1   **Running head:** Prezygotic isolation in Arabidopsis lyrata

2   Limited phenological and pollinator-mediated isolation among selfing and outcrossing

3   *Arabidopsis lyrata* populations

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27   **Abstract**

28   Transitions from outcrossing to selfing have been a frequent evolutionary shift in plants and

29   clearly play a role in species divergence. However, many questions remain about the initial

30   mechanistic basis of reproductive isolation during the evolution of selfing. For instance, how

31   important are prezygotic pre-pollination mechanisms (e.g. changes in phenology and pollinator

32   visitation) in maintaining reproductive isolation between newly arisen selfing populations and

33   their outcrossing ancestors? To test whether changes in phenology and pollinator visitation

34   isolate selfing populations of *Arabidopsis lyrata* from outcrossing populations, we conducted a

35   common garden experiment with plants from selfing and outcrossing populations as well as their

36   F1 hybrids. Specifically, we asked whether there was isolation between outcrossing and selfing

37   plants and their F1 hybrids through differences in 1) the timing or intensity of flowering; and/or

38   2) pollinator visitation. We found that phenology largely overlapped between plants from

39   outcrossing and selfing populations. There were also no differences in pollinator preference

40   related to mating system. Additionally, pollinators preferred to visit flowers on the same plant

41   rather than exploring nearby plants, creating a large opportunity for self-fertilization. Overall,

42   this suggests that prezygotic pre-pollination mechanisms do not strongly reproductively isolate

43   plants from selfing and outcrossing populations of *Arabidopsis lyrata*.

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45   **Key words:** *Arabidopsis lyrata*, mating system, phenology, pre-pollination, prezygotic isolation,

46   pollinators, reproductive isolation, selfing

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49 **Background**

50   Mating-system transitions from obligate outcrossing to predominantly selfing have arisen

51 repeatedly across almost all major plant lineages [1]. Up to 15% of seed plants are predominantly

52 selfing and many share a relatively recent common ancestor with outcrossing species [2]. The

53 transition from outcrossing to selfing is usually associated with convergent evolution of a flower

54 morphology that optimizes self-pollination and resource use (e.g. smaller flower size and/or a

55 reduction in pollen, nectar, and scent production), termed the "selfing syndrome" [3–5]. These

56 types of changes in flowering likely contribute to the reproductive isolation of selfing lineages

57 [6], but it is unclear if they or their subsequent effects on pollinators are the main drivers of

58 reproductive isolation in incipient selfing species.

59   Reproductive barriers are essential to the maintenance of evolutionary independence of

60 parapatric populations (i.e. populations with slightly overlapping ranges). Reproductive isolation

61 can be partial or complete, and most plant species are isolated by a combination of pre- and

62 postzygotic barriers [6–8]. In plants, prezygotic mechanisms are generally more important than

63 postzygotic mechanisms in contributing to the total reproductive isolation of species [6,8–10].

64 Post-pollination mechanisms such as genetic incompatibilities can cause differences in seed

65 number and/or seed viability, but pre-pollination mechanisms generally contribute more to the

66 total reproductive isolation of plant species [6,9,10]. Although rarely addressed, this principle

67 appears to hold for cases where a shift to self-fertilization has played a part in speciation. For

68 example, in two closely related *Mimulus* species with a contrasting mating system, differences in

69 mean flowering date and floral display contributed the most to reproductive isolation compared

70 to other pre- and post-pollination mechanisms [11]. However, whether the transition to self-

71   fertilization could also promote rapid prezygotic reproductive isolation via changes in floral

72   morphology and associated shifts in pollinator preferences [7] has rarely been studied.

73   Plant phenological traits and the behaviour of pollinators could readily interact. For

74   example, pollinator behaviour and the number of flowers should both play a large role in

75   determining the opportunity for the flowers of self-compatible plants to be fertilized by a

76   different flower on the same plant (i.e. geitonogamy). Furthermore, geitonogamy could help to

77   reproductively isolate self-compatible individuals. For instance, if pollinators commonly visit

78   multiple flowers on the same individual, it could facilitate higher selfing rates of self-compatible

79   individuals [12]. Different types of pollinators, such as flies vs. bees, could also differ greatly in

80   their pollination strategies [13]. Additionally, if pollinators more commonly visit plants in very

81   close proximity, this could contribute to greater population viscosity and result in more matings

82   among closely related individuals that share the same mating system [14–16]. Furthermore, due

83   to flower attractiveness, pollinators might preferentially and repeatedly visit plants exhibiting a

84   particular mating system type. Alternatively, at the earliest stages of divergence, pollinators

85   might show limited or no ability to differentiate between plants with alternative mating types.

86   Here, we use *Arabidopsis lyrata* ssp. *lyrata* (L.) to examine the role of differences in

87   phenology and pollinator attraction as mechanisms of reproductive isolation in a recently

88   diverged selfing lineage. In several populations across the range of this normally outcrossing

89   species (multi-locus outcrossing rate: $0.83 < T_m < 0.99$), all plants are self-compatible, have low

90   outcrossing rates, and therefore reproduce primarily through selfing (multi-locus outcrossing

91   rate: $0.09 < T_m < 0.41$) [17–19]. The selfing and outcrossing populations are geographically

92   interspersed, therefore secondary contact following evolutionary divergence in parapatry is

93   likely. Also, the transition to selfing in these populations is thought to have happened < 10,000

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94   years ago because 1) the range now occupied by outcrossing and selfing populations was mostly

95   covered by ice during the last glacial maximum [19], and 2) the selfing populations have not

96   developed a selfing syndrome [20]. This raises the question of whether selfing populations have

97   diverged from their outcrossing ancestors in traits conferring reproductive isolation. Similarly,

98   given that outcrossing and selfing populations are at least partly interfertile and can regularly

99   produce healthy offspring [21,22], F1 hybrids may be a critical factor in determining whether

100   secondary contact would lead to coalescence of the diverged populations or alternatively

101   reinforce their evolved differences.

