbreeding depression and/or seed discounting might also be ameliorated by a shift to annuality (Morgan et al. 1997, Lloyd 2002, Lesaffre and Billiard 2019). Furthermore, in selfing lineages, mutations that reduce lifespan could accumulate due to neutral processes such as the buildup of drift load due to the lower effective population size associated with selfing (Heller and Smith 1978, Wright et al. 2013). Additionally, population bottlenecks before or during the formation of new selfing populations (Willi et al. 2013) may have fixed mutations that reduce lifespan. The roles of inbreeding depression and drift load in favoring the annual habit in perennial species that have transitioned to selfing can be evaluated by comparing crosses within and between populations that differ in their selfing rates. We are unaware of any experimental studies that have examined whether perennial species transition towards an annual habit after evolving to self.

Here, we used *Arabidopsis lyrata* to examine whether the evolution of selfing is followed by a transition towards the annual habit in newly diverged selfing lineages. This species is largely an obligate (self-incompatible) outcrosser but contains several self-compatible populations with high selfing rates (Foxe et al. 2010). These selfing populations likely originated from outcrossing ancestors <10,000 years ago, as they occur in previously glaciated habitat and lack a clear selfing syndrome (Carleial et al. 2017a). Additionally, genetic evidence indicates selfing in these populations originated multiple times (Appendix B) (Hoebe et al. 2009, Foxe et al. 2010). Studies both in the laboratory and/or under relatively mild environmental conditions have shown that both self-incompatible and selfing plants readily survive for multiple years (Willi 2013a, Buckley et al. 2019). Therefore, there is substantial evidence that all *A. lyrata* are ancestrally self-incompatible perennials and that novel selfing populations retain this perennial habit.

We monitored survival of progeny from crosses within and between selfing populations and crosses within and between outcrossing populations in a common garden within their native range over two years. Specifically, we asked: 1) whether plants from selfing populations had reduced over-winter survival compared to plants from outcrossing populations; and 2) whether between-population cross-progeny had higher survival than within-population cross-progeny (heterosis), and if this differed between selfing and outcrossing populations. Additionally, we asked 3) whether the selfed progeny of plants from selfing populations suffered from inbreeding depression. Furthermore, to test for a potential trade-off between survival and reproduction, we asked 4) whether plants that had a higher reproductive investment (i.e. flower and seed production) had lower survival.

Methods

Study system

Arabidopsis lyrata spp. *lyrata* (L.) is a perennial native to North American rock outcrops and sand dunes. Most populations are self-incompatible and highly outcrossing (multi-locus outcrossing rates t_m>0.8, Appendix B), with only a low frequency of self-compatible individuals (Mable et al. 2017). However, self-compatibility has become fixed in some populations now characterized by high selfing rates (Mable et al. 2005, Foxe et al. 2010) (t_m: 0.1-0.4; Appendix B).

Crossing designs

Our crossing designs are described in more detail elsewhere (Carleial et al. 2017b, Li et al. 2019, Gorman et al. 2019). In brief, crosses involved plants (raised from field-collected seeds) from 12 *A. lyrata* populations with known mating systems: six populations of self-incompatible plants displaying high outcrossing rates (hereafter SI populations) and six populations of self-compatible plants displaying low outcrossing rates (hereafter SC populations) (Foxe et al. 2010). These populations likely represent multiple transitions to selfing (Hoebe et al. 2009, Foxe et al. 2010). We performed two consecutive rounds of crosses, each with different sets of parental plants. First, using five to eight plants per population, we performed within-population crosses to generate the following cross types:

SI×SI$_{\text{within}}$, SC×SC$_{\text{within}}$, and SC×self. Second, for a further 6 plants per population, we performed within-population crosses (giving increased sample sizes for the SI×SI$_{\text{within}}$ and SC×SC$_{\text{within}}$ cross types) and between-population crosses (giving the two additional cross types SI×SI$_{\text{between}}$ and SC×SC$_{\text{between}}$ for testing heterotic effects, i.e., whether drift load affected survival). Crosses yielded 600 seed families in total.

Common garden experiment

To evaluate whether the evolution of selfing is accompanied by a shift to the annual habit, we set up a common garden experiment at Trent University in Peterborough, Ontario, Canada. This location is at an intermediate latitude within the geographic range of the source populations (Appendix B). From March 20-22, 2018, we sowed up to 50 seeds per seed family on peat-based substrate (one pot per family) in climate chambers (11-hour days, 21°C/18°C day/night cycles, and 95% humidity). We planned to transplant three seedlings from each seed family, but three seedlings were not always available due to insufficient germination of 279 families. When seedlings developed at least two true leaves (April 18-May 1), we transplanted one to three haphazardly chosen seedlings from each germinated seed family to individual pots, resulting in 915 experimental plants. On May 10, prior to flowering, plants were moved to the common garden and organized in a randomized block design with three replicates of three 3m×6m blocks. Plants from each seed family and cross type were evenly distributed among the blocks.

Winterization

On November 9, 2018, plants were winterized. To simulate natural winter conditions of snow cover that insulates meristems from deep-freezing temperatures, pots were packed closely together and then covered with a frost blanket and straw layer. A tarp was placed over the plants to hold the straw in place. On April 7, 2019, the plants were unpacked and returned to the original blocks.

Survival and trade-offs with reproductive investment

To test whether plants from selfing populations showed lower survival to the second year than plants from outcrossing populations, we scored overwinter survival immediately after unpacking in 2019. Plants were considered to have survived when they had green living tissue or turgid roots. To test for a trade-off between survival and reproductive investment, we used the previous year's flowering data. For each plant, we had recorded whether it flowered and, if so, estimated how many flowers it produced throughout the season (Gorman et al. 2019). Additionally, to test for a trade-off between survival and reproductive output (i.e., first year seed production), we estimated individuals' total seed production by multiplying fruit number and average seed number per fruit for each plant (Appendix F).

Statistics

All statistics were performed in R 3.6.1 (R Core Team 2019). To test whether plants from selfing populations had lower survival than plants from outcrossing populations, and whether there were trade-offs between reproduction and survival, we used mixed-effects binary logistic regression models implemented in lme4 (Bates et al. 2014). These models included *plant survivorship* (response variable), *cross type* (fixed effect), and *maternal population* and *paternal population* (random effects). To test whether survival depended on flowering in the first year, we included *flowering in 2018* (binary) as a fixed effect. For the subset of plants that flowered, we tested whether survival depended on *flowering intensity* (estimated from the sum of all flowers produced in 2018; standardized using the *scale* function in R) included as a fixed effect. Additionally, to test whether survival depended on seed production in 2018, we included *total seed number* (standardized using the *scale* function in R) as a fixed effect. To assess significance of the fixed terms, we re-fitted models using maximum likelihood (ML) and compared models with and without fixed terms using likelihood-ratio tests (LRTs (Zuur et al. 2013); Table 1). For cross type, we defined a contrast matrix for post-hoc comparisons with the *glht* function in the *multcomp* package (Table 2) (Hothorn et al. 2008). We calculated these contrasts for the model of survival including only *cross type* as a fixed effect because models that included additional fixed effects related to reproductive investment and their interactions with *cross type* were not significantly better at explaining survival (Table 3.1). Specifically, we made four comparisons to test whether: (C1) crosses between populations resulted in higher survival than crosses within populations (heterosis); (C2) C1 depended on mating system; (C3) survival of progeny from self-compatible plants depended on whether they were produced by selfing or outcrossing (inbreeding depression); (C4) crosses among plants from selfing populations (SC×SC crosses) had lower survival than crosses among plants from outcrossing populations (SI×SI crosses).

Table 3.1 Mixed model analysis of overwinter survival of selfing and outcrossing *Arabidopsis lyrata*. Significance of fixed effects was evaluated using model comparisons with likelihood-ratio tests.

All experimental plants (n= 915)				**Subset of plants that flowered (n= 549)**							
Fixed effects	df	LRT	Pr(Chi)	**Fixed effects**	df	LRT	Pr(Chi)	**Fixed effects**	df	LRT	Pr(Chi)
Flowered 2018 (FL)	1	0.648	0.421	**Flowering intensity 2018 (FL_INT)**	1	0.136	0.713	**Seed production 2018 (SP)**	1	0.455	0.500
Cross type (CT)	**4**	**21.307**	**<0.001**	**Cross type (CT)**	**4**	**15.118**	**0.004**	**Cross type (CT)**	**4**	**15.240**	**0.004**
FL:CT	4	1.193	0.879	**FL_INT:CT**	4	1.202	0.878	**SP:CT**	4	1.007	0.909
Random effects	df	SD explained ($df_{residual}$= 903)		**Random effects**	df	SD explained ($df_{residual}$= 537)		**Random effects**	df	SD explained ($df_{residual}$= 537)	
Maternal population	1	0.447		**Maternal population**	1	0.366		**Maternal population**	1	0.364	
Paternal population	1	0.300		**Paternal population**	1	0.258		**Paternal population**	1	0.248	

Results

Cross type had a significant effect on overwinter survival. However, survival was not affected by any of our measures of first year reproductive investment (Table 3.1). Plants resulting from between-population crosses had a significantly higher survival than plants from within-population crosses (significant effect of C1; Table 3.2; Figure 3.1). This effect did not significantly differ between SC and SI populations (no significant effect of C2; Table 3.2). Within SC populations, progeny produced by selfing had similar survival as progeny produced by outcrossing (no significant effect of C3; Table 3.2). Overall, plants from outcrossing populations (i.e., SI×SI crosses) had significantly higher overwinter survival than plants from selfing populations (i.e., SC×SC crosses) (45% versus 27%; significant effect of C4; Table 3.2, Figure 3.1).

Table 3.2 Post-hoc hypothesis testing for survival using a contrast matrix (*glht* function in the multcomp package (Hothorn et al. 2008)). Abbreviations: SE= standard error; z= Wald statistic, SI = self-incompatible, and SC = self-compatible. Significant effects are highlighted in **bold**.

