# Sexual selection, sexual conflict and mating systems: the *triumvirate* ruling over plant (reproduction) evolution



Přírodovědecká fakulta

HABILITAČNÍ PRÁCE

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"That's what I mean by an unstoppable team, one that brings diverse gifts to bear on the team's goals through a shared sense of purpose and a deep commitment to each other. You can assemble as many individual superstars as you'd like, but they won't become unstoppable unless they believe in each other and in their collective mission."

*"Unstoppable Teams", Alden Mills.*

# Table of Contents

# Acknowledgements

No one is self-made, and I am no exception. A Japanese psychological method called *Naikan* aims at making us realize this, by reflecting on all the things that others had brought to us. This list goes on forever in my case, and I feel extremely blessed it is this way.

To start with, the research I performed so far follows the heritage of past (sometimes centuries-old) scientific works and ideas. It is also the fruit of discussions, experiments, analyses, thoughts, feedback, published works from team members and contemporary colleagues, either from the same lab or more distant ones. It is well known that there is a time when all the elements are gathered for new ideas to emerge, explaining why some discoveries happen at the same time in different parts of the world. Because of this reason, there is probably no such thing as "my "ideas, but instead, there are fruits of past legacy and contemporary exchanges of thoughts. Someone said that creativity boils down to a simple equation: copy + paste + transform. I think this is particularly true in science.

Then, the road from being a PhD student to a PI is full of banana peels and turtle shells. On this road, companions make the journey much safer and easier. Two major companions are Roswitha Schmickl and Marek Slovák, with whom I built Lab Alliance, a safe haven for students and young researchers to learn, make mistakes, and grow. Having companions with shared values and fights are important to construct and sustain positive change, and Matyáš Fendrych has been a really valuable road mate in this respect, with whom I have been creating small and bigger projects. Thanks to Marian Novotný too, for being a great guide and discussion partner about Czech science and education. Finally, thanks to companions who shared a longer piece of the road with me, back from Sweden, and with whom I've experienced similar dreams, issues and solutions transitioning from being a postdoc having fun to becoming an overstressed young PI: Iva Mozgová and Duarte Figueiredo. Especially thanks to Iva, who helped me settle in the wild Czech Republic and its specificities.

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# List of selected articles

*Team members are indicated in bold.*

**Paper I.** İltaş Ö, Svitok M, Cornille A, Schmickl R, **Lafon Placette C**\*. 2021. Early evolution of reproductive isolation: a case of weak inbreeder/strong outbreeder leads to an intraspecific hybridization barrier in *Arabidopsis lyrata*. Evolution. **\*corresponding author**

**Paper II. Lafon Placette C**\*. 2020. Endosperm genome dosage, hybrid seed failure, and parental imprinting: sexual selection as an alternative to parental conflict. American Journal of Botany 107: 17–19. **\*corresponding author**

**Paper III. Lafon-Placette C**, Hatorangan MR, Steige KA, Cornille A, Lascoux M, Slotte T, Köhler C. 2018. Paternally expressed imprinted genes associate with hybridization barriers in Capsella. Nature Plants 4: 352.

**Paper IV. Lafon-Placette C**, Johannessen IM, Hornslien KS, Ali MF, Bjerkan KN, Bramsiepe J, Glöckle BM, Rebernig CA, Brysting AK, Grini PE, et al. 2017. Endosperm-based hybridization barriers explain the pattern of gene flow between *Arabidopsis lyrata* and *Arabidopsis arenosa* in Central Europe. Proceedings of the National Academy of Sciences 114: E1027–E1035.

**Paper V. Lafon-Placette C**, Köhler C. 2014. Embryo and endosperm, partners in seed development. Current Opinion in Plant Biology 17: 64–69.

**Paper VI. Lafon-Placette C**, Köhler C. 2016. Endosperm-based postzygotic hybridization barriers: developmental mechanisms and evolutionary drivers. Molecular Ecology 25: 2620–2629.

**Paper VII.** Rebernig CA, **Lafon-Placette C**, Hatorangan MR, Slotte T, Köhler C. 2015. Non-reciprocal Interspecies Hybridization Barriers in the Capsella Genus Are Established in the Endosperm. PLOS Genetics 11: e1005295.

**Paper VIII.** Dziasek K, Simon L, **Lafon-Placette C**, Laenen B, Wärdig C, Santos-González J, Slotte T, Köhler C. 2021. Hybrid seed incompatibility in Capsella is connected to chromatin condensation defects in the endosperm. PLOS Genetics 17: e1009370.

**Paper IX.** Kučera J, Štubňová EG, Svitok M, Martónfiová L, **Lafon Placette C** and Slovák M (2021). Eunuchs or females? Causes and consequences of gynodioecy on morphology, ploidy and ecology of Stellaria graminea L. (Caryophyllaceae). *Front Plant Sci*, doi: 10.3389/fpls.2021.589093.