102   In a common-garden experiment set within the native range of *A. lyrata*, we simulated

103   two phases of secondary contact between selfing and outcrossing populations. The first phase

104   corresponds to initial contact between parental plants from selfing and outcrossing populations.

105   The second phase corresponds to secondary contact between admixed plants (hybrids between

106   populations) and parental plants. This allowed us to test whether the evolution of selfing has led

107   to pre-pollination isolation through divergence in phenology and/or insect pollinator attraction.

108   Specifically, we asked whether there was reproductive isolation between outcrossing and selfing

109   plants and their F1 hybrids through differences in 1) the timing or intensity of flowering; and 2)

110   pollinator visitation rates and paths. Based on this, we tested whether phenological differences

111   and pollinator behaviour reduced the opportunities for pollen exchange between mating systems.

112   Moreover, as geitonogamy can also contribute to reproductive isolation, we quantified the

113   opportunities for geitonogamy.

114   **Methods**

115   Study system

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116   *Arabidopsis lyrata* spp. *lyrata* (L.) is a small, short-lived perennial that is native to North

117   America. It occurs in dry-mesic habitats with shallow soils, such as rock outcrops and sand

118   dunes. Individual plants can produce several stems that terminate in racemes of numerous (>20)

119   small white flowers. The primary pollinators of *A. lyrata* are small solitary bees and hoverflies,

120   which are attracted to the nectar and pollen of the flowers. The ancestral condition in

121   *Arabidopsis lyrata* is self-incompatibility, however the barrier to self-fertilization has broken

122   down in several North American populations [17]. Additionally, many of these newly self-

123   compatible populations have evolved high selfing rates [19]. There is some variation in floral

124   traits, such as flower size and pollen:ovule ratios, among selfing and outcrossing populations,

125   which is primarily explained by population genetic background and not mating system [20].

126   <u>Crossing designs</u>

127   To generate the material needed to simulate secondary contact between diverged selfing

128   and outcrossing populations, we sowed field-collected seeds from 12 North

129   American *A. lyrata* populations with known breeding and mating systems [19] (seeds were

130   kindly provided by Barbara Mable, University of Glasgow). These included six populations

131   characterized as outcrossing (high outcrossing rates, high frequency of self-incompatible

132   individuals, hereafter referred to as SI populations) and six populations characterized as selfing

133   (low outcrossing rates, high frequency of self-compatible individuals, hereafter referred to as SC

134   populations) (Table S1).

135   In 2012 and 2013, we then produced seeds by manually cross- and self-pollinating up to

136   eight plants per population. To perform the pollinations, we emasculated a flower prior to anther

137   dehiscence, or the same individual in 'selfed' crosses, and rubbed a freshly dehisced anther from

138   a haphazardly chosen plant from the same population over its stigma. Progeny were produced

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139   with the following cross-types: within SI population (SI-within), and within SC population both

140   by crossing (SC-within) and by selfing (SC-self).

141   Then to generate the material needed to simulate admixture between the parental

142   populations and their F1 hybrids, we performed a full diallel cross in 2014 and 2015 with six

143   plants of each of the six SI and six SC populations. This cross produced additional progeny of

144   the SI-within and SC-within cross types, as well as the following cross types: between SI

145   population (SIxSI), between SC population (SCxSC), between SC and SI population reciprocally

146   (SIxSC or SCxSI). All crosses were reciprocal and yielded a total of 1032 seed families (Table

147   S2).

148   <u>Experimental design of common garden experiment</u>

149   To test whether differences in phenology and flower-visitor attraction can reproductively

150   isolate plants from selfing populations, we set up a common garden experiment at Trent

151   University in Peterborough, Ontario, Canada. This location is at an intermediate latitude within

152   the geographic range of the source populations (Fig. 1). From March 20 to 22, 2018, for each

153   seed family, up to 50 seeds were sown on a moistened peat-based substrate in one pot. Plants

154   were grown in climate chambers with 11-hour days and a 21°C/18°C day/night cycle at 95%

155   humidity. Between April 18 and May 1, when seedlings had developed at least two true leaves,

156   we transplanted three haphazardly chosen seedlings from each seed family to individual Stuewe

157   and Sons Ray Leach "Cone-tainers" ™ [Tangent, Oregon, USA] with the same peat-based

158   substrate. On May 10, plants were moved to the common garden, prior to any flowering.

159   Within the common garden, plants were organized in a randomized block design. There

160   were three replicates of three 3x6m blocks. Each of the nine resulting blocks contained between

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161   150-180 individual plants distributed randomly over 180 positions within 9 cone-tainer trays

162   with plants from each seed family and cross type evenly distributed among the blocks. In total,

163   1509 plants were raised in the common garden. Sample sizes for the cross types were: SI-within

164   (n=172), SC-within (n=175), SC-self (n=65), SIxSI (n=203), SCxSC (n=296), SIxSC (n=314),

165   SCxSI (n=284).

166   <u>Phenological data</u>

167   To test for potential reproductive isolation between the cross types due to differences in

168   phenology, we recorded daily for each plant whether it flowered and how many open flowers it

169   had. Open flowers were defined as flowers with visible reproductive organs (stigma and anthers)

170   and that still had petals attached to the flower. Besides calculating opportunities for pollen-

171   transfer between outcrossing and selfing populations, this allowed us to compare the time to

172   onset of flowering, flowering duration, time of peak flowering, and the maximum flower number

173   (i.e., the number of flowers on the day of peak flowering) for each individual for each of the

174   cross-types.