Post-hoc comparison	**Estimate**	**SE**	**z**	**Pr(>|z|)**	**Contrast definitions**				
					Outcrossing mating system		Selfing mating system		
					SI×SI within	SI×SI between	SC×self	SC×SC within	SC×SC between
C1 – Between- vs. within- population crosses (heterosis)	**0.616**	**0.165**	**3.734**	**<0.001**	-1/2	1/2	0	-1/2	1/2
C2 – Interaction of mating system with C1	0.010	0.164	0.061	1.000	1/2	-1/2	0	-1/2	1/2
C3 – Difference between progeny produced by selfing and outcrossing (inbreeding depression)	0.146	0.348	0.418	0.988	0	0	-1	1	0
C4 – Difference between mating system (SI vs. SC)	**0.913**	**0.349**	**2.617**	**0.035**	1/2	1/2	-1/3	-1/3	-1/3

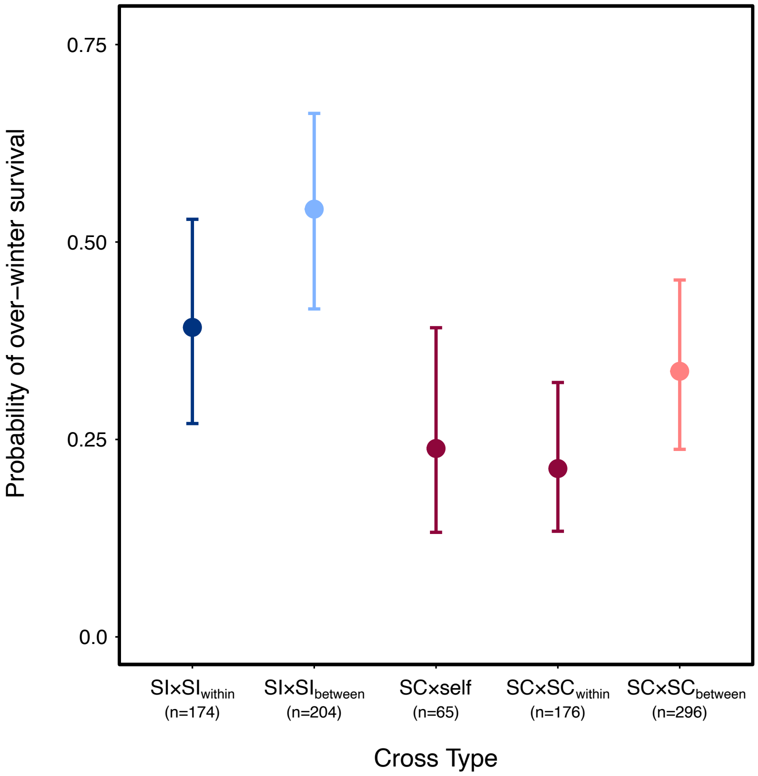

Figure 3.1 Effect plot of overwinter survival by cross type, n = 915. Predictions from the binary logistic regression model were obtained with the *ggpredict* function in the *ggeffects* R package (Lüdecke 2018). Colored points are predicted values and error bars represent 95% confidence intervals. The between-population cross types (SI×SI$_{\text{between}}$ and SC×SC$_{\text{between}}$) had significantly higher survival than their respective within-population cross types ($p < 0.001$). The outcrossing mating system (represented by the cross types SI×SI$_{\text{within}}$ and SI×SI$_{\text{between}}$) had significantly higher survival than the selfing mating system (represented by cross types SC×self, SC×SC$_{\text{within}}$, and SC×SC$_{\text{between}}$) ($p = 0.035$).

Discussion

Our key finding is that plants from selfing populations of *A. lyrata* had a 39% decreased over-winter survival rate as compared to plants from outcrossing populations, which was not explained by differences in first-year reproductive investment. Below we discuss the potential neutral and non-neutral drivers of this shift towards an annual life cycle.

Neutral processes could be influencing the genetic background of selfers and facilitating selection towards a more annual habit. Selfing populations often experience genetic degradation through processes such as inbreeding depression and the accumulation of drift load. Although inbreeding depression has been identified for *A. lyrata* vegetative biomass (Carleial et al. 2017b, Li et al. 2019), SC×self and SC×SC$_{\text{within}}$ crosses did not differ in survival. Therefore, our results are not consistent with inbreeding depression affecting over-winter survival. It is also likely that *A. lyrata* populations have gone through repeated cycles of colonization and extinction (Mable and Adam 2007, Wright et al. 2013) and thus locally contain an excess of deleterious alleles (Peischl and Excoffier 2015). In line with this, we found that between-population crosses resulted in significant heterosis in survival. This heterosis was of similar magnitude for SI and SC populations, and thus did not restore SC plant survival to the levels observed in crosses between SI plants. This suggests that SI and SC populations harbor similar levels of drift load. While our results agree with previous findings that drift processes are important in *A. lyrata* [20], drift alone seems unlikely to explain the observed differences in overwinter survival between selfing and outcrossing populations.

Among the potential non-neutral drivers, trade-offs between reproduction and meristems needed for overwinter survival are often presumed to be a critical factor in driving transitions to annuality (Friedman and Rubin 2015). However, our data are not consistent with reproductive trade-offs explaining a shift towards annuality in *A. lyrata* selfing populations. Plants that flowered in the first year were as likely to survive overwintering as plants that postponed flowering. Also, survival did not depend on flowering intensity or seed production. Yet, there could be a genetic correlation between floral traits and lifespan, so that selection for increased pollen:ovule ratios (Carleial et al. 2017a) indirectly selected for reduced lifespan. However, this would not explain why previous work in non-native, more-temperate environments found no differences in overwinter survival between selfing and outcrossing *A. lyrata* (Willi 2013a, Buckley et al. 2019). Furthermore, greenhouse experiments with common agricultural pests have shown that SC plants are not more susceptible to infection or herbivory than SI plants (Hoebe et al. 2011, Joschinski et al. 2015). Therefore, we suggest that environmental conditions specific to our experiment led to the reduced lifespan in selfers. These conditions could include biotic factors such as native herbivores or pathogens, and/or abiotic factors such as the extreme fluctuations in temperature that characterize the continental climate of southeastern Canada. How environmental factors interact with the genetic consequences of selfing to cause the reduced overwinter survival of selfers remains to be investigated.

General Discussion

The research presented aimed to determine whether recently evolved self-compatible populations have diverged from self-incompatible populations in traits that confer reproductive isolation or differences in life histories in the North American plant species *Arabidopsis lyrata*. In Chapter 1, I set up a common garden experiment to test whether self-compatible and self-incompatible populations are reproductively isolated from each other via prezygotic pre-pollination mechanisms (i.e., differences in phenology and/or pollinator visitation). In Chapter 2, I used seeds produced from controlled crosses within and between self-compatible and self-incompatible populations to determine whether self-compatible and self-incompatible populations are reproductively isolated from each other by pre- or postzygotic post-pollination barriers (i.e., differences in seed number and seed weight). In Chapter 3, I monitored the survival of plants produced by controlled crosses within and between self-compatible and self-incompatible populations over two years in a common garden environment to test whether self-compatible and self-incompatible plants have diverged in life history traits (i.e., whether self-compatible plants have developed an annual habit). I found that while self-compatible and self-incompatible populations are not strongly reproductively isolated by any of the examined pre- or post-pollination mechanisms, they are divergent in life history traits. This line of research has important implications for the field of mating system evolution, as I found that traits that are often thought to confer reproductive isolation in the early stages of a shift to self-compatibility may not always be the first to evolve after a shift to self-compatibility.

Limited evidence for prezygotic pre-pollination reproductive isolation

In this common garden study, I found little evidence that self-compatible and self-incompatible *A. lyrata* populations are reproductively isolated via prezygotic pre-pollination mechanisms. Phenology mostly overlapped between plants from self-compatible and self-incompatible populations. Furthermore, neither solitary bees nor hoverflies displayed any preferences related to mating system. However, both types of pollinators strongly preferred to visit flowers on the same plant (~50% of all pollinator movements) rather than exploring nearby plants. This foraging behavior created a large opportunity for geitonogamous self-fertilization in my experiment, which could isolate self-compatible plants to an extent. There were also equal opportunities for pollen-transfer within- and among mating systems, as revealed by models of pollen-transfer probabilities, which integrated differences in phenology (timing and intensity of flowering). Overall, my results suggest that prezygotic pre-pollination mechanisms do not strongly reproductively isolate plants from self-compatible and self-incompatible populations of *A. lyrata*.

No evidence for pre- or postzygotic post-pollination reproductive isolation

I found that plants from self-compatible and self-incompatible populations of *A. lyrata* are neither reproductively isolated via post-pollination prezygotic mechanisms, nor reproductively isolated by early acting postzygotic mechanisms. In contrast, both for the set of self-incompatible and the set of self-compatible populations, I found that seed number and seed weight increased when plants from different populations were crossed (i.e., there was often heterosis for these traits). Heterosis often accelerates gene flow between populations (Ingvarsson & Whitlock, 2000; Bierne et al., 2002). Thus, I essentially found the opposite of what I would expect if self-compatible and self-incompatible populations were reproductively isolated by post-pollination mechanisms. Overall these results suggest that despite the differences in mating system, newly evolved self-compatible populations in *A. lyrata* show little to no evidence of post-pollination reproductive isolation from self-incompatible populations.

Evidence for life-history divergence

In this common garden experiment, I found that despite their recent divergence from self-incompatible populations, plants from self-compatible populations exhibited a 39%

decrease in over-winter survival after the first year compared to plants from self-incompatible populations. Furthermore, the difference in survival was not explained by reproductive investment in the first year (i.e., flower or seed production), inbreeding depression, or unequal consequences of genetic drift. Instead, my results suggest that environmental conditions specific to my common garden positioned in the native range of *A. lyrata*, interacted with the genetic consequences of selfing to cause the reduced lifespan of self-compatible plants. Overall, these results suggest that the transition to self-compatibility in perennial *A. lyrata* may be accompanied by a shift towards the annual habit. However, the drivers of the process in this species require further investigation.

