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Early evolution of reproductive isolation: a case of weak inbreeder/strong outbreeder leads to an intraspecific hybridization barrier in *Arabidopsis lyrata*.

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### Authorship Contributions

CLP, AC and RS designed the research. Öİ performed the research and collected data. Öİ, MS and CLP analyzed the data. Öİ, MS, CLP, AC and RS interpreted the data. Öİ, MS and CLP wrote the manuscript.

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### **Data availability**

All data used in this article are published in it (gathered in Table S1).

### **Early evolution of reproductive isolation: a case of weak inbreeder/strong outbreeder leads to an intraspecific hybridization barrier in *Arabidopsis lyrata*.**

Type of article: Original Article

Running title: Incipient speciation in *Arabidopsis lyrata*

### **Abstract**

Reproductive strategies play a major role in plant speciation. Notably, transitions from outcrossing to selfing may lead to relaxed sexual selection and parental conflict. Shifts in mating systems can affect maternal and paternal interests, and thus parent-specific influence on endosperm development, leading to reproductive isolation: if selfing and outcrossing species hybridize, the resulting seeds may not be viable due to endosperm failure. Nevertheless, it remains unclear how the switch in mating systems can impact reproductive isolation between recently diverged lineages, i.e., during the process of speciation. We

investigated this question using *Arabidopsis lyrata*, which recently transitioned to selfing (10,000 years ago) in certain North American populations, while European populations remain outcrossing. We performed reciprocal crosses between selfers and outcrossers, and measured seed viability and endosperm development. We show that parental genomes in the hybrid seed negatively interact, as predicted by parental conflict. This leads to extensive hybrid seed lethality associated with endosperm cellularization disturbance. Our results suggest that this is primarily driven by divergent evolution of the paternal genome between selfers and outcrossers. In addition, we observed other hybrid seed defects, suggesting that sex-specific interests are not the only processes contributing to postzygotic reproductive isolation.

### **Keywords**

Hybrid seed lethality; Endosperm Balance Number; *Arabidopsis lyrata*; grey zone of speciation; mating system divergence.

### **Main text**

#### **Introduction**

The evolutionary interests of female and male parents do not always align with regard to the production of high fitness offspring (Parker and Partridge 1998). When sexes differ in their contribution to offspring provisioning, sex-specific selective interests are likely to diverge, and may even be in conflict. Selection through maternal interests will favor allocating resources equally among offspring, as maternal parents are equally related to each embryo (Smith and Fretwell 1974). In contrast, when multiple mating is the rule, each paternal contributor competes for maternal resources allocated to its offspring. Therefore, promoting more resource allocation to their respective progeny may bring a selective advantage to paternal contributors (Trivers 1974; Rice 1982). Thus, the apparent collaboration between parental genomes may instead be a fragile equilibrium between the antagonistic influences of maternal (resource limitation) and paternal (resource drive) genomes. This conflict between maternal and paternal interests is well-known as “parental conflict” (Haig and Westoby 1989). In angiosperms, seeds arise as the product of double fertilization, whereby most of the seed, i.e., the embryo and its nourishing tissue, the endosperm, inherit both the maternal and paternal genomes (Baroux et al. 2002). The interaction of the two parental genomes seems

robust and concerted as it usually leads to viable seeds. Nevertheless, females are the only resource providers for the embryo, while the paternal contribution to the endosperm may allow its influence in maternal resource allocation to the developing offspring, laying the groundwork for parental conflict to occur.

Conflict among parental interests in resource allocation to offspring is predicted to be observed most strongly in the endosperm, as this tissue nourishes the embryo and is contributed to by both maternal and paternal genomes (Haig and Westoby 1989). These two contributions are quantified under the theoretical concept of the Endosperm Balance Number (EBN) (Johnston and Hanneman 1980, 1982). The EBN concept proposes that the endosperm relies on the equilibrium between paternal and maternal molecular factors (determining the EBN) to be functional. If the EBN of one lineage is higher than the EBN of another, the hybrid seed between the two will show failure of endosperm development that will cause embryo defects and result in seed lethality (Johnston and Hanneman 1980, 1982; Roth et al. 2018). Most angiosperms have a nuclear endosperm and in this case, hybrid endosperm defects encompass parent-of-origin disturbance in the timing of endosperm cellularization, a crucial developmental transition for embryo survival (Hehenberger et al. 2012). When the pollen donor has a higher EBN than the maternal plant, endosperm cellularization is delayed, while in the reciprocal cross it occurs precociously (Lafon-Placette et al. 2017). Changing the ploidy of one parental species can restore hybrid seed viability and endosperm development, supporting the idea that this hybridization barrier is underlain by a quantitative imbalance between paternal and maternal EBN factors (Johnston and Hanneman 1982; Lafon-Placette et al. 2017). Imprinted genes (genes expressed from either the maternally- or paternally-inherited allele) are strong candidates for the enigmatic “EBN parental factors” (Garner et al. 2016). Indeed, imprinted genes are implicated in hybrid seed lethality in multiple angiosperms, providing a potential molecular basis linking EBN and speciation (Wolff et al. 2015; Florez-Rueda et al. 2016; Lafon-Placette et al. 2018).