**Paper X.** Bachmann JA, Tedder A, Laenen B, Fracassetti M, Désamoré A, **Lafon Placette C**, Steige KA, Callot C, Marande W, Neuffer B, Bergès H, Köhler C, Castric V, Slotte T (2019). Genetic basis and timing of a major mating system shift in *Capsella*. *New Phytol* 224: 505-517.

**Paper XI. Lafon-Placette C**, Vallejo-Marin M, Parisod C, Abbott RJ and Köhler C (2016). Current plant speciation research: unravelling the processes and mechanisms behind the evolution of reproductive isolation barriers. *New Phytol* 209(1):29-33.

**Paper XII.** Morgan EJ, Čertner M, Lučanová M, Deniz U, Kubíková K, **Venon A**, Kovářík O, **Lafon Placette C**, Kolář F (2021). Disentangling the components of triploid block and its fitness consequences in natural diploid-tetraploid contact zones of Arabidopsis arenosa. *New Phytol*, doi: 10.1111/nph.17357.

**Paper XIII.** Svitok M, **Venon A**, Morgan E, **Chimetto G**, Deniz U, Kolář F, **Lafon Placette C**\*. Embryo and endosperm development remains robustly interconnected despite genetic, ploidy and environmental variation in *Arabidopsis arenosa*. *Under revision in New Phytologist.* \* **corresponding author**.

**Paper XIV. İltaş Ö**, Slovák M, **Lafon Placette C***. The coevolution between pollen tube growth and style length: a molecular love story. *To be submitted to Molecular Ecology.* * **corresponding author**.

# Introduction

Is sex in plants comparable in any way to what happens in animals? In a sense, not really: sex in plants includes a myriad of different forms, which are way more diverse than in animals – hermaphroditism, dioecy, outbreeding, selfing or apomixis, to cite only a few. On the other hand, reproductive processes – from gamete production, mating, fertilization and even viviparity – are actually analogous in flowering plants and animals. Therefore, to which extent school-case evolutionary processes in animals, such as sexual selection and sexual conflict, are significant enough to consider in plants? Do they substantially shape the evolution of sexual traits, genes & genomes in plants? Does this have a general impact on plant evolution and the emergence of new species? Finally, does the diversity of sexual strategies found in plants affect sexual selection and conflict? Or vice versa?
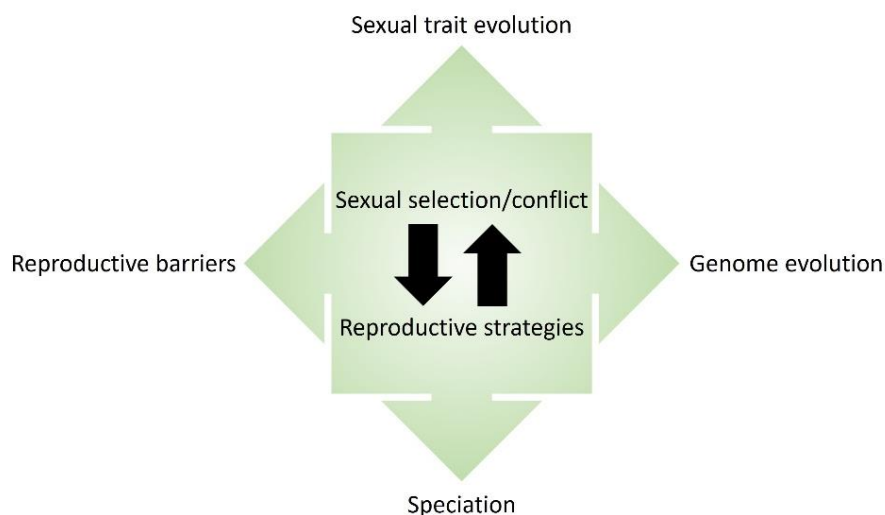


**Figure 1. Core questions of the Plant Evo Repro lab research.**

The line of research presented in this thesis has been revolving around these questions, and is about plant reproduction and its interlink with plant evolution and speciation (Figure 1). During my career, I first focused on a specific aspect of these processes, namely hybrid seed lethality. From this phenomenon, the research of my team, the Plant Repro Evo lab (https://lab-allience.natur.cuni.cz/plantreproevo), has now expanded to broader evolutionary processes related to sexual reproduction, namely sexual selection, sexual conflict and shifts in reproductive strategies. Across all my career, I have made a point to provide answers from different fields of research, at different time and space scales, and using different methods, in order to bring a holistic view on the studied phenomena. This thesis reflects this intellectual journey. Finally, I want to emphasize that this work, spanning a decade or research, is the fruit of collaborations, discussions, inspiration from co-workers, peers, and students.