Speciation by selfing

Together, my results provide no evidence of speciation by selfing in North American *Arabidopsis lyrata*. This is in contrast to a growing body of evidence that the transition from self-incompatibility to self-compatibility is associated with rapid speciation of highly selfing lineages (Foxe et al. 2009, Guo et al. 2009, Briscoe Runquist et al. 2014). The transition from self-incompatibility to self-compatibility often has profound influences on gene flow and mating patterns between self-compatible and self-incompatible lineages, that should theoretically lead to the evolution of reproductive barriers and ultimately speciation. For instance, the shift to self-compatibility is associated with a selfing syndrome, or specific morphological and functional changes that promote self-fertilization, (Darwin 1876, Sicard and Lenhard 2011), and shifts in phenology and life history (Barrett et al. 1996, Goodwillie et al. 2010). Together, these changes can restrict gene flow (Martin and Willis 2007, Fishman et al. 2015) between self-compatible and self-incompatible lineages and lead to reproductive isolation. However, despite the high selfing rates in natural populations of self-compatible *A. lyrata*, I found no evidence for pre- or post-pollination barriers to reproduction between plants from self-compatible populations and plants from self-incompatible populations.

There are several potential explanations for the lack of current reproductive barriers between self-compatible and self-incompatible populations of *A. lyrata*. Unlike other examples of a transition to self-compatibility being associated with rapid speciation (Foxe et al. 2009, Guo et al. 2009, Briscoe Runquist et al. 2014), *A. lyrata* has not developed the stereotypical selfing syndrome (Carleial et al. 2017a). The transition to self-compatibility in *A. lyrata* is also more recent (<10,000 years) (Foxe et al. 2010) than these other examples (*Capsella,* <20,000 years, Foxe *et al.* 2009; *Mimulus* <65,000 years, Briscoe Runquist *et al.* 2014). Thus, it seems possible that there has not been enough time for the evolution of reproductive barriers between self-compatible and self-incompatible *A. lyrata* populations.

Another possibility is that the traits that are thought to most strongly confer reproductive isolation in the initial stages of a shift to self-compatibility (e.g., phenology, attractiveness to pollinators, etc.) are not the first traits to diverge after this intraspecific transition to self-compatibility. Perhaps selection is driving populations apart in other ways, such as divergent life histories. In fact, I found that self-compatible plants have shorter lifespans than self-incompatible plants, and therefore that the evolution of self-compatibility in *A. lyrata* has likely been accompanied by a shift towards the annual habit. If self-compatible and self-incompatible populations continue to diverge in lifespan and potentially other aspects of life history (e.g., differences in phenology become more pronounced), reproductive barriers could accumulate and/or become stronger. Future studies should continue to examine the evolution of reproductive barriers between self-compatible and self-incompatible *A. lyrata* as this system provides us with a unique perspective into the earliest dynamics of a transition to self-compatibility.

Potential consequences of secondary contact

I found that plants resulting from between mating system crosses had intermediate phenological traits and seed numbers compared to plants resulting from within-population crosses. I also found that pollinator visitation and seed number were similar in plants resulting from between mating system crosses and plants resulting from within-population crosses. This suggests that self-compatible and self-incompatible populations would likely merge in a scenario of secondary contact of previously geographically isolated populations, because between population hybrids would likely function as a bridge to gene flow between the parental populations (Rhymer and Simberloff 1996). Therefore, *A. lyrata* would likely maintain a mixed-breeding system (i.e., populations with both self-incompatible plants and self-compatible plants with variable selfing rates) in the initial stages of secondary contact. Whether such admixed populations would evolve to become predominantly self-compatible or self-incompatible on longer timescales remains to be tested.

The dynamics of secondary contact could also depend heavily on the environment in which the contact takes place. For example, the ecological conditions of my common garden would likely favor self-incompatible plants, as plant densities were high (10 plants per m^2), and pollinators were abundant. In other words, there was ample opportunity for cross-pollination. Additionally, I found that self-compatible plants have shorter lifespans than self-incompatible plants. Together, this suggests that if secondary contact occurred in an environment with abundant conspecifics and pollinators, self-incompatible plants would likely have an advantage over self-compatible plants. However, in natural populations, the availability of conspecifics and/or pollinators could be highly variable. For instance, there is evidence that plant densities as compared to the densities examined in the common garden are much lower in natural populations (~7.2 plants per m^2 (Mable and Adam 2007)), which could impact pollinator visitation rates (Grindeland et al. 2005, Bernhardt et al. 2008). If secondary contact took place in an environment with low plant density and a lack of pollinators, self-compatible plants would likely have an advantage over self-incompatible plants (Baker 1955). This prediction is further supported by my finding that pollinator behavior provides large opportunities for geitonogamous self-fertilization in *A. lyrata*. In other words, when pollinators do visit *A. lyrata*, they prefer to visit flowers on the same plant versus exploring other plants nearby which should aid self-fertilization.

It is also important to consider how the evolutionary consequences of selfing would influence the mating system of *A. lyrata* in a scenario of secondary contact. For instance, inbreeding depression for vegetative biomass is relatively weak in *A. lyrata* (Carleial et al. 2017b, Li et al. 2019). Moreover, I found no evidence that inbreeding depression influences lifespan in *A. lyrata*. Therefore, self-compatible plants may be initially favored in a scenario of secondary contact due to the inherent transmission advantage of selfing (Fisher 1941). However, selfing might be disadvantageous on longer timescales, as deleterious mutations may accumulate in self-compatible populations due to genetic drift (Willi 2013a). In line with this, I found that drift processes influence survival in both self-compatible and self-incompatible populations. Over time, these negative consequences of drift may accumulate faster in self-compatible populations due to their lower effective population size as compared to self-incompatible populations (Heller and Smith 1978, Wright et al. 2013). An essential first step to elucidating the evolutionary fate of self-compatible and self-incompatible *A. lyrata* populations under a scenario of secondary contact would be to monitor the performance and mating system of experimentally admixed populations in a common environment over multiple years.

Conclusions

I found no strong evidence of pre- or post-pollination reproductive isolation between self-compatible and self-incompatible *A. lyrata* populations. However, I did find unambiguous evidence for divergence in life histories. This work has important implications for the field of mating system evolution, as I found that traits that are thought to most strongly confer reproductive isolation in the initial stages of a shift to self-compatibility may not always be the first to evolve after a shift to self-compatibility. Instead, my results suggest that selection is driving self-compatible and self-incompatible populations apart in other ways, such as divergence in life histories. My work indicates that future studies should aim to provide a more comprehensive understanding of the early dynamics of the evolutionary transition from self-incompatibility to self-compatibility.

Future Directions

This work opens the door to several new lines of research. For instance, future studies could investigate whether prezygotic pre-pollination mechanisms of reproductive isolation are stronger in natural populations than in my common garden. It would also be interesting to examine whether pollinator behavior promotes opportunities for geitonogamous self-fertilization in natural populations. It would also be appealing to determine outcrossing rates of both self-compatible and self-incompatible plants when they are grown together in a common environment. This would determine whether self-compatible plants maintain high selfing rates after secondary contact, and potentially shed light on the processes that drive the transition to self-compatibility in *A. lyrata.* Furthermore, future studies should monitor the performance and mating system of experimentally admixed populations in a common environment over multiple years. These data would inform our predictions on the likely evolutionary fate of self-compatibility after secondary contact with self-incompatible populations. Importantly, my study provided the first empirical evidence of a shift towards the annual habit after the evolution of self-compatibility in an ancestrally perennial and self-incompatible species. Future studies should investigate whether this has happened in other species with a recent shift to self-compatibility. It would also be important to understand how the environment interacts with the genetic consequences of selfing to reduce the lifespan of self-compatible *A. lyrata*. Finally, synthetic series of studies that explore the dynamics of self-compatibility, reproductive isolation, and life history will continue to contribute to a more comprehensive understanding of mating system evolution.

References

Baack, E., M. C. Melo, L. H. Rieseberg, and D. Ortiz-Barrientos. 2015. The origins of reproductive isolation in plants. New Phytologist 207:968–984.

Baker, H. G. 1955. Self-compatibility and establishment after “long-distance” dispersal. Evolution 9:347.

Barrett, S. C. H. 2002. The evolution of plant sexual diversity. Nature Reviews Genetics 3:274–284.

Barrett, S. C. H., and L. D. Harder. 2017. The ecology of mating and its evolutionary consequences in seed plants. Annual Review of Ecology, Evolution, and Systematics 48:135–157.

Barrett, S. C. H., L. D. Harder, and A. G. Worley. 1996. The comparative biology of pollination and mating in flowering plants. Philosophical Transactions of the Royal Society B: Biological Sciences 351:1271–1280.

Barrett, S. C. H., A. J. Richards, M. W. Bayliss, D. Charlesworth, and R. J. Abbott. 2003. Mating strategies in flowering plants: The outcrossing-selfing paradigm and beyond. Pages 991–1004 *in* H. G. Dickinson, S. J. Hiscock, and P. R. Crane, editors. Philosophical Transactions of the Royal Society B: Biological Sciences.

Bates, D., M. Mächler, B. M. Bolker, and S. C. Walker. 2014. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software 67.

Bateson, W. 1909. Heredity and Variation in Modern Lights. Cambridge University Press.

Bernhardt, C. E., R. J. Mitchell, and H. J. Michaelsy. 2008. Effects of population size and density on pollinator visitation, pollinator behavior, and pollen tube abundance in *Lupinus perennis*. International Journal of Plant Sciences 169:944–953.

Bierne, N., T. Lenormand, F. Bonhomme, and P. David. 2002. Deleterious mutations in a hybrid zone: Can mutational load decrease the barrier to gene flow? Cambridge University Press.

Brandvain, Y., and D. Haig. 2005. Divergent mating systems and parental conflict as a barrier to hybridization in flowering plants. American Naturalist 166:330–338.

Briscoe Runquist, R. D., E. Chu, J. L. Iverson, J. C. Kopp, and D. A. Moeller. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. Evolution 68:2885–2900.

Brunet, J., and D. Charlesworth. 2006. Floral sex allocation in sequentially blooming plants. Evolution 49:70.

Brys, R., A. Vanden Broeck, J. Mergeay, and H. Jacquemyn. 2014. The contribution of mating system variation to reproductive isolation in two closely related *Centaurium* species (Gentianaceae) with a generalized flower morphology. Evolution 68:1281–1293.