Parental conflict may act as a selective pressure on these parental factors (potentially imprinted genes) to increase their role and serve antagonistic parental interests over endosperm resource accumulation (Brandvain and Haig 2005; Lafon-Placette et al. 2018). This conflict is dependent on the degree to which a species is outcrossing, as parental conflict is driven by sire-sire competition for maternal resources. Indeed, across angiosperms, many lineages have shifted from an outcrossing mating system to various degrees of selfing

(Wright et al. 2013). In a self-pollinating species, every offspring has the same father and, further, the father and mother are the same individual, which together lead to the absence of conflict. In contrast, outcrossed offspring generally have multiple different fathers, setting the stage for intense conflict (Queller 1984). Consequently, the stronger selective pressure for parental influence in outcrossing compared to selfing species can explain why outcrossers are mostly found to have a higher EBN than their selfer counterparts, a phenomenon coined as “Weak Inbreeder/Strong Outbreeder”, later referred to as “WISO” (Brandvain and Haig 2005; Lafon-Placette and Köhler 2016). It is interesting to note that a higher EBN in outcrossers compared to selfers may well be explained by another parent-specific selective pressure. Namely, EBN may be a trait related to male-male competition for maternal resources and mate choice by females, i.e., sexual selection (Burley and Willson 1984; Lafon Placette 2020). Indeed, a repressive influence on endosperm by the maternal genome may act as selection of paternal contributors able to counteract the maternal influence (“good gene-like” selection; Lafon Placette 2020).

The higher EBN in strictly outcrossing lineages compared to selfers often leads to the inviability of outcrosser  $\times$  selfer reciprocal seeds, thus limiting hybridization and contributing to the ongoing divergence of such lineages (Brandvain and Haig 2005; Rebernig et al. 2015). Nevertheless, with limited divergence time (recently diverged lineages) or limited mating system divergence (e.g. mixed mating/partial selfing) between two lineages, the divergence of EBN may not be large enough to lead to seed lethality (Willi 2013; Raunsgard et al. 2018). With limited EBN divergence, crossing a pollen donor with a higher EBN (the more outcrossing lineage) compared to the maternal plant may yield larger seeds than normal, and the reciprocal cross ( $EBN_{\text{♀}} > EBN_{\text{♂}}$ ) may lead to smaller seeds (Willi 2013; Raunsgard et al. 2018). This is consistent with a stronger selective pressure in outcrossers compared to selfers on male progenitors to promote resource accumulation and for an antagonistic maternal influence (Brandvain and Haig 2005). Divergence in mating systems associated with EBN differences were shown for cases of either well-separated species and extensive EBN divergence/hybrid seed lethality (Brandvain and Haig 2005; Rebernig et al. 2015) or cases of closely related intraspecific populations showing mild EBN divergence and hybrid seed size modulation with no impact on viability (Willi 2013; Raunsgard et al. 2018). Besides mating systems, it remains unclear whether other factors contribute to EBN evolution.

Additional case studies of EBN divergence associated with hybrid seed inviability, especially between recently diverged lineages, are therefore required to disentangle contributing factors.

*Arabidopsis lyrata* is a plant species that occurs in Eurasia as well as North America (Al-Shehbaz and O’Kane 2002; Schmickl et al. 2010). It is assumed to have colonized North America from ancestral European populations (Ross-Ibarra et al. 2008; Schmickl et al. 2010), and the divergence between the Eurasian and North American lineages has been dated to around 35,000 years ago (Ross-Ibarra et al. 2008). The colonization of North America was accompanied by a strong bottleneck, leading to genetically less diverse *A. lyrata* populations in North America compared with Europe (Schmickl et al. 2010). European populations of *A. lyrata* (*A. lyrata* subsp. *petraea*) have been found to be exclusively outcrossing (self-incompatible) (Schierup 1998; Koch et al. 1999; Nasrallah et al. 2000). On the other hand, North American populations of *A. lyrata* (*A. lyrata* subsp. *lyrata*) are still predominantly self-incompatible, but a loss of self-incompatibility occurred multiple times in populations located in the Great Lakes region (Mable et al. 2005; Hoebe et al. 2009; Foxe et al. 2010). The loss of self-incompatibility is associated with low outcrossing rates (Foxe et al. 2010). A previous study showed that North American *A. lyrata* outcrossers have a higher EBN than their selfer counterparts (Willi 2013). This affected hybrid seed size in a cross-direction dependent manner, but the EBN divergence did not seem to be large enough to cause any loss of viability of the hybrid seeds. In this case, therefore, mating system shift does not seem to have led to substantial hybrid seed defects. Nevertheless, coupled with different demographic histories, such mating system shifts could lead to hybridization barriers between the North American selfers and other outcrossing populations across *A. lyrata*. Consistent with this idea, hybrid seeds between outcrossing Russian and North American populations of *A. lyrata* showed parent-of-origin-specific reduction in germination rate, pointing to a divergence in EBN between outcrossing populations from North America and Eurasia (Hämälä et al. 2017). Nevertheless, factors other than EBN divergence, such as the random accumulation of incompatible alleles during genetic divergence, may well explain the reduced germination of hybrid seeds, which will require further investigation.

Here, we investigated the early evolution of reproductive isolation, potentially involving the combination of EBN divergence and other factors such as genetic divergence and demographic history in hybrid seed lethality between outcrossing and selfing populations of *A. lyrata*. We performed manual reciprocal crosses between North American selfing and

Central European outcrossing populations of *A. lyrata*, measured hybrid seed viability, and monitored hybrid endosperm development. Our results suggest that changes in mating system accompanied with the successive colonization history of the species from Europe to North America appears to have induced a significant enough EBN divergence to cause extensive hybrid seed lethality within *A. lyrata*.

## Material and methods

### Plant material and growth conditions

In this study, both selfing and outcrossing populations of *A. lyrata* were used. Seeds were collected from Point Pelee, Canada (GPS coordinates 41°55'40.0"N 82°30'58.0"W) for the selfer *A. lyrata* subsp. *lyrata*; for the outcrosser *A. lyrata* subsp. *petraea*, seeds were collected from Nová Ves u Oslavan, Czech Republic (GPS coordinates 49°07'03.1"N 16°18'21.4"E).