# Part I – Hybrid seed lethality: a model to study the role of sexual conflict in plant reproduction evolution and speciation

## A longstanding interest from geneticists and breeders

The first human-made interspecific plant hybrid date back to 1717. It was created by an English gardener, Thomas Fairchild, who crossed two species of Dianthus, *D. caryophyllus* and *D. barbatus* to produce the hybrid known as "Fairchild's Mule" (Figure 2; Leapman, 2001). Beyond the obvious revolution this finding would become for plant breeding, Fairchild crossing experiment was no less than the discovery of plant sex. To his own expenses, Mr Fairchild most probably faced and discovered another phenomenon: hybrid seed lethality. While he did not mention it, later work, crossing *D. caryophyllus* and *D. barbatus*, reported only a few viable hybrid seeds out of 200 hand pollinations (Guobao *et al.*, 1995). Fairchild's mule, therefore, must have been the fruit of hard, tedious work, guided by Mr Fairchild's perseverance, which is a lesson to learn in itself.



**Figure 2. The first human-made hybrid, Fairchild's mule.** A) Dianthus caryophyllus; B) D. barbatus; C) and D) *D. caryophyllus* × *D. barbatus* hybrid (Fairchild's mule), alive plants and Fairchild's herbarium specimens, respectively. Adapted from https://www.ft.com/content/64451cc4-07f3-11e7-ac5a-903b21361b43.

During his life, Mr Fairchild produced other interspecific hybrids, and this would become, and still is, a major strategy in plant breeding. Consequently, hybrid seed lethality being a widespread phenomenon across angiosperms (Gärtner, 1827; Gill & Waines, 1978; Lafon-Placette & Köhler, 2016; Florez-Rueda *et al.*, 2016), it has been, and to a certain extent still is, a major obstacle in these breeding strategies. Hybrid seed lethality gained scientific interest soon after Fairchild's work, notably with Gärtner conceptualizing the potential causes of the inviability of *Primula* hybrid seeds (Gärtner, 1827). The hybrid seed lethality scientific "buzz" would come however at the beginning of the 20th century, where important discussions around genetic incompatibilities (Bateson, 1909), the Modern Synthesis (Huxley, 1942) and the concept of species (Mayr, 1963) led to seminal works on the physiological and genetic basis of hybrid seed incompatibility (Sansome *et al.*, 1942; Cooper & Brink, 1945; Brink & Cooper, 1947; Wangenheim, 1957; Woodell & Valentine, 1961; Valentine & Woodell, 1963; Williams & White, 1976; Gill & Waines, 1978; Johnston & Hanneman, 1982), later refined and further investigated, especially at the genomic level, in recent years (**Papers III, VII**; Burkart-Waco *et al.*, 2012; Garner *et al.*, 2016; Florez-Rueda *et al.*, 2016; Roth *et al.*, 2019; Coughlan *et al.*, 2020; Kinser *et al.*, 2021).

Therefore, the longstanding interest in hybrid seed lethality provides a solid knowledge on its key genetic and developmental patterns, helping to investigate the many remaining shadows of this phenomenon. In particular, my work is part of recent scientific efforts in the improvement, expansion and revival of old key concepts explaining hybrid seed lethality, especially exploring the interconnection between genomic mechanisms and evolutionary processes (**Papers I, II, III, IV, VI, VII**). The next sections will summarize the knowledge acquired so far.

## Largely shared developmental, molecular and genetic causes across angiosperms

One may intuitively think that the processes underlying hybrid seed lethality, just as any genetic incompatibility, are specific to each plant family or even to each sister pair. It is not so. Probably the most striking aspect of hybrid seed lethality is that it shares developmental, genetic, molecular and evolutionary components across the whole angiosperm clade (**Papers III, VI**; Gill & Waines, 1978; Johnston & Hanneman, 1982; Roth *et al.*, 2019; Städler *et al.*, 2021). An important theoretical aspect of my efforts has been to unify available evidence concerning hybrid seed lethality, and to clarify the patterns common to all known angiosperm cases (**Papers I, II, III, IV, VI**). These patterns are explained below.

### a) Developmental aspects: why the hybrid endosperm fails to play its role

Angiosperms are characterized by double fertilization (Navashin, 1898; Guignard, 1899), which means that a pollen grain contains two sperm cells (male), and one of these cells will merge with the egg cell (female) to form a zygote – the progeny, while the other sperm cell will fuse with the central cell to form the endosperm, a 'dead end' tissue whose function is to support and nourish the growing embryo. Embryo and especially endosperm development varies between families. For example, in the so-called "nuclear" type of endosperm development, endosperm may first be present as a syncytium, where nuclei freely divide without cytokinesis, and only later in seed development, cell walls may form around each nucleus. This type of endosperm is found in *Brassicaceae* or *Poaceae*, for example (Ishikawa *et al.*, 2011; Stoute *et al.*, 2012). In another type of endosperm development, so called "cellular", as the name indicates the endosperm is cellular *ab initio*, i.e. each nuclear division is always followed by cytokinesis (Baroux *et al.*, 2002). This type of endosperm is found in *Solanaceae*, for example (Roth *et al.*, 2019). Also, the endosperm may persist until seed maturity and provide nutrients during seed germination, as in *Poaceae*, or vice versa, it may be completely absorbed by the growing embryo upon seed maturity, like in legumes.