175   <u>Flower visitor observations</u>

176   To test for differences in insect attraction and flower visitor movements within and

177   between plants, we recorded flowers with GoPro Hero Session® [San Mateo, California, USA]

178   cameras. Specifically, we tested whether there were differences in the potential for

179   geitonogamous selfing (visitor movement within the same plant), and for outcrossing (i.e., visitor

180   movement between plants) within and between mating system. To standardize the recording

181   procedure, 4-6 flowering plants (depending on their size) were taken from their blocks and

182   placed in a tray located at the front of their respective blocks. This method ensured clear video

183   footage of multiple focal plants simultaneously, while minimizing interfering with the visual

184   context of the pollinators provided by the configuration of plants in the block design. To make

185   sure that focal plants had a different set of neighbors for each set of observations, we combined

186   flowering individuals systematically according to their position in the block, going through the

187   block in three different ways: 1) taking consecutive plants in a vertical direction, 2) taking

188   consecutive plants in a horizontal direction, and 3) taking plants from the same position but in

189   different trays. Due to this approach, the cross type of the focal plants combined in the video-

190   frames was random.

191   We recorded 12-15-minute-long videos that were later trimmed to the central 10 minutes

192   to exclude potential effects of disturbance during starting and stopping the cameras. In total, 500

193   videos were taken throughout the flowering period, of which a random subset of 140 videos

194   (23.3 hours of video) were analysed by the first author in a random order. In total, these videos

195   included 379 unique individuals (41% of all flowering individuals in the common garden), and

196   123 plants were observed in multiple videos. For each visitor, we recorded whether it was a

197   solitary bee or hoverfly, the duration of the visit and the path it took (see below). Finer

198   taxonomic identification was not possible due to the video resolution, but we took high quality

199   photographs to identify the most common visitors: hoverflies (Syrphidae) *Eristalis arbustorum*,

200   *Syritta pipiens*, *Sphaerophoria* sp. and *Toxomerus marginatus*, and solitary bees from the family

201   Halictidae (kindly identified by Bill Crins, Toronto, Canada).

202   The path that each visitor took after its initial visit to a flower was recorded to test

203   whether plants from selfing populations received fewer visits than those from outcrossing

204   populations as is expected in selfing plants [23]. Moreover, because pollinators will often focus

205   on exploiting one type of flower and/or floral scent, we tested whether visitors were more likely

206   to move to neighbouring plants with the same mating system than to plants with a different

207   mating system, and whether progeny from crosses between mating system received fewer visits

208   than progeny from crosses within the same mating system. We classified visitor paths as: "away"

209   – the visitor left the video frame after an initial visit; "same" – the visitor visited a second flower

210   on the same individual; or to one of the cross types as defined above ("SI-within", "SC-within",

211   "SC-self", "SIxSI", "SCxSC", "SIxSC", "SCxSI") – the visitor went to a flower on a different

212   individual. This allowed us to classify the flight paths of the visitors and compare visitation rates

213   among destinations.

214   <u>Statistical analyses</u>

215   All statistical analyses were done in R 3.5.1 [24]. To test if there were differences in the

216   time of peak flowering and duration of flowering between SI and SC cross types (SI-within, SC-

217   within, SC-self) and between within population cross types and between population cross types

218   (SI-within, SC-within, SC-self vs SIxSI, SCxSC, SIxSC, SCxSI), we used Gaussian linear

219   mixed-effects models implemented in lme4 [25] using *cross type* as a fixed effect, and *maternal*

220   *population* and *paternal population* as random effects. We used "Improper" prior distributions,

221   i.e. distributions with density functions that do not integrate to 1 and are therefore not "proper"

222   probability distributions [26]. Specifically, $p(\beta) \propto 1$ was implemented for the model coefficients

223   and $p(\sigma^2) \propto 1/\sigma^2$ for the variance parameters. To obtain the posterior distribution, 5000 values

224   were directly simulated from the joint posterior distribution of the model parameters using the

225   function sim of the R package 'arm' [27]. The means of the simulated values from the joint

226   posterior distributions of the model parameters were then used as estimates, and the 2.5% and

227   97.5% quantiles were used as the lower and upper limits of the 95% credible intervals to make

228   comparisons among cross types.

229   To test if there were differences in the mean maximum flower number among the cross

230   types, a hurdle model (hurdle function, package 'pscl'; [28,29]) with a negative binomial

231   distribution that included *maximum flower number* as the response variable and *cross type* as a

232   fixed effect was performed. The hurdle model accounts for the excess number of zero counts in

233   the maximum flower number data. This model specifies one process for zero counts and a

234   separate process for positive counts. The zero counts (flower number as either 0 or 1) were then

235   modelled with a binomial logit model and the positive counts (plants that flowered) with a

236   truncated negative binomial model. The hurdle model also allowed us to calculate the probability

237   that individuals from a cross type would flower.