Buckley, J., R. Daly, C. A. Cobbold, K. Burgess, and B. K. Mable. 2019. Changing environments and genetic variation: natural variation in inbreeding does not compromise short-term physiological responses. Proceedings of the Royal Society B: Biological Sciences 286:20192109.

Busch, J. W., and L. F. Delph. 2012. The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. Narnia.

Carleial, S., M. van Kleunen, and M. Stift. 2017a. 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.

Carleial, S., M. van Kleunen, and M. Stift. 2017b. Relatively weak inbreeding depression in selfing but also in outcrossing populations of North American *Arabidopsis lyrata*. Journal of Evolutionary Biology 30:1994–2004.

Caruso, C. M., A. L. Case, and M. F. Bailey. 2012. The evolutionary ecology of cytonuclear interactions in angiosperms. Elsevier.

Charlesworth, D. 2006. Evolution of Plant Breeding Systems. Cell Press.

Charlesworth, D., and J. H. Willis. 2009. The genetics of inbreeding depression. Nature Reviews Genetics 10:783–796.

Coyne, J., and H. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Darwin, C. 1876. The effects of cross and self fertilisation in the vegetable kingdom / by Charles Darwin. Page The effects of cross and self fertilisation in the vegetable kingdom / by Charles Darwin. J. Murray, London :

Dobzhansky, T. 1937. Genetics and the origin of species. Columbia University Press, New York.

Duncan, T. M., and M. D. Rausher. 2013. Evolution of the selfing syndrome in *Ipomoea*. Frontiers in Plant Science 4:301.

Fausto, J. A., V. M. Eckhart, and M. A. Geber. 2001. Reproductive assurance and the evolutionary ecology of self-pollination in *Clarkia xantiana* (Onagraceae). American Journal of Botany 88:1794–1800.

Fisher, R. A. 1941. Average excess and average effect of a gene substitution. Annals of Eugenics 11:53–63.

Fishman, L., P. M. Beardsley, A. Stathos, C. F. Williams, and J. P. Hill. 2015. The genetic architecture of traits associated with the evolution of self-pollination in *Mimulus*. New Phytologist 205:907–917.

Fishman, L., and J. H. Willis. 2001. Evidence for Dobzhansky-Muller incompatibilites contributing to the sterility of hybrids between *Mimulus guttatus* and *M. nasutus*. Evolution 55:1932–1942.

Foxe, J. P., T. Slotte, E. A. Stahl, B. Neuffer, H. Hurka, and S. I. Wright. 2009. Recent speciation associated with the evolution of selfing in *Capsella*. Proceedings of the National Academy of Sciences 106:5241–5245.

Foxe, J. P., M. Stift, A. Tedder, A. Haudry, S. I. Wright, and B. K. Mable. 2010. Reconstructing origins of loss of self-incompatibility and selfing in North American *Arabidopsis lyrata*: A population genetic context. Evolution 64:3495–3510.

Friedman, J., and M. J. Rubin. 2015. All in good time: Understanding annual and perennial strategies in plants. John Wiley & Sons, Ltd.

Gelman, A., and J. Hill. 2006. Data Analysis Using Regression and Multilevel/Hierarchical Models. Page Data Analysis Using Regression and Multilevel/Hierarchical Models. Cambridge University Press.

Gervasi, D. D. L. L., and F. P. Schiestl. 2017. Real-time divergent evolution in plants driven by pollinators. Nature Communications 8:14691.

Goodwillie, C., S. Kalisz, and C. G. Eckert. 2005. The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. Annual Review of Ecology, Evolution, and Systematics 36:47–79.

Goodwillie, C., R. D. Sargent, C. G. Eckert, E. Elle, M. A. Geber, M. O. Johnston, S. Kalisz, D. A. Moeller, R. H. Ree, M. Vallejo-Marin, and A. A. Winn. 2010. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. New Phytologist 185:311–321.

Gorman, C., L. Bond, M. van Kleunen, M. Dorken, and M. Stift. 2019. Limited phenological and pollinator-mediated isolation among selfing and outcrossing *Arabidopsis lyrata* populations. bioRxiv:2019.12.17.879361.

Gorman, C. E., L. Bond, M. van Kleunen, M. E. Dorken, and M. Stift. 2020a. Limited phenological and pollinator-mediated isolation among selfing and outcrossing *Arabidopsis lyrata* populations. Proceedings of the Royal Society B: Biological Sciences 287:20202323.

Gorman, C. E., C. Steinecke, M. van Kleunen, M. E. Dorken, and M. Stift. 2020b. A shift towards the annual habit in selfing Arabidopsis lyrata. Biology Letters 16:20200402.

Grindeland, J. M., N. Sletvold, and R. A. Ims. 2005. Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. Functional Ecology 19:383–390.

Guo, Y. L., J. S. Bechsgaard, T. Slotte, B. Neuffer, M. Lascoux, D. Weigel, and M. H. Schierup. 2009. Recent speciation of *Capsella rubella* from *Capsella grandiflora*, associated with loss of self-incompatibility and an extreme bottleneck. Proceedings of the National Academy of Sciences of the United States of America 106:5246–5251.

Harder, L. D., and S. C. H. Barrett. 1995. Mating cost of large floral displays in hermaphrodite plants. Nature 373:512–515.

Harder, L. D., and S. C. H. Barrett. 1996. Pollen dispersal and mating patterns in animal-pollinated plants. Pages 140–190 Floral Biology. Springer US, Boston, MA.

Harder, L. D., and W. G. Wilson. 1998. A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. American Naturalist 152:684–695.

Heller, R., and J. M. Smith. 1978. Does Muller's ratchet work with selfing? Genetical Research 32:289–293.

Hesse, E., and J. R. Pannell. 2011. Density-dependent pollen limitation and reproductive assurance in a wind-pollinated herb with contrasting sexual systems. Journal of Ecology 99:1531–1539.

Hoebe, P. N., M. Stift, E. B. Holub, and B. K. Mable. 2011. The effect of mating system on growth of *Arabidopsis lyrata* in response to inoculation with the biotrophic parasite *Albugo candida*. Journal of Evolutionary Biology 24:391–401.

Hoebe, P. N., M. Stift, A. Tedder, and B. K. Mable. 2009. Multiple losses of self-incompatibility in North-American *Arabidopsis lyrata*?: Phylogeographic context and population genetic consequences. Molecular Ecology 18:4924–4939.

Holsinger, K. E., M. W. Feldman, and F. B. Christiansen. 1984. The evolution of self-fertilization in plants: A population genetic model. The American Naturalist 124:446–453.

Hothorn, T., F. Bretz, and P. Westfall. 2008, June. Simultaneous inference in general parametric models.

Ingvarsson, P. K., and M. C. Whitlock. 2000. Heterosis increases the effective migration rate. Proceedings of the Royal Society B: Biological Sciences 267:1321–1326.

Jain, S. K. 1976. The Evolution of Inbreeding in Plants. Annual Review of Ecology and Systematics 7:469–495.

Joschinski, J., M. van Kleunen, and M. Stift. 2015. Costs associated with the evolution of selfing in North American populations of *Arabidopsis lyrata*? Evolutionary Ecology 29:749–764.

Karron, J. D., K. G. Holmquist, R. J. Flanagan, and R. J. Mitchell. 2009. Pollinator visitation patterns strongly influence among-flower variation in selfing rate. Annals of Botany 103:1379–1383.

Korner-Nievergelt, F., T. Roth, S. von Felten, J. Guélat, B. Almasi, and P. Korner-Nievergelt. 2015. Bayesian Data Analysis in Ecology Using Linear Models with R, BUGS, and Stan. Elsevier Inc.

Lesaffre, T., and S. Billiard. 2019. The joint evolution of lifespan and self-fertilization. Journal of Evolutionary Biology:jeb.13543.

Levin, D. A. 1971. The origin of reproductive isolating mechanisms in flowering plants. Taxon 20:91–113.

Li, Y., M. van Kleunen, and M. Stift. 2019. Sibling competition does not magnify inbreeding depression in North American *Arabidopsis lyrata*. Heredity 123:723–732.

Lloyd, D. G. 1980. Demographic factors and mating patterns in angiosperms, In Demography and Evolution in Plant Populations, O. T. Solbrig, ed. Page Demography and Evolution in plant populations. Blackwell.

Lloyd, D. G. 2002. Self- and cross-fertilization in plants. II. The Selection of Self- Fertilization. International Journal of Plant Sciences 153:370–380.

Lloyd, D. G., and D. J. Schoen. 1992. Self- and cross-fertilization in plants. I. Functional dimensions. International Journal of Plant Sciences 153:358–369.

Lowe, A. J., and R. J. Abbott. 2004. Reproductive isolation of a new hybrid species, *Senecio eboracensis* Abbott & Lowe (Asteraceae). Heredity 92:386–395.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu, and J. H. Willis. 2008a. Review. The strength and genetic basis of reproductive isolating barriers in flowering plants.

Lowry, D. B., R. C. Rockwood, and J. H. Willis. 2008b. Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. Evolution 62:2196–2214.

Lüdecke, D. 2018. ggeffects: Tidy data frames of marginal effects from regression models. Journal of Open Source Software 3:772.

Mable, B. K., and A. Adam. 2007. Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. Molecular Ecology 16:3565–3580.

Mable, B. K., J. Hagmann, S.-T. T. Kim, A. Adam, E. Kilbride, D. Weigel, and M. Stift. 2017. What causes mating system shifts in plants? *Arabidopsis lyrata* as a case study. Heredity 118:52–63.

Mable, B. K., A. V. Robertson, S. Dart, C. Di Berardo, and L. Witham. 2005. Breakdown of self-incompatibility in the perennial *Arabidopsis lyrata* (Brassicaceae) and its genetic consequences. Evolution 59:1437–1448.

Martin, N. H., and J. H. Willis. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. Evolution 61:68–82.

Mitchell, R. J., R. E. Irwin, R. J. Flanagan, and J. D. Karron. 2009. Ecology and evolution of plant-pollinator interactions. Narnia.

Moore, D. M., and H. Lewis. 1965. The evolution of self-pollination in *Clarkia xantiana*. Evolution 19:104.

Morgan, M. T., D. J. Schoen, and T. M. Bataillon. 1997. The evolution of self-fertilization in perennials. American Naturalist 150:618–638.