In 2019, seeds were placed in paper bags and kept for two months at 4°C to break dormancy. After seed germination on damp soil, seedlings were transferred to pots filled with damp soil and were grown for six weeks in short-day conditions (8h of light per day) and 4°C to induce flowering. Seedlings were then transferred to long-day conditions (16h of light per day) and 21°C. Plants were reciprocally crossed within and between populations, and 6-14 manual pollinations (145-451 seeds) were performed for each type of cross (always noted as ♀ × ♂; selfer × selfer, outcrosser × outcrosser, outcrosser × selfer, selfer × outcrosser). Between two and six different maternal plants and 4-6 different pollen donors were used per type of cross in random combinations (see Supporting Information Table S1 for details).

For all crosses, designated female partners were emasculated before anthesis, and the pistils were hand-pollinated two days after emasculation (same procedure for selfing and outcrossing plants). Fully matured siliques were harvested 4/5 weeks after pollination and stored at 4°C under dry and dark conditions.

### Seed phenotyping

Seeds were arranged on white paper and pictures were taken using a macroscope (Olympus SZX10 connected with a Zeiss AxioCam ERc5s camera system). Non-collapsed seeds were identified as brownish-yellow and plump, with variable size. Collapsed seeds were dark



brown/black, shriveled and irregularly shaped. For measuring the seed size, images were converted to black and white using the “threshold” function on ImageJ (<https://imagej.nih.gov/ij/>) and size was measured using the “Analyze Particles” function.

### **Seed germination assays**

After harvesting, seeds were kept at 4°C for one year. To perform germination assays, seeds were surface sterilized (with 0.5% NaClO and 0.01% (v/v) of Triton X-100) and plated on MS medium (containing: 1 x MS-Salts and 0.8% (w/v) Plant agar, at pH 5.8). Then, plates were transferred and kept for 2 weeks in a growth chamber (12 h at 23°C/day and 12 h at 13°C/night). Germination rates were assessed by counting seeds with a ruptured seed coat and protruding radicles (Supporting Information Figure S1).

### **Assessment of endosperm cellularization**

Between three to four manual pollinations were performed for each type of cross. The same plants were used for control and hybrid crosses. To perform the crosses, flowers were emasculated before anthesis, and pollinated one to two days after. Seeds were collected 13, 16 and 18 days after pollination (DAP) and placed into fixative solution (3:1 v:v ethanol:acetic acid). After overnight incubation at 4°C, the fixative solution was changed to 70% (v/v) ethanol and the samples were kept at -20°C until subsequent analysis.

Seeds were prepared for microscopy with Feulgen staining, as previously described (Braselton et al. 1996). The imaging of optical seed sections was performed using a multiphoton Zeiss LSM880 microscope with an excitation wavelength of 760 nm and emission from 518 nm and upwards. Approximately 15-20 seeds per cross per time point were imaged.

### **Embryo *in vitro* culture**

Mature dry seeds were surfaced sterilized as described above. Seed coat was then removed, and the embryo dissected out using fine needles. The embryos were then placed on plates containing MS media supplemented with 2% sucrose. Plates were put for 2 weeks in a growth

chamber (12 h at 23°C/day and 12 h at 13°C/night), and the number of embryos with a grown radicle and green cotyledons were counted as germinated.

### Statistical analysis

We used generalized linear mixed models – GLMM (Bolker et al. 2009) to test for the interacting effect of male and female mating systems (outcrossing *vs.* selfing) on size, abortion rate and germination rate of the offspring seeds. Identities of maternal plants and pollen donors were included as random intercepts in the models to account for variation among individual plants. We used GLMM with a gamma distribution and a logarithmic link function to model non-negative continuous values of seed sizes (McCullagh and Nelder 1989). Abortion rates, calculated as proportions of collapsed seeds to total seed numbers, were modeled using binomial distribution and a logit link function. Similarly, germination rates were quantified as proportions of germinated to total seeds used in germination assays. However, germination rates did not follow unit dispersion (approx.  $\phi = 2.03$ ) and thus, we used beta-binomial distribution ( $\theta = 7.23$ ) to model these over-dispersed data (Bolker 2008). Likelihood ratio tests were employed to assess significance of the model terms. Nested models with parameters estimated by maximum likelihood and restricted maximum likelihood were compared to test the fixed and random effects, respectively (Pinheiro and Bates 2000). When significant interaction terms were revealed, we performed post-hoc tests with Tukey-adjusted probabilities to compare the estimated marginal means (Lenth 2016).

Finally, we used beta regression (Ferrari and Cribari-Neto 2004) to assess the effect of mating system on endosperm cellularization rate across multiple stages of *A. lyrata* embryo development. Since cellularization rates are bounded between 0 and 100% and their variance is arguably not constant, we assumed that the response follows a beta probability distribution defined by mean and precision (inverse dispersion) parameters that naturally account for non-constant variances (Cribari-Neto and Zeileis 2010). We fitted a three-way full factorial model with the main effects of female mating system, male mating system and embryo developmental stage, and all higher-order terms (interactions). Prior to analysis, measurements from the first two developmental stages were excluded from the dataset since cellularization was recorded only seldom during these early stages. Again, likelihood ratio tests were employed to assess significance of the beta model terms.

The analyses were performed in R (R Core Team 2019) using the libraries betareg (Cribari-Neto and Zeileis 2010), emmeans (Lenth 2019), lmtest (Zeileis and Hothorn 2002), ggplot2 (Wickham 2016) and glmmTMB (Brooks et al. 2017).

## Results

### Seeds from paternal outcrossers are larger

To test for potential different EBN divergence between selfer and outcrosser *A. lyrata* populations, we performed reciprocal crosses between and within a population of selfers (SC) and a population of outcrossers (SI) (Figure 1). The gamma GLMM revealed a significant influence of male mating system on the offspring seed size (Table 1). Plants with outcrossers used as pollen donors had significantly larger seeds than those with selfer pollen donors ( $0.40 \pm 0.04$  vs.  $0.33 \pm 0.03$  mm<sup>2</sup>) (Figure 2a). Female mating system did not appear to play a significant role in seed size.