238   Pollinator visitation rate (per plant) was analysed separately for the two main visitor

239   classes hoverflies and solitary bees. The cross type 'SC-self' was excluded from the analyses of

240   pollinator visitation due to low sample size. To test if there were differences in the frequency of

241   pollinator visits among the cross types, two identical generalized linear mixed-effects models

242   with negative binomial distributions with *number of visits* as the response variable (one model

243   for visits made by hoverflies and another one for solitary bees). The explanatory variables were

244   *cross type* and *flower number* as fixed effects, and *maternal population* and *paternal population*

245   as random effects. In these models, the number of adaptive Gauss-Hermite quadrature points

246   (nAGQ) was set to zero, which optimizes the random effects and the fixed-effects coefficients in

247   the penalized iteratively reweighted least squares step [25]. This results in a faster but less

248   precise parameter estimation for generalized mixed effect models [25]. These models used a log-

249   link function. Improper prior distributions were used, as in the analyses of time of peak

250   flowering and flowering duration.

251   Pollinator visitation paths were analysed in two ways. The probability that a pollinator

252   would make a certain choice after landing on a flower was analysed with a multinomial logistic

253   regression as implemented in the function multinom in the package 'nnet' [30]. *Path* in the

254   multinomial model included all cross types and the same plant (opportunity for geitonogamy) as

255   path options, along with the option of leaving the observation frame. This model included both

256   *cross type* and *flower number* as fixed effects and *path* as the response variable using a logit link

257   function. To further parse the pollinator preference and the effect of flower number and distance

258   between plants in the frame, a conditional logistic regression (function clogit, package 'survival';

259   [31]) was performed. The conditional logistic regression was performed separately for hoverflies

260   and solitary bees and included the insect's selection for any of the cross types in the same video

261   frame as the response variable, as well as *relative flower number*, *relative distance*, and *cross*

262   *type* as fixed effects, and finally *switch ID* as the strata. The strata command specifies the group

263   of observations inherent to our video recordings. The strata in this case specifies the group of

264   choice options for each pollinator in each video. Switch ID was defined as: what the insect

265   selected (1) and everything the insect did not select (0) and incorporated information about the

266   distance to the other individuals and the flower number relative to the other individuals. The

267   cross type 'SI-within' was used as the baseline as this cross type represents the ancestral

268   condition in *A. lyrata*. *Relative flower number* and *relative ranked distance* were obtained by

269   dividing by the maximum value within the same video-frame.

270   Pollen-transfer probabilities

271   To examine whether there were differences in the opportunities for self- or outcross

272   pollination between selfing and outcrossing plants, we used the empirical information on

273   phenology and pollinator behaviour to model opportunities for outcrossing between 'SI-within'

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274   and 'SC-within' plants and for geitonogamous pollen movement within plants. Between

275   population cross types were excluded from these analyses. In terms of outcrossing, we were

276   interested in opportunities for pollen exchange within versus between mating types (e.g., whether

277   plants from self-compatible populations of *A. lyrata* had more opportunity to mate with each

278   other than with plants from self-incompatible populations) as a potential mechanism of

279   reproductive isolation. To do this, we used calculations of K_{ij} - the "pollen transfer

280   probabilities" outlined in [32]. Here, we use the calculation of K to refer to the opportunity for

281   mating between plants from different populations in the common garden, based on the overlap in

282   the number of flowers of each mating type per day.

283   In [32], K_{ij} refers to the probability that flowers at the ith position on an inflorescence are

284   pollinated by flowers on other plants at the jth position. Here, we are not interested in the effects

285   of floral position on pollen transfer probabilities, but in the possible effect of mating type.

286   Accordingly, we estimated opportunities for pollen transfer within versus between self-

287   incompatible and self-compatible mating types by calculating the following values of K:

288   1. K_{ss}

289   2. K_{so}

290   3. K_{os}

291   4. K_{oo}

292   where, K_{ss} refers to the opportunity for plants from self-compatible populations (SC-within

293   cross type) to fertilize flowers on other plants from self-compatible populations, K_{so} refers to the

294   opportunity for plants from self-compatible populations to fertilize flowers on plants from self-

295   incompatible populations (SI-within cross type), and so on.

296   Brunet and Charlesworth define the probability of pollen transfer between flowers of type i and

297   j on day c, K_{ij} as: $\frac{\sum_c f_{cj}^m N_{ci}^f}{\sum_c N_{cj}^m}$ where, $f_{cj}^m = \frac{N_{cj}^m}{\sum_j N_{cj}^m}$.

298   In the above expressions, the superscripts m and f refer to plants in the male and female

299   phases. For *A. lyrata*, which is a simultaneous hermaphrodite, $m = f$, but because we are

300   interested in pollen movement between plants, $i \neq j$. To calculate mating-type specific values of,

301   for example, K_{ss}, we calculated f^m as the proportion of all flowers open per day in the common

302   garden that were from individual plants from self-compatible populations (the SC-within cross

303   type). Therefore, for this calculation, the value of the numerator, N_{cj}^m, was calculated only for

304   those plants. Plants from self-incompatible populations were included in the calculation

305   of N^m for K_{os} and K_{oo}. For all values of K, all plants were included in the calculation of the

306   denominator of f^m, $\sum_j N_{cj}^m$.

307   The calculated values of K are frequency dependent - a small group of plants of one

308   mating type surrounded by plants of the opposite mating type would have more opportunities for

309   between, rather than within mating-type pollen transfer. Because we were specifically interested

310   in opportunities for pollen transfer driven by phenology, not frequency, we used bootstrapping to

311   generate 200 randomly sampled, equal-sized populations of plants of each mating type for the

312   calculation of K. For each mating type, we sampled 200 plants (with replacement) of each cross

313   type for inclusion in each calculation of K.