Muller, H. 1942. Isolating mechanisms, evolution, and temperature. Biol. Symp. 6:71–125.

Nagylaki, T. 1976. A model for the evolution of self-fertilization and vegetative reproduction. Journal of Theoretical Biology 58:55–58.

Peischl, S., and L. Excoffier. 2015. Expansion load: Recessive mutations and the role of standing genetic variation. Molecular Ecology 24:2084–2094.

Porcher, E., and R. Lande. 2005. The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. Journal of Evolutionary Biology 18:497–508.

R Core Team. 2019. R: A language and environment for statistical computing. Vienna, Austria.

Ramsey, J., H. D. Bradshaw, and D. W. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534.

Rhymer, J. M., and D. Simberloff. 1996. Extinction by hybridization and introgression. Annual Review of Ecology and Systematics 27:83–109.

Rieseberg, L. H., and J. H. Willis. 2007. Plant Speciation 10.1126/science.1137729. Science 317:910–914.

Ritland, C., and K. Ritland. 1989. Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). American Journal of Botany 76:1731–1739.

Sicard, A., and M. Lenhard. 2011. The selfing syndrome: A model for studying the genetic and evolutionary basis of morphological adaptation in plants. Annals of Botany 107:1433–1443.

Stebbins, G. L. 1950. Variation and Evolution in Plants. Page Variation and Evolution in Plants. Oxford University Press.

Stebbins, G. L. 1957. Self Fertilization and Population Variability in the Higher Plants. The American Naturalist 91:337–354.

Stebbins, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: Pollination mechanisms. Annual Review of Ecology and Systematics 1:307–326.

Tedder, A., S. Carleial, M. Gołebiewska, C. Kappel, K. K. Shimizu, and M. S. Stift. 2015. Evolution of the selfing syndrome in *Arabis alpina* (Brassicaceae). PLoS ONE

10:e0126618.

Therneau, T. M., and P. M. Grambsch. 2000. Modeling survival data : extending the Cox model. Springer.

Tiffin, P., M. S. Olson, and L. C. Moyle. 2001. Asymmetrical crossing barriers in angiosperms. Proceedings of the Royal Society B: Biological Sciences 268:861–867.

Turelli, M., and L. C. Moyle. 2007. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics 176:1059–1088.

Venables, W. N., and B. D. Ripley. 2002. Modern Applied Statistics with S.

Wendt, T., M. B. F. Canela, D. E. Klein, and R. I. Rios. 2002. Selfing facilitates reproductive isolation among three sympatric species of *Pitcairnia* (Bromeliaceae). Plant Systematics and Evolution 232:201–212.

Widmer, A., C. Lexer, and S. Cozzolino. 2009. Evolution of reproductive isolation in plants. Heredity 102:31–38.

Willi, Y. 2013a. Mutational meltdown in selfing *Arabidopsis lyrata*. Evolution 67:806–815.

Willi, Y. 2013b. The battle of the sexes over seed size: Support for both kinship genomic imprinting and interlocus contest evolution. The American Naturalist 181:787–798.

Willi, Y., P. Griffin, and J. Van Buskirk. 2013. Drift load in populations of small size and low density. Heredity 110:296–302.

Willi, Y., and K. Maattanen. 2011. The relative importance of factors determining genetic drift: Mating system, spatial genetic structure, habitat and census size in *Arabidopsis lyrata*. New Phytologist 189:1200–1209.

Wright, S. I., S. Kalisz, and T. Slotte. 2013. Evolutionary consequences of self-fertilization in plants. Proceedings of the Royal Society B: Biological Sciences 280:20130133–20130133.

Wyatt, R. 1984. The evolution of self-pollination in granite outcrop species of *Arenaria* (Caryophyllaceae). I. Morphological Correlates. Evolution 38:804.

Yost, J. M., and K. M. Kay. 2009. The evolution of postpollination reproductive isolation in *Costus*. Springer.

Zhang, D. Y. 2000. Resource allocation and the evolution of self-fertilization in plants. American Naturalist 155:187–199.

Zuur, A. F., J. M. Hilbe, and E. N. Ieno. 2013. A Beginner's Guide to GLM and GLMM with R. Page Beginner's Guide Series.

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Author Contributions

Chapter 1

M.S. conceived the project. C.G., M.D., M.v.K. and M.S. designed the experiment. C.G. and L.B. collected the data. C.G. and M.D. analyzed the data with input from M.v.K. and M.S., C.G. and M.S. wrote the paper with input from all authors.

Chapter 2

M.S. conceived the project. C.G., Y.L., M.D., and M.S. designed the experiment. C.G. and Y.L. collected the data. C.G. and M.S. analyzed the data with input from M.D. C.G. wrote the paper with input from all authors.

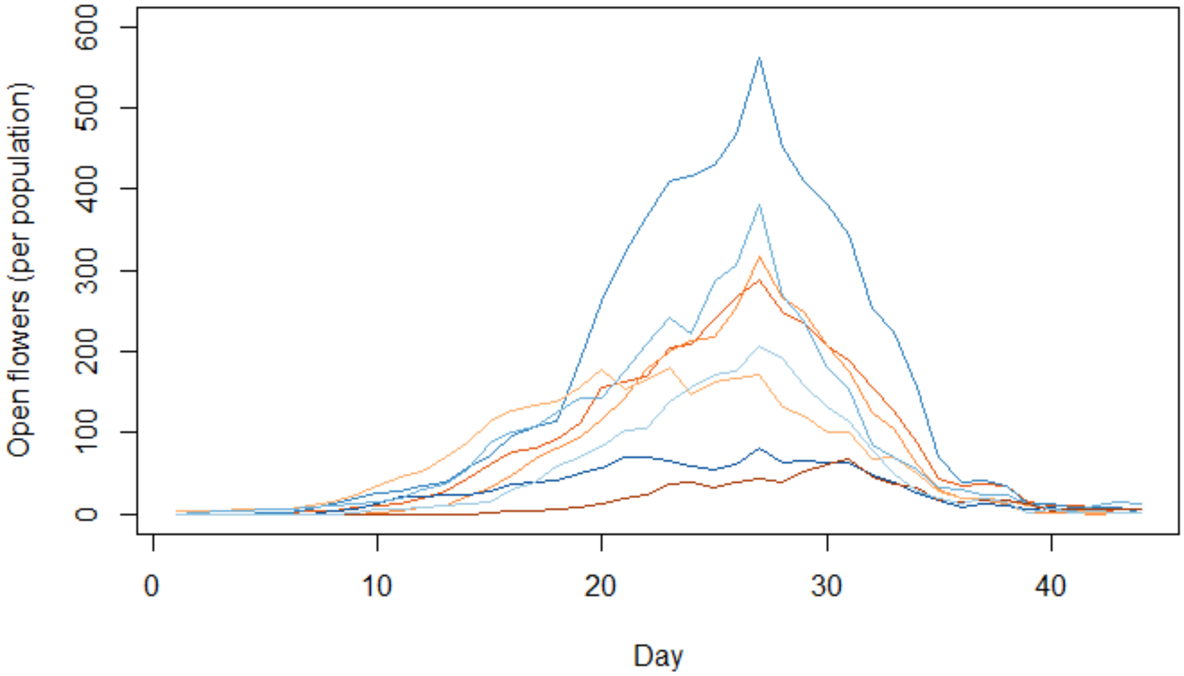
Chapter 3

C.G. and M.S. conceived the study. C.G., M.v.K., M.E.D. and M.S. designed the experiment. C.G. and C.S. collected the data. C.G. and M.S. analyzed the data with input from M.v.K. and M.E.D. C.G. and M.S. wrote the paper with input from all authors.

Appendices

Appendix A: Figure of the number of open flowers (per population) per day over the total flowering period

The number of open flowers (per population) per day over the total flowering period. Blue lines represent selfing populations and orange lines represent outcrossing populations. Only populations for which we had more than 10 plants that flowered are plotted.



Appendix B: Table of background information for populations used in this study

Background information for populations used in this study including outcrossing rates (t_m), frequencies of self-incompatible (SI), self-compatible (SC), and partially compatible (PC) individuals and population genetic cluster (Foxe et al. 2010). The latter was based on a Bayesian clustering analysis of neutral microsatellite variation in 24 populations (Foxe et al. 2010).

Population	Location, State/Province, Country	Population Coordinates		t_m	SI	SC	PC	Cluster
"SC" populations (populations with low outcrossing rates and relatively high frequency of self-compatible individuals)								
PTP	Point Pelee National Park, Ontario, Canada	N 41˚55'40"	W 82˚30'51"	0.09	0	1	0	B
LPT	Long Point Provincial Park, Ontario, Canada	N 42˚34'47"	W 80˚23'15"	0.13	0	1	0	B
TC	Tobermory Cliffs, Bruce Peninsula National Park, Ontario, Canada	N 45˚14'30"	W 81˚31'03"	0.18	0.13	0.88	0	A
RON	Rondeau Provincial Park, Ontario, Canada	N 42˚15'41"	W 81˚50'47"	0.28	0	1	0	B
KTT	Kitty Todd State Nature Preserve, Ohio, USA	N 41˚37'14"	W 83˚47'15"	0.31	0	1	0	C
TSSA	Tobermory Singing Sands Alvar, BPNP, Ontario, Canada	N 45˚11'27"	W 81˚35'26"	0.41	0.38	0.5	0.13	A
"SI" populations (populations with high outcrossing rates and relatively low frequency of self-compatible individuals)								
MAN	Manitoulin Island, Ontario, Canada	N 47˚39'54"	W 82˚15'52"	0.83	0.88	0.13	0	A
PIN	Pinery Provincial Park, Ontario, Canada	N 43˚16'08"	W 81˚49'53"	0.84	1	0	0	B
TSS	Tobermory Singing Sands, BPNP, Ontario, Canada	N 45˚11'33"	W 81˚35'02"	0.91	1	0	0	A
SBD	Sleeping Bear Dunes National Lakeshore, Michigan, USA	N 44˚56'20"	W 85˚52'13"	0.94	0.75	0	0.25	A
PCR	Port Crescent State Park, Michigan, USA	N 44˚00'15"	W 83˚04'26"	0.98	0.75	0	0.25	B
IND	Indiana Dunes National Lakeshore, Indiana, USA	N 41˚37'17"	W 87˚12'44"	0.99	0.86	0.14	0	D