### Crosses between selfer and outcrosser populations of *A. lyrata* lead to seed inviability

The binomial GLMM showed a significant interaction effect of female and male mating systems on the seed abortion rate (Table 1). When selfers were used as maternal plants and outcrossers as pollen donors, the percentage of collapsed seeds was significantly higher ( $57.1 \pm 9.6\%$ ) than in any other combination of parental mating systems (Figure 2b). In contrast, SC  $\times$  SC crosses produced seeds with the lowest abortion rate ( $8.6 \pm 3.2\%$ ).

With germination assays, we then tested whether the morphological defects observed in hybrid seeds were associated with a loss of viability (Supporting Information Figure S1). A significant interaction effect of female and male mating systems was also revealed in the beta-binomial GLMM of germination rates (Table 1). The seeds from selfer parents (SC  $\times$  SC) had significantly higher germination rates ( $83.5 \pm 9.2\%$ ) than those from reciprocal hybrid crosses (SC  $\times$  SI:  $22.7 \pm 14.4\%$ , SI  $\times$  SC:  $0.6 \pm 0.7\%$ ) (Figure 2c).

As SI  $\times$  SC seeds were apparently viable (Figures 1 and 2), we tested whether the low germination of SI  $\times$  SC hybrid seeds could be explained by dormancy rather than inviability. Specifically, the seed coat may participate in establishing the seed dormancy. To test this hypothesis, embryos were dissected out of mature dry seeds and grown on MS medium

complemented with sucrose (Supporting Information Figure S2, see Material and Methods). In parental crosses, embryos germinated well and to similar levels as seeds (Supporting Information Table S2). SC  $\times$  SI embryos also germinated to a large extent (6 out of 9 embryos germinated). However, despite any visible morphological defects, SI  $\times$  SC embryos did not give rise to any seedlings, suggesting that seed coat-related dormancy was not responsible for the low germination rate of SI  $\times$  SC hybrid seeds.

### Hybrid seeds show cross direction-dependent endosperm cellularization disturbance

Hybrid seeds originating from parents with different EBNs usually show a disturbance in the timing of endosperm cellularization, an important developmental transition for embryo survival (Rebernik et al. 2015; Lafon-Placette et al. 2017). We tested whether the hybrid seeds between selfing and outcrossing *A. lyrata* showed such defects.

Across all time points considered (13, 16 and 18 days after pollination, referred to as “DAP” hereafter), the SC  $\times$  SI cross showed delayed endosperm cellularization, as this transition was barely initiated, even at 18 DAP, while cellularization was almost completed in the parents at this time point (Figure 3a). In contrast, in SI  $\times$  SC seeds, most of the endosperm was cellularized at 16 DAP, while the embryo only reached heart stage (Figure 3a).

To account for different development speeds in each type of cross, we used the embryo developmental stage (numbered from 1 to 6, Supporting Information Table S3) as a marker for the physiological age of the seed, and measured the progression of endosperm cellularization for each stage (Figure 3b). Endosperm cellularization rate was significantly influenced by the mating system of parent plants and by the embryo developmental stage ( $\chi^2 = 315.2$ ,  $df = 15$ ,  $p < 0.0001$ ). The influence of both male and female mating system significantly differed among developmental stages (Table 2).

The onset of cellularization (cellularization at stage 3) occurred significantly earlier in seeds of selfer pollen donors compared to those of outcrosser pollen donors (mean  $\pm$  st. error:  $50.1 \pm 5.5$  vs.  $13.2 \pm 4.3\%$ ). However, as the endosperm development progressed, the difference gradually diminished and the seeds of selfer and outcrosser pollen donors eventually reached the same cellularization rate at stage 6 (significant male  $\times$  stage interaction; Figure 3b). In contrast, seeds of outcrosser maternal plants showed an earlier cellularization onset (stage 3) than the seeds of selfer maternal plants even though this effect

was less pronounced ( $37.0 \pm 6.2$  vs.  $16.8 \pm 4.2\%$ ) than that of the pollen donor, and this difference was diminished at stage 6 (significant female  $\times$  stage interaction).

Moreover, the effect of maternal plant mating system depended on the mating system of the pollen donor but this significant female  $\times$  male interaction was independent from the developmental stage (non-significant three-way interaction). Notably, the onset of cellularization in SI  $\times$  SC seeds occurred earlier than in parents, while the onset of cellularization was considerably delayed in crosses with SI pollen donors (Figure 3b). In particular, SC  $\times$  SI hybrid seeds showed consistently delayed cellularization along the developmental trajectory. Nevertheless, the SC  $\times$  SI seeds which eventually reached later stages of embryo development also reached complete endosperm cellularization (Figure 3b).

## Discussion

In this study, we report substantial hybrid seed failure between two populations of *A. lyrata* despite their relatively recent divergence of around 35,000 years ago (Ross-Ibarra et al. 2008). One population, from Central Europe (Czech Republic), is outcrossing while the other, from North America (USA, Great Lakes region), recently transitioned to selfing (10,000 years ago, Foxe et al. 2010). Hybrid seed defects, namely low viability and parent-of-origin-specific endosperm disturbance, are compatible with the divergent evolution of parent-specific traits in response to divergence of mating systems between the two populations. We also uncovered additional failure of the hybrid embryo unrelated to parent-specific traits. This points towards the accumulation of intrinsic genetic incompatibility between the two populations independently of the incompatibility predicted by the parental conflict theory. This further incompatibility could be related to the genetic differentiation between Eurasian *A. lyrata* subsp. *petraea* and North American *A. lyrata* subsp. *lyrata* (Ross-Ibarra et al. 2008; Schmickl et al. 2010; Buckley et al. 2016). Given the relatively short evolutionary time scale, it is striking to observe divergence in parent-specific traits large enough to lead to substantial hybrid seed incompatibility.