314   Two values of K refer to within mating-type fertilization opportunities and two of them

315   to between mating-type fertilization opportunities. To evaluate whether plants from populations

316   of the two different mating types (self-compatible versus self-incompatible) differed in the

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317   proportion of within- versus between mating-type pollen transfer opportunities, we used linear,

318   mixed models for each set of bootstrapped values of K. Population was included as random

319   effect in these models. In the absence of phenological differences between plants from self-

320   compatible and self-incompatible populations, the expected value of the parameter estimate for

321   the mating-type effect is 0. Accordingly, to test whether plants representing the two mating types

322   differed in their opportunities for within- versus between mating-type pollen transfer, we tested

323   whether the distribution of parameter estimates from each set of bootstrapped values of

324   K differed from 0 using a two-tailed t-test.

325   The opportunity for geitonogamous self-pollination is determined by the number of

326   simultaneously open flowers per plant and the likelihood that pollinators will move from one

327   flower to another on the same plant. Videos of pollinator movements provide per-population

328   estimates of that likelihood. We calculated the opportunity for geitonogamous pollen

329   transfer, G_c, for plants with n open flowers as a geometric series of the likelihood of within-plant

330   pollinator movement x. That is, for each day c, $G_c = x + x^2 + x^3 + \cdots + x^{n-1}$. The total

331   opportunity for geitonogamous pollen transfer over the flowering season was calculated as $\sum_c G_c$.

332   **Results**

333   <u>Phenology</u>

334   Of the 1509 plants in the common garden, 938 flowered (62%). The main flowering

335   period lasted six weeks from June 1 to July 14, although a few individuals flowered later (nine

336   individuals flowered a second time and 10 individuals flowered for the first time as late as

337   September) (Fig. S1). The probability of an individual flowering varied by cross type. SI-within

338   and SC-within cross types did not strongly differ from each other in the probability of flowering

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339   (55% and 61%, respectively; CrI overlapping; Fig. 2a), however the probability of flowering of

340   the SC-self cross type (29%) was substantially lower (Fig. 2a). So, while progeny formed by

341   selfing flowered less, merely having the ability to self did not substantially decrease the

342   probability of flowering when compared to individuals from outcrossing populations. The F1

343   hybrid cross types did not differ from the within-population cross types in the probability of

344   flowering (54%-64%; CrIs overlapping; Fig. 2a), with the exception of the SIxSI cross type

345   being more likely (83%) to flower than the other F1 or within-population cross types (Fig. 2a).

346   Additionally, the direction of the cross for SIxSC and SCxSI hybrid F1 crosses did not have an

347   obvious effect on flowering probability, as both cross types had similar probabilities (63% and

348   64% respectively) for flowering (Fig. 2a).

349   The time of peak flowering differed among the cross types. While there were no strong

350   differences in the time of peak flowering between the SI-within and SC-within cross types, the

351   SC-self cross type peaked in flowering about one to two days earlier (average = 27.6 days) than

352   the other within or F1 cross types (however, CrIs overlapped with all cross types except SIxSI;

353   Fig. 2b). The day of peak flowering did not differ between SIxSC and SCxSI plants, indicating

354   that cross direction did not influence the time of peak flowering (CrIs overlapping; Fig. 2b).

355   There were also no strong differences in peak flowering between the within-population and F1

356   cross types (CrIs overlapping), with the exception that the SIxSI cross type tended to reach peak

357   flowering one to two days later (Fig. 2b). Similarly, flowering duration did not strongly differ

358   among the cross types (Fig. 2c), but the SC-self cross type tended to have a shorter duration (6-

359   15 days) than the other within population cross types (11-19 days) or F1 cross types (15-21 days)

360   (Fig. 2c).

361   For flowering plants of all cross types, the mean maximum number of open flowers on a

362   single day ranged from 10.8 to 13.6 flowers. After correcting for zero-inflation in the maximum

363   flower number model, there were no strong differences in maximum flower number among the

364   cross types.

365   <u>Pollinator visitation</u>

366   There were no differences in flower visitation between the within population cross types

367   or between the within population cross types and the F1 hybrids (all CrIs overlapping; Fig. 3a,

368   Fig. 3b). Solitary bees and hoverflies were the predominant visitors, and they had similar

369   visitation frequencies and no clear pattern of preference for any of the cross types (compare Fig.

370   3a and 3b). The behaviour of both types of pollinators appeared to facilitate geitonogamous self-

371   pollination, as ~50% of the movements between flowers were to a different flower on the same

372   plant (Fig. 4). When cases of a pollinator visiting another flower on the same plant were not

373   considered, the odds of an initial visitor moving to a plant in the frame decreased by 89%

374   (solitary bees) and 94% (hoverflies) for each unit increasing relative distance (significantly

375   negative odds-ratios for relative distance; Table 1). In other words, pollinators were more prone

376   to visit the nearest plant, regardless of the cross type or the number of flowers on the

377   neighbouring plant.

378   <u>Opportunities for pollen-transfer</u>

379   Based on a bootstrapping approach that integrated the timing and intensity of flowering

380   throughout the entire flowering period, the opportunity for between vs. within cross type pollen-

381   transfer was nearly equal both for the SI-within and SC-within cross type (Fig. 5). In other

382   words, slight shifts in phenology and flowering intensity (Fig. S1) are unlikely to lead to

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383   reproductive isolation. Additionally, a similar approach that also took into account the pollinator

384   paths showed that the opportunity for geitonogamy arising from plants having multiple flowers

385   open simultaneously and the high frequency of within plant movement of pollinators (Fig. 4) did

386   not differ between SI-within and SC-within plants.