Despite the wide range of variation in endosperm development across angiosperms, the same fact is found across the clade: the defects in inviable hybrid seeds reside in the endosperm, not in the embryo. This conclusion is supported by decades of a breeding method called "embryo rescue" as a way to bypass hybrid seed lethality and produce hybrid plants (**Papers I, IV**; Inomata, 1993): if hybrid embryos are excised from the hybrid seed and cultivated on "synthetic" endosperm (*in vitro* medium containing all necessary nutrients), these embryos survive and gives rise to healthy plants. Again, endosperm failure is a common cause to most known cases of angiosperm hybrid seed lethality (**Papers III, IV, VI**; Cooper & Brink, 1945; Ishikawa *et al.*, 2011; Roth *et al.*, 2019). This suggests that hybrid seed lethality is caused by ancestral mechanisms related to the core endosperm development common to all angiosperms.
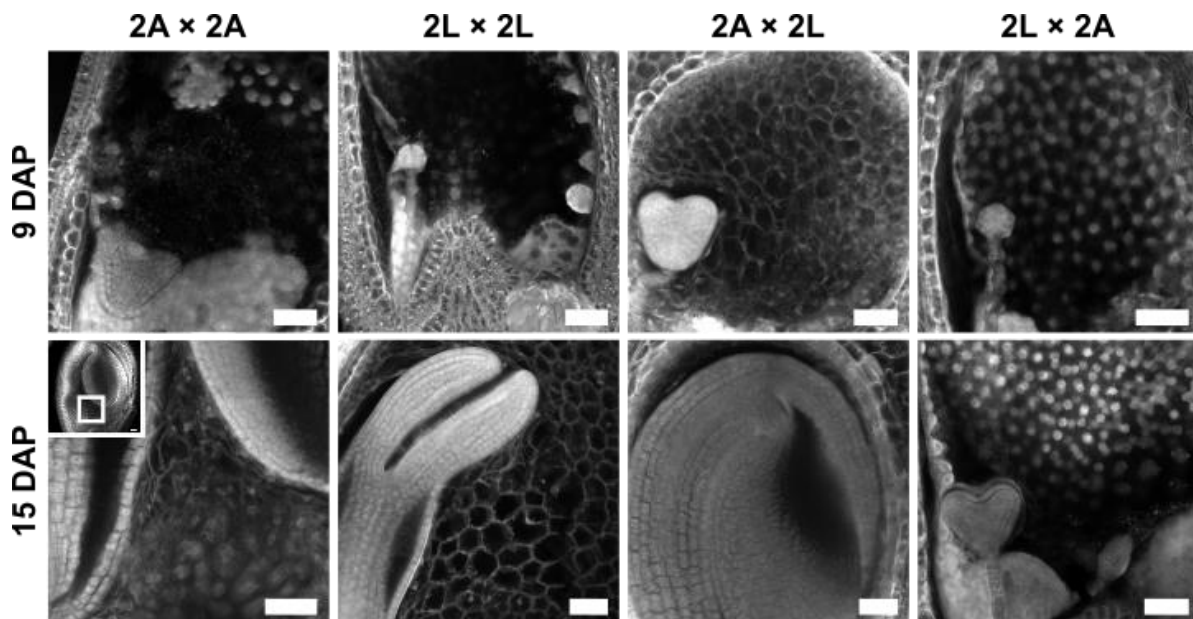
**Figure 3. Impaired viability of diploid *A. lyrata* × *A. arenosa* reciprocal hybrid seeds is correlated with asymmetric endosperm cellularization defects.** Feulgen-stained seeds of *A. lyrata*, *A. arenosa*, and reciprocal hybrids are depicted. 2A: diploid *A. arenosa*; 2L: diploid *A. lyrata*. Early (9 DAP) and late (15 DAP) time points are displayed. At 9 DAP, *A. arenosa* × *A. lyrata* seeds show precocious endosperm cellularization while it is not initiated in any of the two parents. Vice versa, at 15 DAP, *A. lyrata* × *A. arenosa* seeds did not initiate endosperm cellularization, while it is completed in both parents. For a better display of the remaining endosperm in *A. arenosa*, the larger inlay represents the entire seed and the smaller inlay represents the zoomed-in endosperm region. Scale bars = 50 μm. Adapted from (**Paper IV**).