Appendix C: Detailed explanation of crossing designs

A. Detailed explanation of all crosses.

Within- and between-population crosses with 6 plants (A, B, C, D, E, F) per population

6 outcrossing populations (IND, MAN, PCR, PIN, SBD, TSS)

6 selfing populations (KTT, LPT, PTP, RON, TC, TSSA)

Total: 12 populations x 6 plants = 72 parents

Within population: AxB, AxC, BxC, DxE, DxF, ExF (reciprocal)

For example, plant IND A was crossed with:

x IND-B
x IND-C

Between population - within mating system: AxA, BxB, CxC, DxD, ExE, FxF (reciprocal)

For example, plant IND A was crossed with:

x MAN-A
x PCR-A
x PIN-A
x SBD-A
x TSS-A

Between population - between mating system: AxA, BxB, CxC, DxD, ExE, FxF (reciprocal)

For example, plant IND A was crossed with:

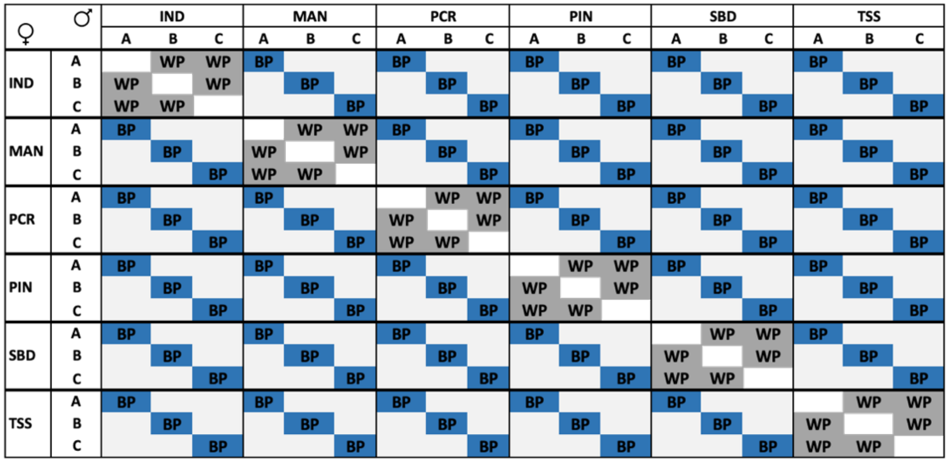
x KTT-A
x LPT-A
x PTP-A
x RON-A
x TC-A
x TSSA-A

Additional within population crosses with 5-12 extra plants per population.

(exact crosses given in C)

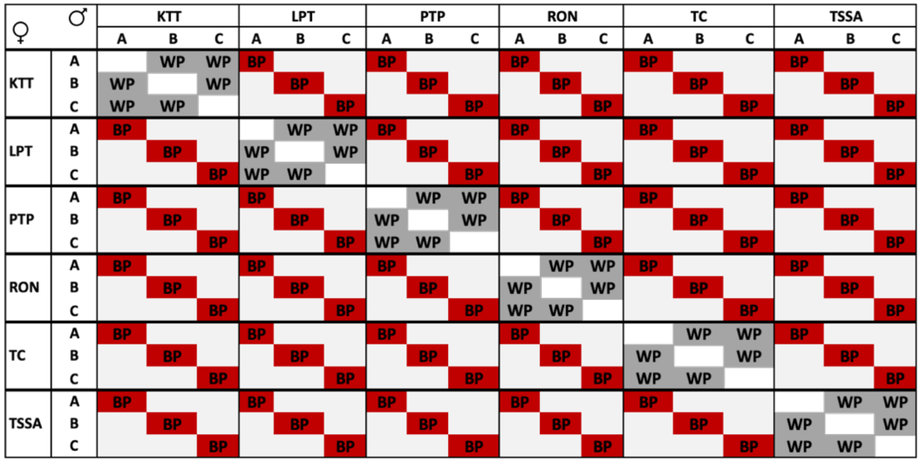
B. Schematic of the crossing design with three plants per population (A, B, and C). For simplicity, plants D, E, and F from each population are not shown. Blue (BP) cells represent crosses between SI populations. Red (BP) cells represent crosses between SC populations. Pink (S) cells represent selfed crosses. Grey (WP) cells represent within population crosses for both SI and SC populations. Purple (BMS) cells represent crosses between mating systems.

SI×SI within and between



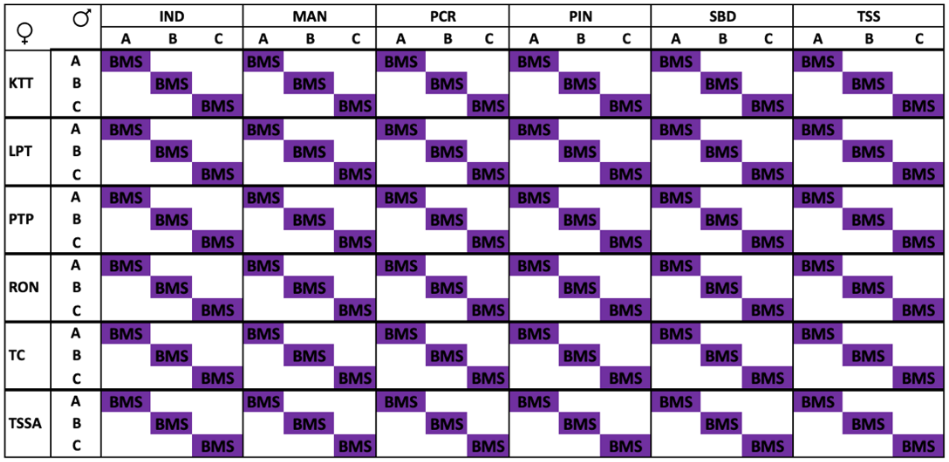
♀	♂	IND			MAN			PCR			PIN			SBD			TSS		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
IND	A		WP	WP	BP			BP			BP			BP			BP		
	B	WP		WP		BP			BP			BP			BP			BP	
	C	WP	WP				BP			BP			BP			BP			BP
MAN	A	BP				WP	WP	BP			BP			BP			BP		
	B		BP		WP		WP		BP			BP			BP			BP	
	C			BP	WP	WP				BP			BP			BP			BP
PCR	A	BP			BP				WP	WP	BP			BP			BP		
	B		BP			BP		WP		WP		BP			BP			BP	
	C			BP			BP	WP	WP				BP			BP			BP
PIN	A	BP			BP			BP				WP	WP	BP			BP		
	B		BP			BP			BP		WP		WP		BP			BP	
	C			BP			BP			BP	WP	WP				BP			BP
SBD	A	BP			BP			BP			BP				WP	WP	BP		
	B		BP			BP			BP			BP		WP		WP		BP	
	C			BP			BP			BP			BP	WP	WP				BP
TSS	A	BP			BP			BP			BP			BP				WP	WP
	B		BP			BP			BP			BP			BP		WP		WP
	C			BP			BP			BP			BP			BP	WP	WP	

SC×SC within and between



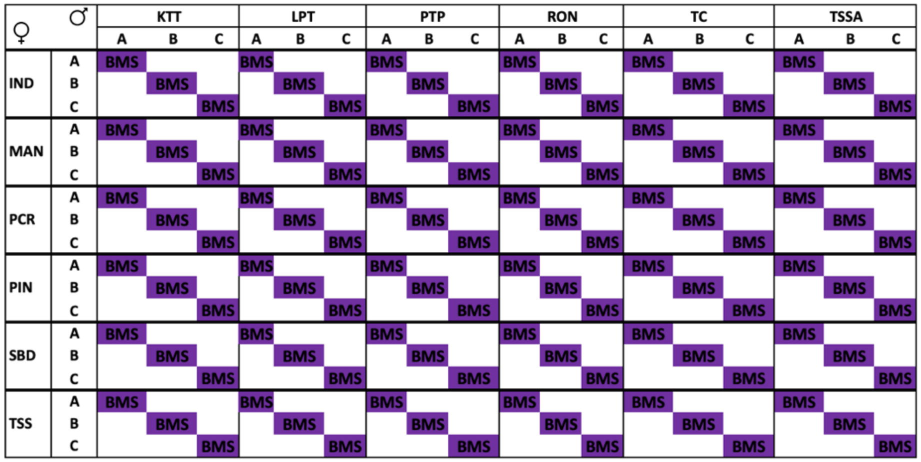
♀	♂	KTT			LPT			PTP			RON			TC			TSSA		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
KTT	A		WP	WP	BP			BP			BP			BP			BP		
	B	WP		WP		BP			BP			BP			BP			BP	
	C	WP	WP				BP			BP			BP			BP			BP
LPT	A	BP				WP	WP	BP			BP			BP			BP		
	B		BP		WP		WP		BP			BP			BP			BP	
	C			BP	WP	WP				BP			BP			BP			BP
PTP	A	BP			BP				WP	WP	BP			BP			BP		
	B		BP			BP		WP		WP		BP			BP			BP	
	C			BP			BP	WP	WP				BP			BP			BP
RON	A	BP			BP			BP				WP	WP	BP			BP		
	B		BP			BP			BP		WP		WP		BP			BP	
	C			BP			BP			BP	WP	WP				BP			BP
TC	A	BP			BP			BP			BP				WP	WP	BP		
	B		BP			BP			BP			BP		WP		WP		BP	
	C			BP			BP			BP			BP	WP	WP				BP
TSSA	A	BP			BP			BP			BP			BP				WP	WP
	B		BP			BP			BP			BP			BP		WP		WP
	C			BP			BP			BP			BP			BP	WP	WP	

SC×SI between



♀	♂	IND			MAN			PCR			PIN			SBD			TSS		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
KTT	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
LPT	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
PTP	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
RON	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
TC	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
TSSA	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS

SI×SC between



♀	♂	KTT			LPT			PTP			RON			TC			TSSA		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
IND	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
MAN	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
PCR	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
PIN	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
SBD	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS
TSS	A	BMS			BMS			BMS			BMS			BMS			BMS		
	B		BMS			BMS			BMS			BMS			BMS			BMS	
	C			BMS			BMS			BMS			BMS			BMS			BMS

C. Exact crossing scheme of the additional within population plants. Outcrosses (producing the $\mathrm{SI}\times\mathrm{SI}_{\text{within}}$ and $\mathrm{SC}\times\mathrm{SC}_{\text{within}}$ cross types) are highlighted in green and self-fertilizations (producing the SC×self cross type) are highlighted in orange. Parents that are connected with a line are siblings of each other.