### A case consistent with the weak inbreeder/strong outbreeder theory

Brandvain and Haig (2005) proposed under the WISO hypothesis that outcrossing plant species may show stronger parental influence on endosperm (aka EBN) compared to selfing

species, as the selective pressure on parental interests (i.e., promoting resources for paternal interests and antagonistic influence for maternal interests) is stronger in outcrossers than in selfers. According to this hypothesis, in the hybrid endosperm obtained from crosses between a selfing and an outcrossing species, the imbalance between EBN factors from the selfing and outcrossing parents is expected to have parent-of-origin specific deleterious effects on endosperm development and result in seed lethality (Brandvain and Haig 2005; Rebernig et al. 2015). Our results are consistent with this hypothesis: while SC  $\times$  SI endosperm suffered from delayed cellularization, a typical consequence of the paternal contributor (SI) having a higher EBN compared to the maternal plant (Rebernig et al. 2015; Lafon-Placette et al. 2017, 2018), the reciprocal was also consistent, with SI  $\times$  SC seeds showing precocious endosperm cellularization. Our results are therefore consistent with the idea that divergent mating systems are associated with divergent selective pressure, such as parental conflict and/or sexual selection (Brandvain and Haig 2005; Lafon Placette 2020), leading to quantitative EBN differences between selfing and outcrossing lineages. Our results indicate that such divergence can occur rapidly since the two *A. lyrata* populations in this study diverged recently yet strong EBN divergence is already measurable. Prior to this study, the earliest reported divergence between a selfer and outcrosser causing EBN divergence and hybrid seed lethality was between *Capsella grandiflora* and *C. rubella* (Rebernig et al. 2015), which diverged around 50,000-100,000 years ago (Guo et al. 2009).

Interestingly, the effect of mating system divergence on EBN was more extreme on the paternal side. Indeed, with the outcrosser being the pollen donor, seeds were larger, independent of the mating system of the maternal parent. Similarly, the SI paternal genome delayed the onset of endosperm cellularization, independent of the mating system of the maternal genome (although this effect was stronger with a selfing maternal plant). This suggests that over this short evolutionary time scale, the maternal genomes of selfers and outcrossers have not diverged for their EBN as much as the paternal genomes have. This implies that maternal and paternal EBN factors are not always in full balance at a given evolutionary time point, which is consistent with an arms race between the two parental genomes (Haig and Westoby 1989). Also, our results suggest that the paternal EBN factors evolve faster than the maternal ones, which is consistent with several studies reporting positive selection acting on paternally expressed genes (Hatorangan et al. 2016; Tuteja et al. 2019), while no footprints of selection on maternally expressed genes were

found. The divergent evolution of seed size between the European outcrossers and North American selfers could also be explained by ecological factors (local adaptation; Metz et al. 2010). Nevertheless, we would expect a stronger maternal effect over seed size, as the maternal plant shares the same local environment as the progeny, while it is not necessarily the case for paternal contributors. However, we cannot rule out an important role of the paternal contribution in this process. In general, whether seed size evolution in response to ecological factors happens via parent-of-origin specific molecular mechanisms such as genomic imprinting is a fascinating perspective.

### **The evolution of seed inviability depends on factors besides EBN**

Interestingly, both cross directions suffered low germination rates, although only the SC  $\times$  SI crosses were noticeably inviable. SI  $\times$  SC seeds appeared viable and their embryos were fully formed with no apparent morphological defect or arrest, suggesting that their precocious endosperm cellularization did not have a major impact on embryo development, similar to other studies (Rebernik et al. 2015). SI  $\times$  SC seeds nevertheless mostly did not germinate, and the physiological reason remains unclear. We show that the low germination rate of SI  $\times$  SC seeds cannot be explained by seed coat dormancy. As we germinated the seeds a relatively long time after they were obtained (a one-year gap), it is possible that the hybrid embryos exhibited reduced longevity, explaining the low germination rate. Consistently, the absence of germination even after embryo dissections suggest an incompatibility which manifests itself in the embryo. We cannot rule out strong physiological dormancy in SI  $\times$  SC embryos. However, physiological dormancy appears as an unlikely scenario: the seeds were stored at 4°C during the year gap, which should have been long enough to break physiological dormancy. This suggests that postzygotic incompatibilities affect embryo lifespan, which could be related to the genetic divergence between Eurasian and North American *A. lyrata* (Ross-Ibarra et al. 2008; Schmickl et al. 2010; Buckley et al. 2016), independently of their mating system divergence.

It was previously shown that when North American outcrossing populations of *A. lyrata* are used as paternal donors with North American selfing *A. lyrata* as maternal plants, hybrid seed size was enlarged, supporting the WISO hypothesis (Willi 2013). Consistent with that study (Willi 2013), when we used European outcrossers as paternal donors, seed size was larger. Nevertheless, no loss of viability was found by Willi (2013), suggesting that the EBN