Nevertheless, it may seem, at least at first sight, that hybrid endosperm problems are specific to the type of endosperm development. Indeed, in all species with the "nuclear" type of endosperm, the same defect is found: the developmental transition ending the syncytial phase is disturbed (Figure 3; **Paper III, IV**; Ishikawa *et al.*, 2011; Stoute *et al.*, 2012). The timing of this developmental is crucial for the nourishment and survival of the embryo, and its impairment is believed to be the main cause for hybrid embryo lethality (**Paper V**; Hehenberger *et al.*, 2012). In addition, in extreme cases, the expansion and nuclei proliferation of the hybrid endosperm is found to be impaired (**Papers III, IV**). This may explain why *in vitro* "synthetic" endosperm can recue these hybrid embryos. The hybrid endosperm defects in species with the "cellular" type of endosperm are somewhat different, and also less well studied, therefore less clear (Roth *et al.*, 2018; Städler *et al.*, 2021). In this case, the endosperm defects include problems of cell division, cell size, and cell vacuolation (Roth *et al.*, 2018; Kinser *et al.*, 2021). Small cells are usually found in meristematic tissues, while mature cells are usually large and vacuolated (Karlsson *et al.*, 2000), suggesting that the hybrid endosperm in the 'cellular' type encounters problem of cell division and expansion/maturation. Therefore, the problems in the "nuclear" and "cellular" hybrid endosperms seem different, but actually converge: both encounter a disturbance in their growth, involving problems related to nuclear/cell division or expansion. The problems also converge at the molecular level: genes regulating endosperm development, known as *AGAMOUS-LIKE* transcription factors, are found misregulated both in "nuclear" and "cellular" hybrid endosperm (**Paper VII**; Roth *et al.*, 2019). Another important pathway impaired in both types of hybrid endosperm is auxin production and signalling, a phytohormone known for its role in cell expansion and division (Roth *et al.*, 2019; Batista *et al.*, 2019a).

Therefore, while more work is required, especially to characterize hybrid endosperm defects with species having a cellular endosperm, it does seem that a core physiological process can explain hybrid seed lethality across angiosperms, affecting endosperm expansion, proliferation and maturation.

### b) A genetic dosage hypothesis unifying interploidy and interspecific hybrid seed failure

The similarities of hybrid seed defects across angiosperms do not stop here. They also encompass genetic processes and involves a specific phenomenon: the genome dosage sensitivity of the endosperm. In most angiosperm species, the endosperm requires a specific ratio between maternal and paternal genome contribution. This ratio is in most species a 2:1 maternal:paternal ratio, meaning that the endosperm contains twice as many chromosomes coming from the maternal parent compared to the paternal one. This ratio is in most cases a requirement: if the balance shifts, such as after the hybridization between parents of different ploidies, then the endosperm shows developmental defects resulting in inviable seeds, a phenomenon coined as "triploid block" (Köhler *et al.*, 2010). This dosage requirement has been proposed to rely on paternal and maternal genomes bringing different components, namely gene products, that are complementary and probably act either antagonistically or as protein complexes to regulate endosperm development (Johnston *et al.*, 1980; Vinkenoog *et al.*, 2003; Dilkes & Comai, 2004).
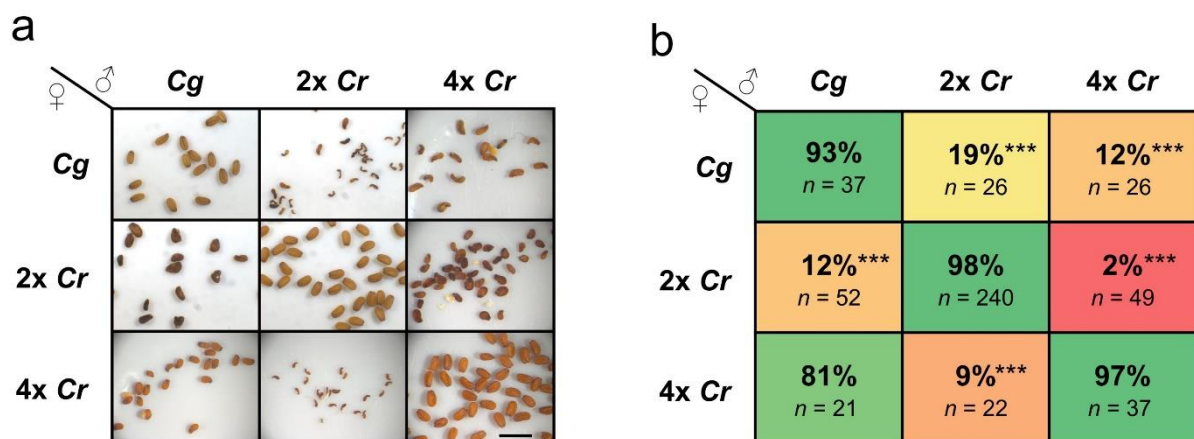


**Figure 4. Rescue of *C. rubella* × *C. grandiflora* hybrid seeds by *C. rubella* tetraploidization. a**, pictures of intra- and interspecific seeds involving the tetraploid *Cr* parent. The scale bar represents 2 mm. **b**, germination rates of the seeds shown in (a). The color gradient is relative to the germination rate from 0% (dark red) to 100% (dark green). *Cg*: *C. grandiflora*; *Cr*: *C. rubella*. 2x: diploid; 4x: tetraploid. n represents the number of seeds for each cross. P-values for one-sided Chi-square test are shown between the midparent (average of parental species) and the hybrid seeds value. Adapted from **Paper III**.