IND (SI) ♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
G			G × I																	
H				H × J																
I																				
J																				
K						K × L														
L					L × K															
M								M × N												
N																				
O											O × Q									
P												P × R								
Q									Q × O											
R																				
S																				S × Z
T															T × U					
U																				
V																		V × X		
W																			W × Y	
X																X × V				
Y																				
Z																				

MAN (SI)

♀ ♂	G	H	I	J	K	L	M	N	O	P
G									G×O	
H			H×I		H×K					
I				I×J						
J										
K		K×H								
L							K×M			
M						M×L				
N				N×J						N×P
O	O×G									
P								P×N		

PCR (SI)

♀ ♂	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
G			G×I	G×J										
H														
I														
J	J×G													
K			K×I											
L									L×O					
M								M×N						
N							N×M							
O														
P												P×R		
Q												Q×R		
R														
S														S×T
T													T×S	

PIN (SI) ♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R
G				G×J								
H			H×I									
I		I×H										
J	J×G											
K										K×P		
L							L×M					
M						M×L						
N												N×R
O											O×Q	
P					P×K							
Q									Q×O			
R								R×N				

SBD (SI) ♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R
G		G×H										
H	H×G											
I										I×P		
J												
K				K×J								
L				L×J								
M											M×Q	
N							N×M					
O												O×R
P			P×I									
Q							Q×M					
R									R×O			

TSS (SI) ♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
G		G×H												
H	H×G													
I													I×S	
J														
K							K×M							
L														L×T
M					M×K									
N				N×J										
O										O×P				
P									P×O					
Q												Q×R		
R											R×Q			
S			S×I											
T						T×L								

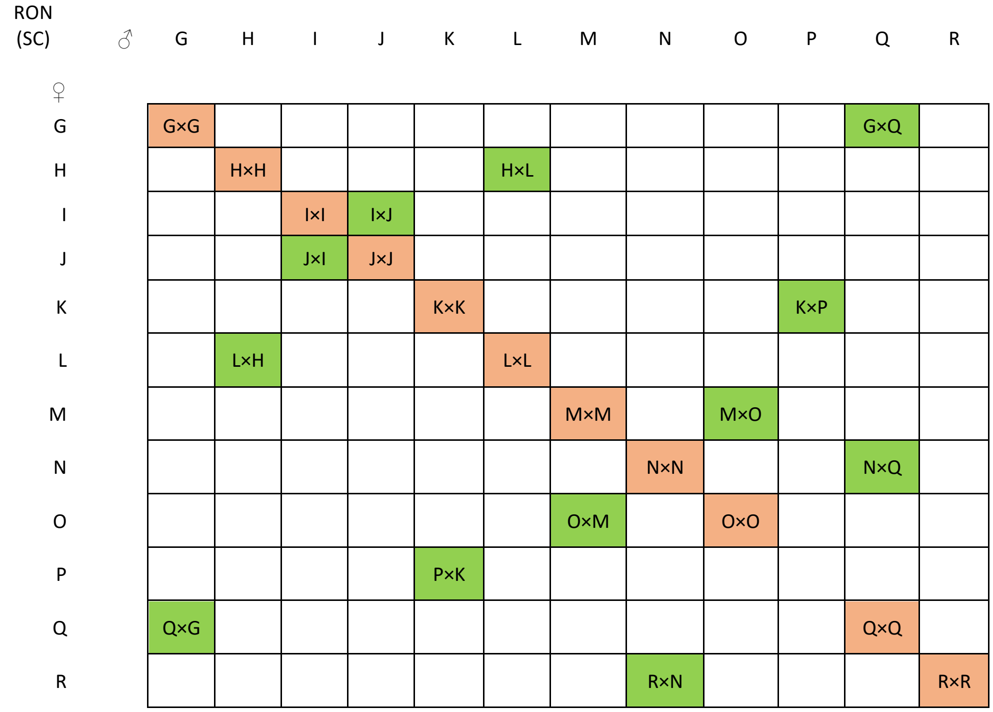
KTT (SC) ♂ ♀	G	H	I	J	K
G	G×G			G×J	
H		H×H			
I			I×I		
J	J×G			J×J	
K					K×K

LPT
(SC)

♂ ♀	G	H	I	J	K	L	M	N	O	P
G	G×G									
H		H×H								
I			I×I		I×K					
J				J×J				J×N		
K			K×I		K×K					
L						L×L				
M							M×M			
N										
O									O×O	O×P
P									P×O	P×P

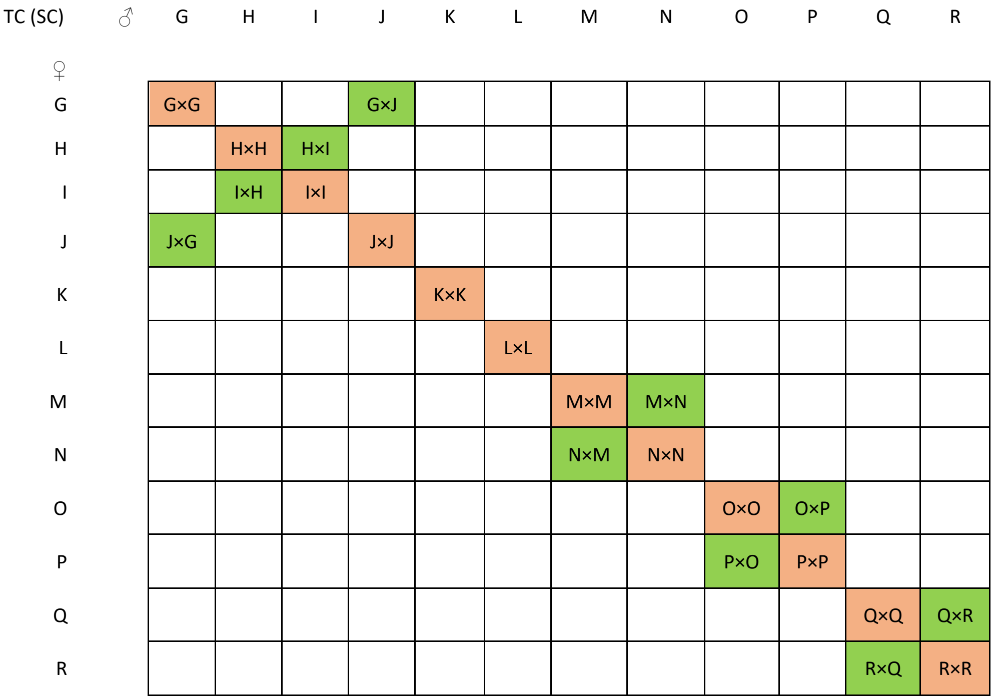
PTP
(SC)

♂ ♀	G	H	I	J	K	L	M
G	G×G			G×J			
H		H×H		H×J			
I			I×I				
J	J×G	J×H		J×J			
K					K×K		
L					L×K	L×L	
M							M×M



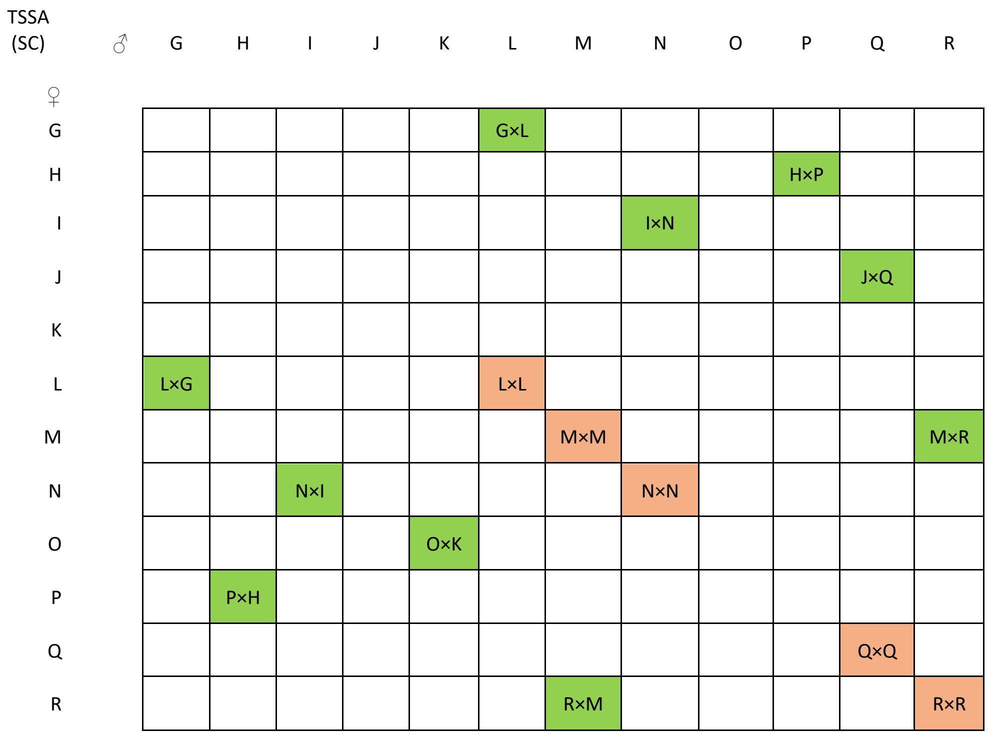
RON (SC)

♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R
G	G×G										G×Q	
H		H×H				H×L						
I			I×I	I×J								
J			J×I	J×J								
K					K×K					K×P		
L		L×H				L×L						
M							M×M		M×O			
N								N×N			N×Q	
O							O×M		O×O			
P					P×K							
Q	Q×G										Q×Q	
R								R×N				R×R



TC (SC)

♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R
G	G×G			G×J								
H		H×H	H×I									
I		I×H	I×I									
J	J×G			J×J								
K					K×K							
L						L×L						
M							M×M	M×N				
N							N×M	N×N				
O									O×O	O×P		
P									P×O	P×P		
Q											Q×Q	Q×R
R											R×Q	R×R



TSSA (SC) ♂ ♀	G	H	I	J	K	L	M	N	O	P	Q	R
G						G×L						
H										H×P		
I								I×N				
J											J×Q	
K												
L	L×G					L×L						
M							M×M					M×R
N			N×I					N×N				
O					O×K							
P		P×H										
Q											Q×Q	
R							R×M					R×R

Appendix D: Supplementary methods for calculating pollen-transfer probabilities

We use the calculation of $\boldsymbol{K}$ to refer to the opportunity for mating between plants from different populations in the common garden, based on the overlap in the number of flowers of each mating type per day.