difference between North American outcrossers and selfers is limited. Here, using an outcrossing population from Central Europe and the same selfing population as Willi (2013), we find a strong reduction in hybrid seed viability, suggesting a significantly higher EBN of Central European *A. lyrata* outcrossers than the North American ones. While we did not test this directly, hybrid seeds between outcrossing Russian and North American populations of *A. lyrata* showed parent-of-origin-specific reduction in germination rate (Hämälä et al. 2017), suggesting a divergence in EBN between the two outcrossing populations. EBN divergence-related hybrid seed lethality has been reported between other outcrossing species (Lafon-Placette et al. 2017; Roth et al. 2018; Coughlan et al. 2020). This supports the idea that besides mating system divergence, additional factors may explain EBN divergence between lineages. The genetic divergence between Eurasian *A. lyrata*, to which the Russian population of Hämälä et al. (2017) belong, and North American *A. lyrata* (Ross-Ibarra et al. 2008; Schmickl et al. 2010) is likely one of them. Also, ongoing gene flow between selfers and outcrossers in the Great Lakes region, a likely scenario since the selfers still outcross to a certain extent (Fuxe et al. 2010), may slow down the emergence of incompatibilities between the two lineages. Nevertheless, different EBNs implies a quantitative/hierarchical divergence rather than a qualitative one, unlike a “classic” case of BDM incompatibility. Instead, it has been suggested that even among outcrossing species, the intensity or efficacy of parental conflict differs as it does between selfing and outcrossing species, and this leads to a quantitative difference of EBN between these species. *Arabidopsis lyrata* went through a strong bottleneck during the colonization of North America, leading to reduced genetic diversity of the North American populations (Schmickl et al. 2010), and through additional bottlenecks during multiple transitions to selfing (Fuxe et al. 2010; Willi et al. 2018). It was recently shown that genetic diversity of a given lineage positively correlates with its EBN (Coughlan et al. 2020). The authors of the study suggested that genetic diversity determines the effective number of paternal contributors in a population, which can affect the strength of parental conflict. Alternatively, we propose that this may be explained by the role of effective population size on selection efficiency (Chen et al. 2017), including the efficiency of parental conflict/sexual selection as selective processes. In other words, the higher EBN in Central European *A. lyrata* outcrossers compared to the North American outcrossers may be explained by a reduced selection efficiency on EBN in the North Americans due to the bottlenecks they experienced. Nevertheless, our study included only one selfing and one outcrossing *A. lyrata* population. Thus, a more exhaustive study across the distribution range



of *A. lyrata* in combination with demographic inference of the selected populations is now required to reconstruct the EBN evolutionary history across the species and its potential consequence on intraspecific hybridization barriers.

In conclusion, we show that despite a relatively short divergence time, a strong postzygotic barrier affects hybrid seed viability between two intraspecific *A. lyrata* lineages. Hybrid seed inviability can therefore evolve rapidly, and in the case of *A. lyrata*, this rapid evolution may be driven by divergence in mating systems and EBN.

### Conflict of Interest

None of the authors have a conflict of interest to declare.

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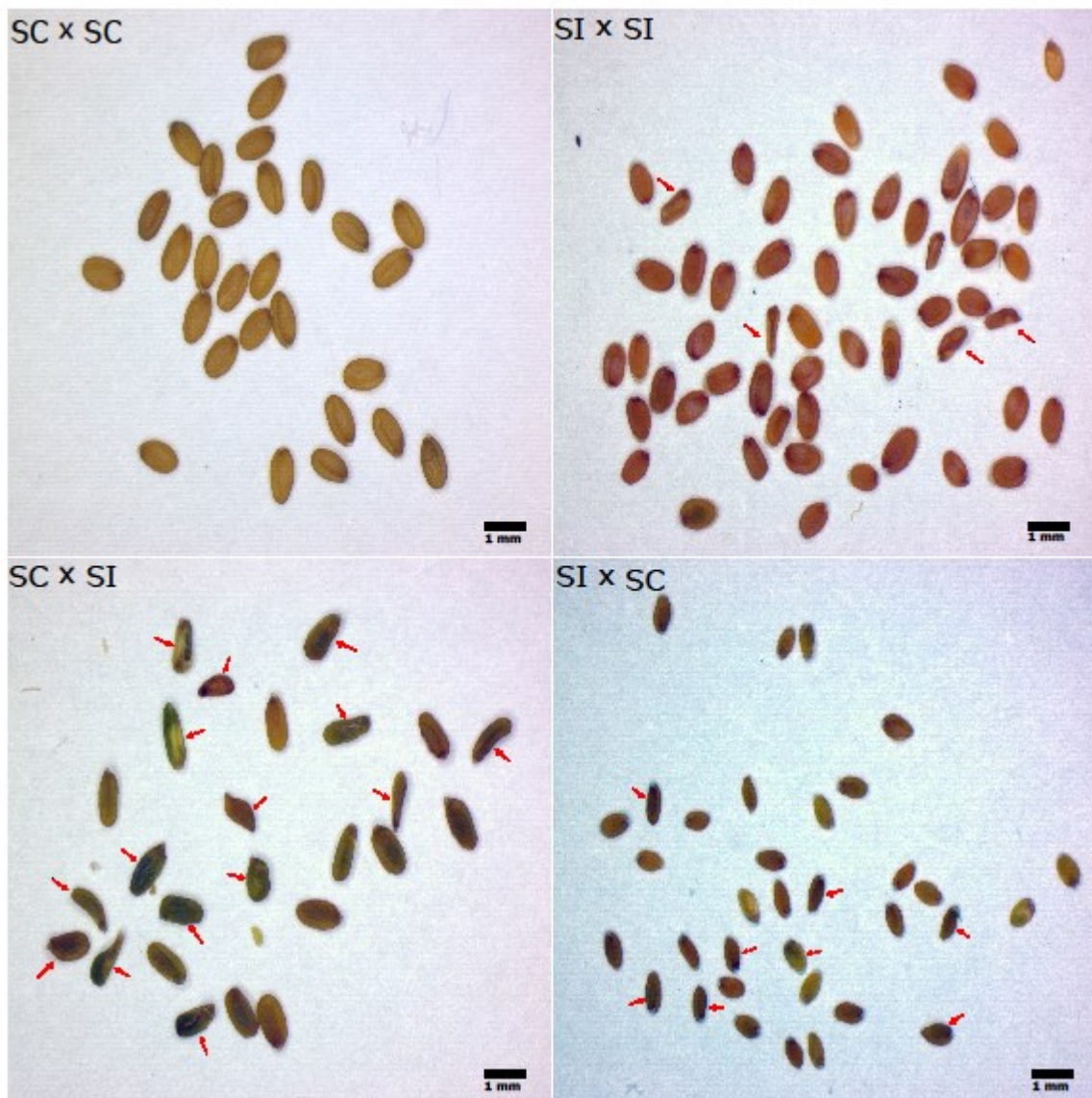
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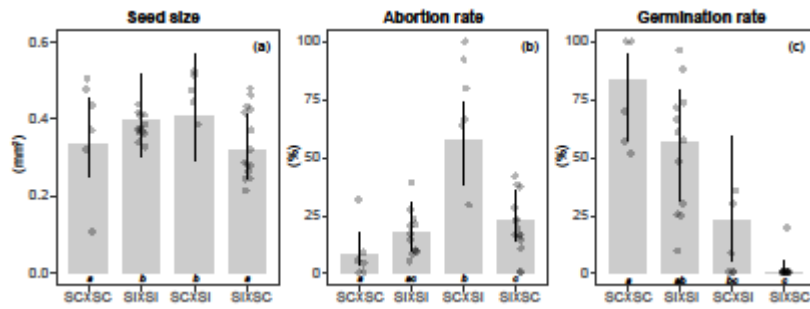
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### Figure legends