Now comes an interesting fact: the endosperm defects observed when using parents of different ploidies (= genome dosage imbalance aka triploid block) are nearly the same as when crossing two homoploid species (Köhler *et al.*, 2021). Suggesting from this fact that homoploid hybrid endosperm shows genome dosage imbalance would be relatively shaky without another set of evidence: homoploid hybrid seed lethality (and related endosperm defects) can be bypassed by doubling the number of chromosomes of one parental species and using it for the cross (Figure 4; **Papers III, IV**; Johnston & Hanneman, 1982; Ishikawa *et al.*, 2011). Doubling the chromosomes of the other parental

species does not rescue the cross, rather the opposite: it leads to even more severe hybrid seed "symptoms" (**Paper IV**; Johnston & Hanneman, 1982). This set of evidence suggests that the failure of homoploid hybrid endosperm is due to a dosage imbalance between paternal and maternal genomes. In other words, two given species may show a quantitative difference in their genomes that manifest itself in the endosperm, one having a higher dosage than the other. This concept has been coined as Endosperm Balance Number or EBN (Johnston *et al.*, 1980). As both species have the same number of chromosomes, it cannot be an imbalance affecting the whole genome, but most probably, an imbalance between a reduced set of genes, or, at least, a reduced set of genetic loci (potentially noncoding, see below). The nature of these genetic loci is explored in following sections of this thesis.

Importantly, these observations are valid across angiosperms, including rice, Brassicaceae, or Solanaceae, suggesting again that hybrid seed lethality shares a core mechanism inherent to the dosage sensitivity of the endosperm, an ancestral property common to most angiosperms (excluding apomictic species, which lost such a property).

### c) Genomic imprinting: the usual suspect

Another common feature of hybrid seed lethality across all angiosperms is its parent-of-origin pattern: the defects of the hybrid seed will be different if one of the species acts as maternal or paternal parent (**Papers III, IV**; Cooper & Brink, 1945; Woodell & Valentine, 1961; Valentine & Woodell, 1963; Ishikawa *et al.*, 2011; Roth *et al.*, 2018; Kinser *et al.*, 2021). The defects affecting the seed, and more precisely the endosperm, are even opposite depending on the cross direction. For example, my work and the work of my research team has shown that in Capsella or Arabidopsis, in one cross direction, the endosperm is abnormally enlarged and fails to transition from a syncytium to a cellular endosperm, while in the other direction of the cross, the endosperm cellularization happens precociously, accompanied with reduced endosperm growth (**Papers I, III, IV**). In Solanum, one cross direction gives rise to small condensed endosperm cells, while the other direction gives enlarged vacuolated cells (Roth *et al.*, 2018). Both cross directions nevertheless lead to the same consequence, i.e. the endosperm failing its nourishing function and thus lethality of the embryo, but via antagonistic physiological means.

In any case, the parent-of-origin aspect of hybrid endosperm defects led to propose early on that the paternal and maternal genomes do not play the same function in the endosperm (Haig & Westboy, 1991): despite being in the same nucleus, each likely provides different gene products, i.e. they express a different set of genes. At the molecular level, this suggests that paternal and maternal genomes in the endosperm show a different epigenetic landscape, and this phenomenon has been coined as genomic imprinting.
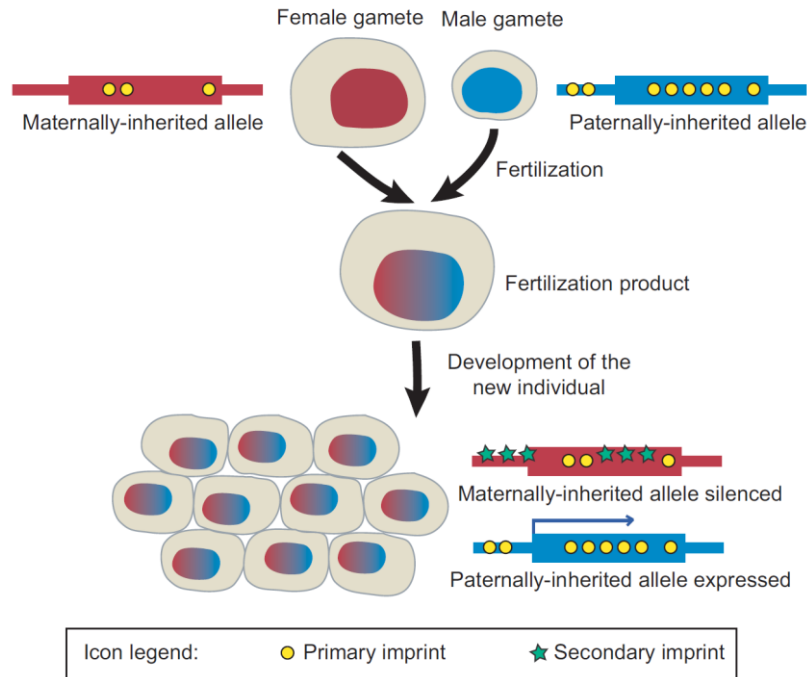
**Figure 5. Schematic representation of genomic imprinting.** It illustrates that central cell (female) and sperm cell (male) genomes are epigenetically marked differently prior to fertilization. Once merged in the endosperm, their epigenetic differences are enforced via secondary epigenetic imprints, altogether leading to paternally and maternally expressed genes (in the figure, the example of a paternally expressed gene only is shown). Adapted from (Rodrigues & Zilberman, 2015).