In [31], $\boldsymbol{K_{ij}}$ refers to the probability that flowers at the *i*th position on an inflorescence are pollinated by flowers on other plants at the *j*th position. Here, we are not interested in the effects of floral position on pollen transfer probabilities, but in the possible effect of mating type. Accordingly, we estimated opportunities for pollen transfer within versus between self-incompatible and self-compatible mating types by calculating the following values of $\boldsymbol{K}$:

1. $\boldsymbol{K_{ss}}$
2. $\boldsymbol{K_{so}}$
3. $\boldsymbol{K_{os}}$
4. $\boldsymbol{K_{oo}}$

where, $\boldsymbol{K_{ss}}$ refers to the opportunity for plants from self-compatible populations (SC-within cross type) to fertilize flowers on other plants from self-compatible populations, $\boldsymbol{K_{so}}$ refers to the opportunity for plants from self-compatible populations to fertilize flowers on plants from self-incompatible populations (SI-within cross type), and so on.

Brunet and Charlesworth define the probability of pollen transfer between flowers of type $\boldsymbol{i}$ and $\boldsymbol{j}$ on day $\boldsymbol{c}$, $\boldsymbol{K_{ij}}$ as: $\frac{\sum_c \boldsymbol{f_{cj}^{m} N_{ci}^{f}}}{\sum_c \boldsymbol{N_{cj}^{m}}}$ where, $\boldsymbol{f_{cj}^{m}} = \frac{\boldsymbol{N_{cj}^{m}}}{\sum_j \boldsymbol{N_{cj}^{m}}}$.

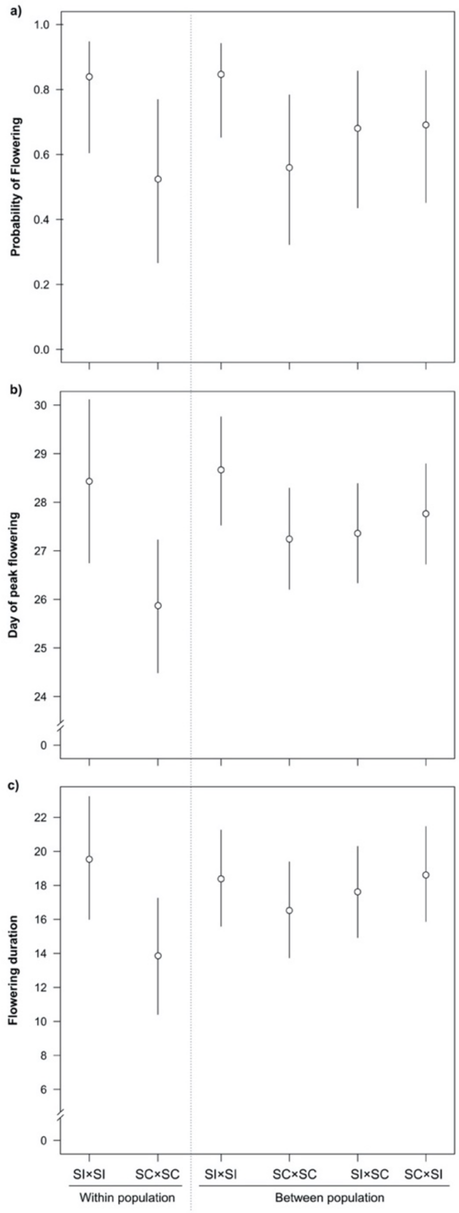
In the above expressions, the superscripts $\boldsymbol{m}$ and $\boldsymbol{f}$ refer to plants in the male and female phases. For *A. lyrata*, which is a simultaneous hermaphrodite, $\boldsymbol{m} = \boldsymbol{f}$, but because we are interested in pollen movement between plants, $\boldsymbol{i} \neq \boldsymbol{j}$. To calculate mating-type specific values of, for example, $\boldsymbol{K_{ss}}$, we calculated $\boldsymbol{f^m}$ as the proportion of all flowers open per day in the common garden that were from individual plants from self-compatible populations (the SC-within cross type). Therefore, for this calculation, the value of the numerator, $\boldsymbol{N^m_{cj}}$**,** was calculated only for those plants. Plants from self-incompatible populations were included in the calculation of $\boldsymbol{N^m}$ for $\boldsymbol{K_{os}}$ and $\boldsymbol{K_{oo}}$. For all values of $\boldsymbol{K}$, all plants were included in the calculation of the denominator of $\boldsymbol{f^m}$, $\sum_j \boldsymbol{N^m_{cj}}$.

The calculated values of $\boldsymbol{K}$ are frequency dependent - a small group of plants of one mating type surrounded by plants of the opposite mating type would have more opportunities for between, rather than within mating-type pollen transfer. Because we were specifically interested in opportunities for pollen transfer driven by phenology, not frequency, we used bootstrapping to generate 200 randomly sampled, equal-sized populations of plants of each mating type for the calculation of $\boldsymbol{K}$. For each mating type, we sampled 200 plants (with replacement) of each cross type for inclusion in each calculation of $\boldsymbol{K}$.

Two values of $\boldsymbol{K}$ refer to within mating-type fertilization opportunities and two of them to between mating-type fertilization opportunities. To evaluate whether plants from populations of the two different mating types (self-compatible versus self-incompatible) differed in the proportion of within- versus between mating-type pollen transfer opportunities, we used linear mixed models for each set of bootstrapped values of $\boldsymbol{K}$. Population was included as random effect in these models. In the absence of phenological differences between plants from self-compatible and self-incompatible populations, the expected value of the parameter estimate for the mating-type effect is 0. Accordingly, to test whether plants representing the two mating types differed in their opportunities for within- versus between mating-type pollen transfer, we tested whether the distribution of parameter estimates from each set of bootstrapped values of $\boldsymbol{K}$ differed from 0 using a two-tailed t-test.

Appendix E: Results of the analyses of phenological traits without the individuals from the additional within population crosses

Results of the analyses of phenological traits without the individuals from the additional within population crosses that were performed to increase sample sizes for the SI×SI$_{\text{within}}$ and SC×SC$_{\text{within}}$ cross types (and to generate the SC-self cross type). The SC-self cross type is not present here as it was generated by the extra within population crosses.



Appendix F: Description of the method for seed number estimation

To estimate the total reproductive output (seed production) per plant in the first year, we counted the fruits produced by each plant after flowering ceased (July 7-25, 2018) and multiplied this with a plant-specific estimate of the mean seed number per fruit. To obtain this estimate, we collected fruits from each plant over five time points (from July 12-31) in the following way. At the first collection time point, we collected all unopened ripe fruits from each individual. To save time, for the following four time points, we collected up to 10 ripe but unopened fruits from the central inflorescence of each plant, starting with the lowest positioned one. If 10 fruits were not available from the central inflorescence, we collected the remaining fruits from another haphazardly selected inflorescence (again bottom to top). This resulted in 1-64 total fruits (mean 5.6 fruits per plant), which were pooled for each plant. This strategy ensured that the total number of collected fruits per plant reflected the total number of fruits produced per plant (Fig. 1, Fig.2). We then estimated the seed number per fruit per plant using the following strategy: 1) to obtain the mean seed weight, we weighed a subset of 100 seeds; 2) to obtain the total seed weight, we weighed all seeds; 3) to obtain the number of collected seeds per plant, we divided the total seed weight by the mean seed weight; 4) to obtain the seed number per fruit per plant, we divided the mean seed weight by the number of collected fruits.

Figure 1. Distribution of the total number of fruits counted for each plant.

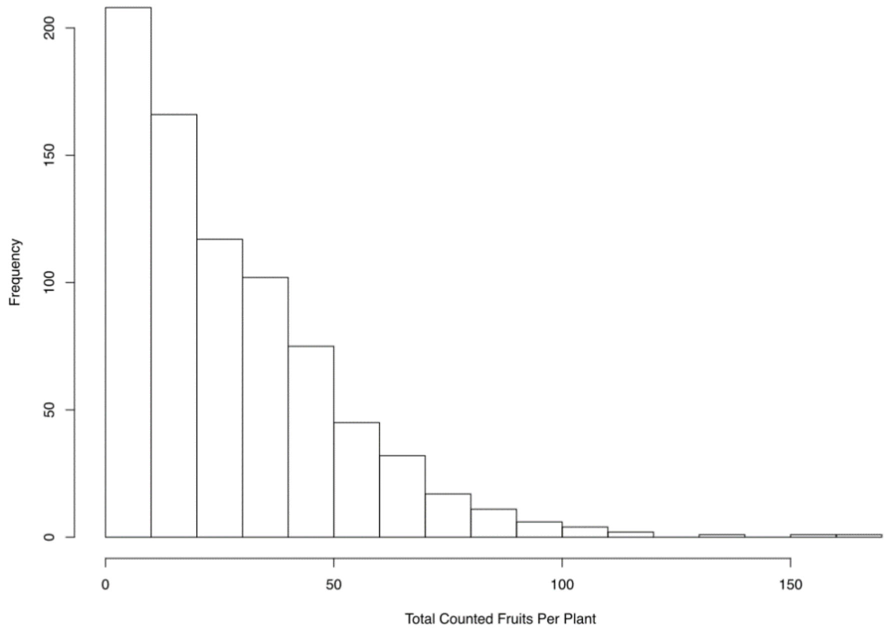


Figure 2. Distribution of the number of collected fruits per plant.