**Figure 1. Phenotypic defects of *Arabidopsis lyrata* hybrid seeds.** Pictures of seeds obtained from parental and reciprocal hybrid crosses between selfer (SC) and outcrosser (SI) populations of *A. lyrata*. By convention, crosses are always noted as ♀ × ♂. Arrows show seeds considered as aborted. Scale bars: 1 mm.

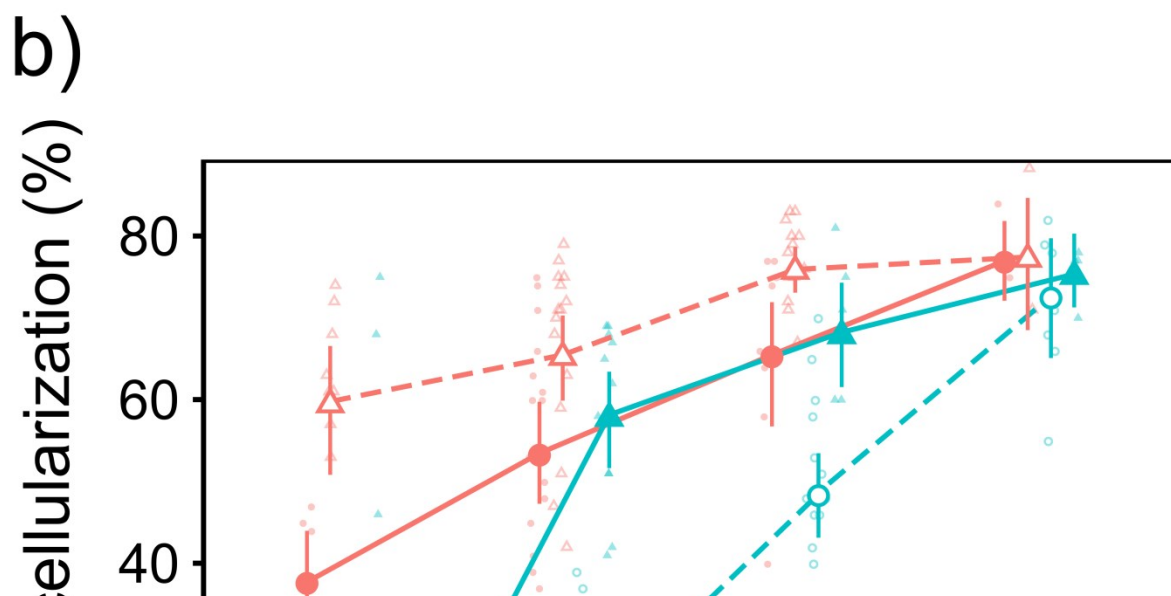
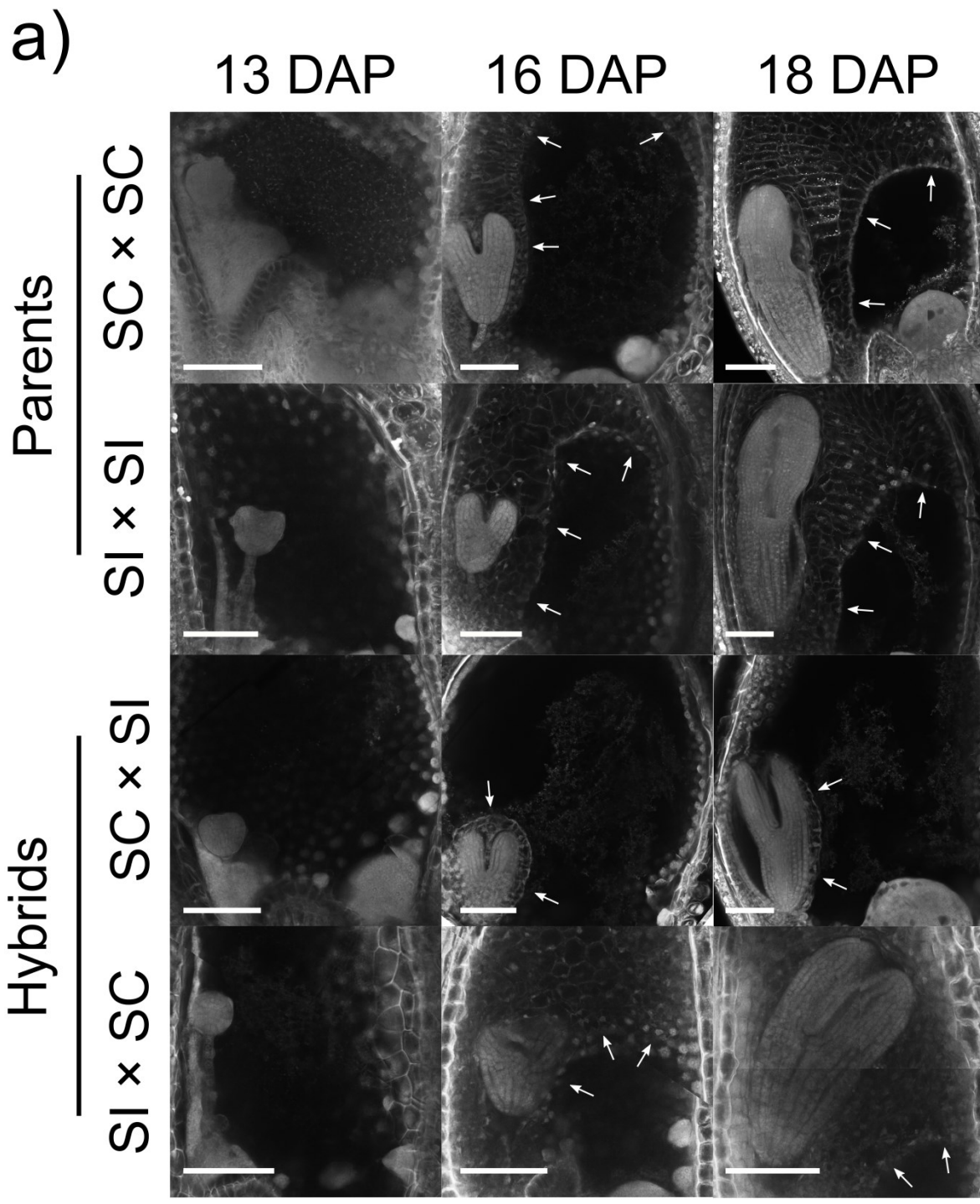