387   **Discussion**

388   Our common garden experiment simulating secondary contact between outcrossing and

389   selfing populations showed that phenology largely overlapped between plants from outcrossing

390   and selfing populations. There were also no differences in pollinator preference related to mating

391   system. Regardless of mating system, pollinators tended to move between flowers on the same

392   plant, thus facilitating opportunities for geitonogamy. Our models of pollen-transfer

393   probabilities, which integrated differences in phenology (timing and intensity of flowering),

394   revealed equal opportunities for pollen-transfer within- and among mating systems. Together,

395   this suggests that prezygotic pre-pollination mechanisms do not strongly reproductively isolate

396   plants from selfing and outcrossing populations of *Arabidopsis lyrata*. However, because plants

397   simultaneously open multiple flowers, and pollinators predominantly move from flower to

398   flower on the same plant, our data suggest that there is a large opportunity for geitonogamy in

399   this system. Such geitonogamy could isolate selfers to some extent.

400   Pollinator visitation

401   Our results show that differences in pollinator preference do not play a large role in the

402   reproductive isolation of the newly diverged selfing populations. We found no differences in the

403   preferences of the two main pollinator types, hoverflies and solitary bees, among the cross types.

404   Additionally, when given a choice pollinators preferred to stay on the same individual versus

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405   exploring nearby plants regardless of cross type. When they did choose to visit another plant,

406   pollinators preferred to go to the closest plant regardless of how many flowers it had or what

407   cross type it was. This lack of pollinator preference could be due to the pollinators simply being

408   generalists, as previous studies have shown that hoverflies, for instance, are not very choosy with

409   respect to the plants they visit [13]. Additionally, there could be a lack of floral differences

410   because selfing individuals still need pollinators to transfer their pollen as they are not

411   completely autonomous [20]. Overall, pollinator preference seems to be playing little role in

412   differentiating the selfing from outcrossing populations of *A. lyrata*.

413   Nevertheless, the behaviour of the pollinators could favour selfing for several other

414   reasons. For instance, we found that pollinators often visit different flowers on the same

415   individual, irrespective of mating system. This should provide ample opportunity for within-

416   individual pollen transfer [3,33], and thus for self-compatible individuals to self-fertilize through

417   geitonogamy. Moreover, when pollinators moved between plants, they mainly moved between

418   nearby individuals. Given that *A. lyrata* seeds have no mechanisms to promote dispersal, and

419   plants can produce over 1000 seeds per season, neighbouring plants could be highly related to

420   each other [34]. As a consequence, the observed behaviour of pollinators could cause mate

421   limitation in self-incompatible plants, making the transfer of cross-pollen rare and/or mainly

422   from incompatible partners (e.g., from relatives that share S-alleles). In self-compatible plants,

423   on the other hand, geitonogamy may help overcome this mate limitation and provide

424   reproductive assurance [35]. Even without mate limitation, where geitonogamy does not provide

425   reproductive assurance but rather results in seed discounting, selfing could still be favoured due

426   to the associated inherent transmission advantage [36]. The transmission advantage would favour

427   selfers if inbreeding depression is low. Indeed, the relatively low estimates of inbreeding

428   depression for our study populations [37] imply that such transmission advantage alone could be

429   sufficient to drive the evolution of selfing. There is usually a low frequency of self-compatible

430   individuals in outcrossing populations [19,38]. Since the observed pollinator behaviour should

431   promote geitonogamous selfing, it remains enigmatic why selfing has not evolved in all North

432   American populations of *A. lyrata*.

433   <u>Potential consequences of admixture</u>

434   We found that F1 hybrid cross types had a similar phenology and pollinator visitation as

435   the parental cross types. Earlier studies have shown that hybrids between outcrossing and selfing

436   plants can be intermediate for phenological traits. For instance, in the genus *Clarkia,*

437   hybridization between selfing and outcrossing populations resulted in floral characteristics and

438   flowering times that were intermediate between the parental populations [39]. Our results show

439   similar relationships between F1 hybrids and the parental populations in regard to flowering

440   probability and time of peak flowering. This suggests that in a scenario of secondary contact, F1

441   hybrids would likely function as a bridge to further gene exchange between selfing and

442   outcrossing plants, which could potentially lead to the parental populations merging [40].

443   Whether the resulting admixed populations will maintain a mixed mating system [2], or evolve

444   to become predominantly selfing or outcrossing remains to be tested. Initially, as inbreeding

445   depression tends to be low [37,41], selfing may be favoured due to the associated inherent

446   transmission advantage. However, on longer timescales, expression of drift load may select

447   against selfing as shown in selfing populations of *A. lyrata* [22](but see [42]). It would therefore

448   be of interest to monitor the performance and mating system of admixed populations over

449   multiple years.

450   <u>Conclusions</u>

451   Our common garden experiment showed that, although pollinator behaviour may isolate

452   selfers by promoting geitonogamy, outcrossing and selfing *A. lyrata* populations are only weakly

453   reproductively isolated via pre-pollination mechanisms. These findings differ from findings in

454   other systems with a recent transition to selfing (e.g., [43,44]). The weak isolation between

455   selfing and outcrossing populations of *A. lyrata* is likely because its transition to selfing is even

456   more recent, and has not led to evolution of a selfing syndrome [20]. Future studies could

457   investigate if reproductive isolation due to prezygotic pre-pollination mechanisms are larger in

458   natural populations, giving specific attention to parapatric selfing and outcrossing populations.

459   Moreover, to what extent other mechanisms such as niche differentiation and genetic

460   incompatibilities contribute to reproductive isolation remains to be investigated.