Genomic imprinting is an epigenetic phenomenon leading to parentally biased gene expression, and this phenomenon predominantly occurs in the endosperm (Batista & Köhler, 2020). It is the result of the regulation of noncoding elements, namely transposable elements (TEs), during gametogenesis and sexual reproduction, presumably whose role is to reinforce TE silencing and maintain genome integrity from a generation to the next (Batista & Köhler, 2020). In short, the maternal genome in the central cell (the female cell contributing to the endosperm) undergoes a hypomethylation prior to fertilization, and this hypomethylation targets preferentially TEs (Figure 5; Ibarra *et al.*, 2012; Rodrigues & Zilberman, 2015). To the contrary, DNA methylation in sperm cells is reinforced during male gametogenesis, especially at TE loci (Calarco *et al.*, 2012). Thus, the two parental genomes show different epigenetic landscapes prior to fertilization, and this epigenetic difference between maternal and paternal genomes is then maintained in the endosperm (Ibarra *et al.*, 2012; Batista & Köhler, 2020). The parent-specific epigenetic landscape and regulation of TEs eventually affect neighbouring genes, which become expressed in a parent-of-origin-specific manner = imprinted (Batista & Köhler, 2020). Genes can be maternally or paternally expressed, meaning that either the maternal or the paternal allele of the endosperm is expressed, respectively.

From numerous studies of imprinted genes across angiosperms, it appears clear that maternally expressed genes have different biological functions compared to paternally expressed ones (Hatorangan *et al.*, 2016; Chen *et al.*, 2018), consistent with the idea that paternal and maternal genomes are complementary, providing different gene products, and this may explain the parent-of-origin feature of hybrid seed lethality (Batista & Köhler, 2020; Städler *et al.*, 2021). Another evidence indirectly supports this idea: genomic imprinting is extremely variable between species and even within such. In other words, the set of genes that are imprinted is very different between lineages

within the same species or between closely related species, not even mentioning between more distant species (Pignatta *et al.*, 2014; Hatorangan *et al.*, 2016; Chen *et al.*, 2018). This suggests that in the case of a hybridization event, the maternally expressed genes from one lineage may not be able to complement the function of the paternally expressed ones from the other lineage. As imprinted genes are often involved in transcription regulation, and even regulate each other's expression (Hatorangan *et al.*, 2016; Batista *et al.*, 2019b), this lack of complementarity between parental genome may lead to a domino effect, with broad deregulation of imprinted genes and the genes they regulate, overall leading to the observed pleiotropic endosperm developmental defects. This is what I and co-workers suggested, as we showed that imprinted genes are preferentially deregulated in hybrid seeds between Capsella species (**Paper III**).
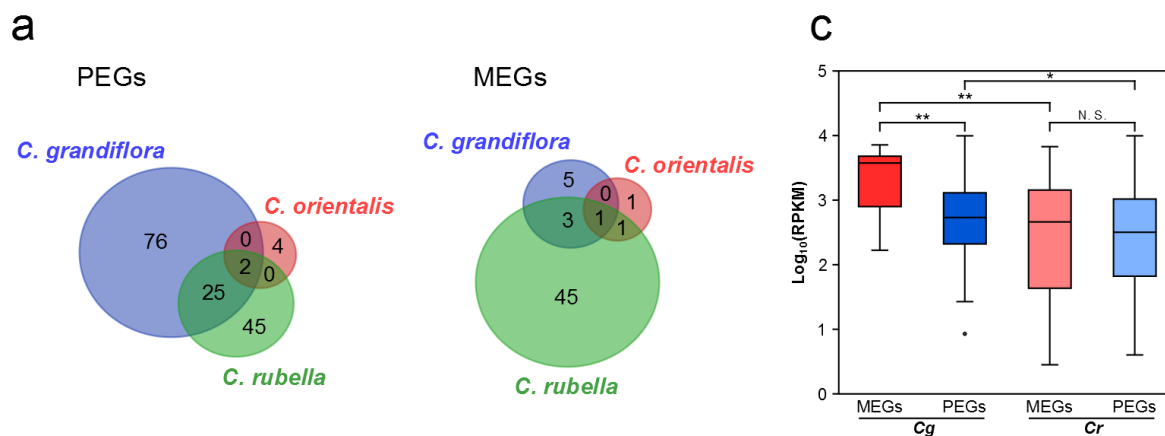
**Figure 6. Species-specific number and expression of paternally expressed imprinted genes is an indicator for endosperm effective ploidy (EBN).** The EBN ranking goes *C. grandiflora > C. rubella >> C. orientalis*. **a**, Total number and conservation of imprinted genes for the three Capsella species. **c**, Boxplot showing expression levels of *Cg* and *Cr* MEGs and PEGs in *Cg* and *Cr* seeds, respectively. Data are displayed as $\log_{10}$(RPKM). The n number of imprinted genes is the same as in a. Boxes bound the interquartile range divided by the median, and whiskers extend to the minimal and maximal values of the non-outlier range. * $P < 0.05$ and ** $P < 0.01$ based on two-sided Mann–Whitney test (**c**) are shown. *Cr*: *C. rubella*; *Cg*: *C. grandiflora*. PEGs: Paternally expressed genes; MEGs: Maternally expressed genes. Adapted from **Paper III**.