**Figure 2. Hybrid seeds show impaired viability.** Differences in seed size (a), seed abortion rate (b) and seed germination rate (c) among parental crosses (noted ♀ × ♂) of selfer (SC) and outcrosser (SI) populations of *Arabidopsis lyrata* (n = 145 seeds from 6 crosses for SC × SC; n = 451 seeds from 12 crosses for SI × SI, n = 193 seeds from 7 crosses for SC × SI and n = 268 seeds from 14 crosses for SI × SC). GLMM-based estimates (gray bars) are displayed along with their 95% confidence intervals (error bars) and observed values (dots). Groups labeled with different lowercase letters are significantly different from each other ( $\alpha = 5\%$ ).



**Figure 3. Hybrid seeds show reciprocal endosperm cellularization disturbance.** a) Examples of embryo and endosperm development in parental and hybrid seeds across the surveyed time points (13-18 DAP). Arrows indicate the border between cellularized and uncellularized endosperm. DAP: days after pollination. Scale bars: 100  $\mu\text{m}$ ; b) Differences in endosperm cellularization rate among parental crosses (noted  $\text{♀} \times \text{♂}$ ) of selfer (SC) and outcrosser (SI) populations of *Arabidopsis lyrata* across four developmental stages (see Supporting Information Table S3 for the description of embryo stages). Beta regression estimates (geom. shapes) are displayed along with their 95% confidence intervals (error bars) and observed values (dots). Stages 1-2 are not represented on the figure as endosperm cellularization was not detected for most of the samples (see Supporting Information Table S1 for details).





## Tables

**Table 1.** Summary of GLMMs testing for the fixed effects of female and male mating system (outcrossing vs. selfing) and their interaction on size, abortion rate and germination rate of seeds for *Arabidopsis lyrata*. Tests of the random effects (identity of maternal plants and pollen donors) are also displayed. Table shows likelihood ratio test statistics ( $\chi^2$ ) and associated probabilities (p). Results significant at  $\alpha = 5\%$  are highlighted in bold.

Model terms	Seed size		Abortion rate		Germination rate	
	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
Fixed effects (mating system)						
Female	0.06	0.8140	0.09	0.7688	<b>4.91</b>	<b>0.0267</b>
Male	<b>6.72</b>	<b>0.0096</b>	3.58	0.0585	0.41	0.5244
Female $\times$ male	0.04	0.8454	<b>24.30</b>	<b>&lt; 0.0001</b>	<b>33.30</b>	<b>&lt; 0.0001</b>
Random effects						
Maternal individual	<b>16.76</b>	<b>&lt; 0.0001</b>	2.17	0.1410	<b>6.13</b>	<b>0.0133</b>
Paternal individual	< 0.01	0.9502	<b>11.96</b>	<b>0.0005</b>	< 0.01	1.0000

**Table 2.** Summary of beta regression model testing for the effect of female and male mating system (outcrossing vs. selfing), embryo developmental stage (stages 3 – 6) and their interactions on endosperm cellularization rate for *Arabidopsis lyrata*. Table shows likelihood ratio test statistics ( $\chi^2$ ), degrees of freedom (df) and associated probabilities (p). Results significant at  $\alpha = 5\%$  are highlighted in bold.

Model terms	$\chi^2$	df	p
Female	<b>21.9</b>	<b>1</b>	<b>&lt; 0.0001</b>
Male	<b>60.1</b>	<b>1</b>	<b>&lt; 0.0001</b>
Stage	<b>165.5</b>	<b>3</b>	<b>&lt; 0.0001</b>
Female $\times$ stage	<b>9.4</b>	<b>3</b>	<b>0.0249</b>
Male $\times$ stage	<b>86.0</b>	<b>3</b>	<b>&lt; 0.0001</b>

Female × male	<b>6.8</b>	<b>1</b>	<b>0.0090</b>
Female × male × stage	5.7	3	0.1299

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