461   **Acknowledgements**

470   **Data Accessibility**

471   Data will be made available from the Dryad Digital Repository upon acceptance.

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595   **Figure Captions**

596   Figure 1: Map of the location of the common garden experiment in relation to the source

597   populations. Gray circles represent selfing populations and black diamonds represent outcrossing

598   populations. The black X represents the location of the common garden at Trent University,

599   Peterborough, Ontario.

600   Figure 2: Panel of effect plots for the differences in phenological traits among the cross types

601   (n=1509). a) The probability of flowering per cross type. Fitted values are obtained from the

602   hurdle model. b) The day of peak flowering per cross type. This was calculated as the day where

603   each individual had the highest number of flowers during the total flowering period. Fitted

604   values obtained are from the Gaussian linear model that included *day of peak flowering* as the

605   response variable. c) The flowering duration (days) per cross type. Fitted values obtained are

606   from the Gaussian linear model that included *flowering duration* as the response variable.

607   Vertical bars in all figures represent the 95% credible intervals. SI-within= within outcrossing

608   population cross type, SC-within= within selfing population cross type, SC-self= selfed, SI= self-

609   incompatible, SC= self-compatible. The probability of flowering and time of peak flowering

610   varied among the cross types. Flowering duration did not strongly differ among the cross types.

611   Figure 3: Differences in pollinator visitation (number of visits) by a) solitary bees and b)

612   hoverflies among the cross types (n=502). The fitted values obtained are from the generalized

613   linear models with negative binomial distributions described in the methods. Vertical bars

614   represent the 95% credible intervals. SI-within= within outcrossing population cross type, SC-

615   within= within selfing population cross type, SI= self-incompatible, SC= self-compatible. The

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616   number of visits did not strongly differ among the cross types and pollinator identity had a minor

617   influence on the number of visits. Solitary bees made slightly more visits overall.

618   Figure 4: Stacked bar plot representing the probability of pollinators making a choice to visit an

619   individual of any cross type after visiting an individual of a certain cross type. Probabilities were

620   obtained from the multinomial model (and thus corrected for the number of available flowers on

621   each plant in the array). Pollinators could also choose to visit a flower on the same plant

622   ("same") or to leave the experimental setup ("away"). The cross types on the x-axis represent the

623   cross type of the initial visit. The stacked blue bars represent the probability of that cross type

624   being selected after a visit to the cross type on the x-axis. SI-within= within outcrossing

625   population cross type, SC-within= within selfing population cross type, SI= self-incompatible,

626   SC= self-compatible.

627   Figure 5: Representative bootstrapped run of the distribution of the self- and outcross pollen

628   transfer opportunities for self-compatible and self-incompatible individuals. The parameter

629   estimate for the difference in transfer-probability for the shown run was 0.024, and thus close to

630   the mean value of 0.020 across all the bootstrapped samples. There were no significant

631   differences in the opportunities for pollen transfer.
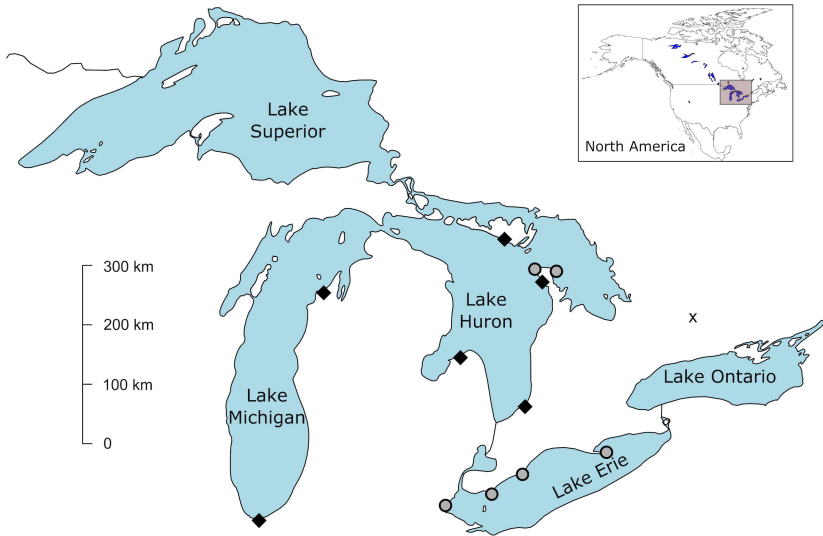

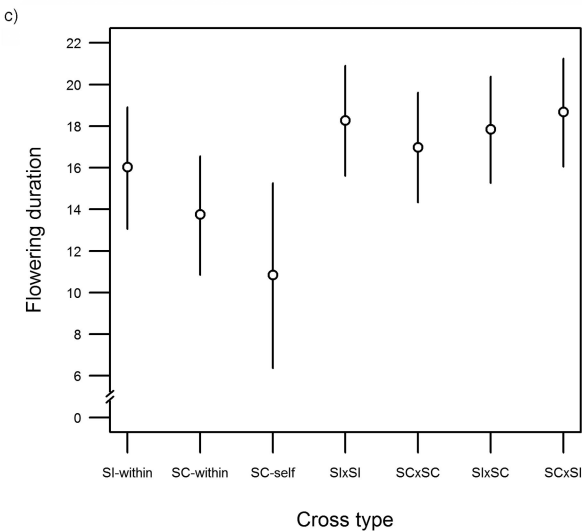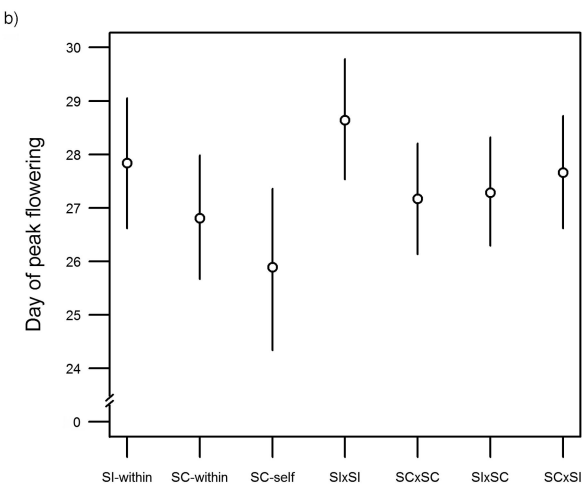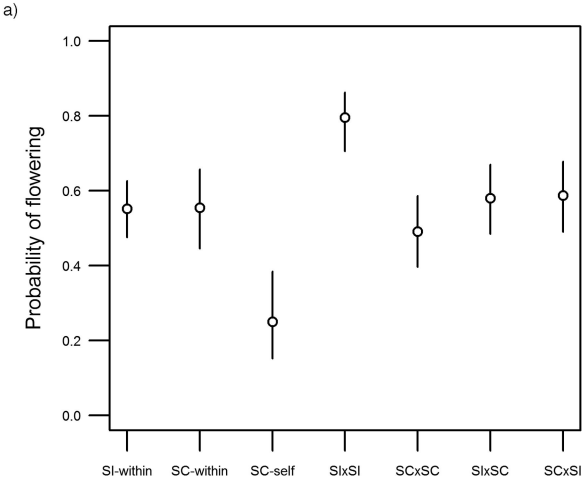632

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636   Table 1. Summary of results from the conditional logistic regression model that analyzed

637   whether *relative flower number*, *relative distance*, or *cross type* influenced the pollination path.

638   Cross type 'SI- within' was used as the baseline. Hoverflies: n=546, number of events = 142,

639   Likelihood ratio test= 62.16 on 7 df, p=<.001*. Solitary Bees: n=541, number of events=140,

640   Likelihood ratio test= 45.24 on 7 df, p=<.001*. Symbols and abbreviations used in the column

641   headings: SE= standard error; z= Wald statistic. For both hoverflies and solitary bees, relative

642   plant distance had the greatest influence on pollinator choice. There was no strong preference for

643   any of the cross types or individuals with more flowers. Significant effects are highlighted in

644   **bold**.

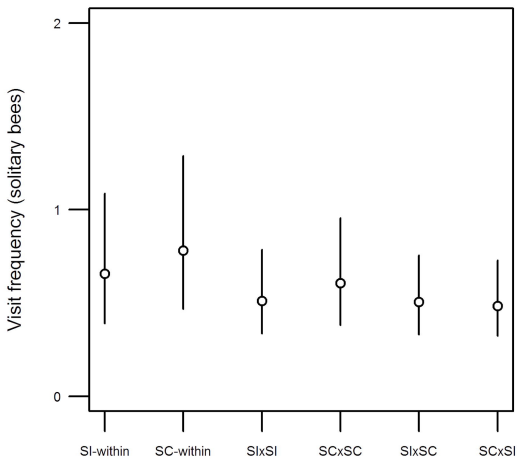| Visitor	Fixed effect	Odds Ratio	SE	z	Pr(>|z|)	95% confidence interval	
Solitary bees	Relative Flower Number	1.10	0.32	0.29	0.77	0.59	2.04
	Relative Distance	**0.11**	**0.38**	**-5.73**	**<.001***	**0.05**	**0.24**
	SC-within	2.01	0.39	1.80	0.07	0.94	4.30
	SIxSI	1.32	0.39	0.71	0.48	0.62	2.82
	SCxSC	0.96	0.39	-0.12	0.91	0.44	2.07
	SIxSC	0.80	0.36	-0.64	0.52	0.40	1.60
	SCxSI	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**
	SC-within	1.06	0.45	0.14	0.89	0.44	2.55
	SIxSI	1.09	0.41	0.21	0.83	0.49	2.44
	SCxSC	1.70	0.40	1.34	0.18	0.78	3.71
	SIxSC	1.03	0.40	0.07	0.95	0.47	2.27
	SCxSI	0.83	0.41	-0.44	0.66	0.37	1.86

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646   * indicate p-values <.05. The 95% confidence interval is the confidence interval of the odds

647   ratio. *Relative flower number* and *relative distance* were estimated relative to the other

648   individuals in the observation. These variables were transformed to range between 0 and 1.
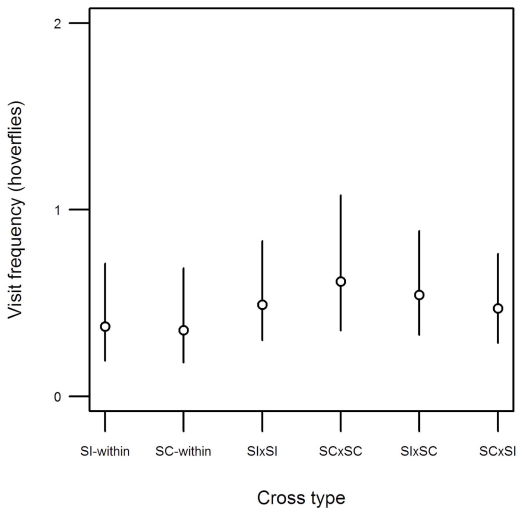
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Lake Superior

North America

Lake Huron

Lake Michigan

Lake Ontario

Lake Erie

300 km

200 km

100 km

0

X

a)

b)

c)

Cross type

a)

b)

Cross type