However, as explained in previous sections of this thesis, the problem in the hybrid endosperm is quantitative: a dosage imbalance between parental genomes, following the EBN concept, is likely to be the primary genetic cause. If imprinted genes are involved therefore, it is thus unlikely that the incompatibility between imprinted genes of two different species is simply a mismatch of functions, but instead, paternally and maternally expressed genes compensate each other in a quantitative way, which is expected, for example, if they play a role in antagonistic molecular pathways. In support with this idea, imprinted genes are causally linked to the endosperm failure caused by an interploidy hybridization in Arabidopsis: knocking down an imprinted gene leads to the suppression of the dosage sensitivity of the endosperm and the survival of triploid seeds (Kradolfer *et al.*, 2013; Wolff *et al.*, 2015). This line of evidence suggests that the imbalance between parental gene products in the hybrid seeds, under the EBN concept, may well happen between products from paternally and maternally expressed genes. In other words, the endosperm genome dosage of a given species may be determined by the number and expression level of imprinted genes, and upon hybridization, the number/expression of

such genes may not match those of another species, leading to a dosage imbalance and endosperm failure. This is what I and co-workers found in Capsella: the species showing the highest EBN/genome dosage also had more imprinted genes, and these genes were expressed to a higher level, compare to species with lower EBN (Figure 6; **Paper III**). Interestingly, this trend was not found in Solanum (Roth *et al.*, 2019), suggesting lineage-specific differences leading to imprinted genome dosage sensitivity.

### d) Causal loci are noncoding, nonrecombining regions. A reproductive barrier driven by selfish elements?

The above hypotheses regarding the role of genomic imprinting in hybrid seed lethality are somewhat not fully convincing in the face of current evidence. Indeed, some works in Solanum or Mimulus suggest that not only the expression of imprinted genes is altered in hybrid seeds, but also their imprinting status itself (Florez-Rueda *et al.*, 2016; Kinser *et al.*, 2021). In other words, genes being imprinted in the parental species are bi-allelically expressed or even switch from being paternally to become maternally expressed in the hybrid endosperm (Florez-Rueda *et al.*, 2016). This suggests that the epigenetic mechanisms upstream of imprinted gene regulation, and which are responsible for the imprinting itself (see above), are disturbed in hybrid seeds. Efforts to elucidate this question tend to show epigenetic changes in the hybrid endosperm (Kinser *et al.*, 2021; Florez-Rueda *et al.*, 2021), but the results remain equivocal, with no clear cause-consequence scenario emerging so far.

Especially, these studies remain for most parts correlative, and a causal approach is required to pinpoint and find the primary genetic loci causing hybrid seed lethality. A common approach in this direction is the genetic mapping of the incompatibility loci. A few efforts have been led by independent teams in different species (**Paper VIII**; Burkart-Waco *et al.*, 2012; Garner *et al.*, 2016). All these studies suggest that hybrid seed lethality has a multigenic basis, i.e. several small-effect loci are responsible for this phenomenon. A study in Mimulus found that the loci causing the lethality in one cross direction are not the same as the ones causing the lethality in the other direction of the cross, pointing at imprinted loci once again (Garner *et al.*, 2016).

Nevertheless, a striking observation to which I contributed (**Paper VIII**) is that all the loci responsible for hybrid seed lethality in Capsella are found in pericentromeric regions (Figure 7). These regions are typically gene-poor, enriched in transposable elements and show low recombination (Kent *et al.*, 2017). This finding points towards noncoding sequences as primary causes for hybrid seed incompatibility. Actually, recent studies have suggested that sperm cells are "loaded" with TE-generated small RNAs, and these small RNAs are involved in the dosage sensitivity of the endosperm in Arabidopsis (Erdmann *et al.*, 2017; Borges *et al.*, 2018; Martinez *et al.*, 2018). It could thus well be that the imbalance between different genome dosage (EBN) of different species, leading to hybrid endosperm failure, happen between small RNAs and other epigenetic regulators coming from paternal and maternal genomes, rather than a dosage between genes. Of course, this does not rule out imprinted genes as molecular mechanisms behind hybrid seed lethality: most probably, they are involved, but potentially as downstream collateral damage of the imbalance between epigenetic actors such as small RNAs originating in pericentromeric TEs, since imprinted genes are regulated by these epigenetic mechanisms (see above; Batista & Köhler, 2020). This "domino effect" scenario however remains to be fully elucidated.
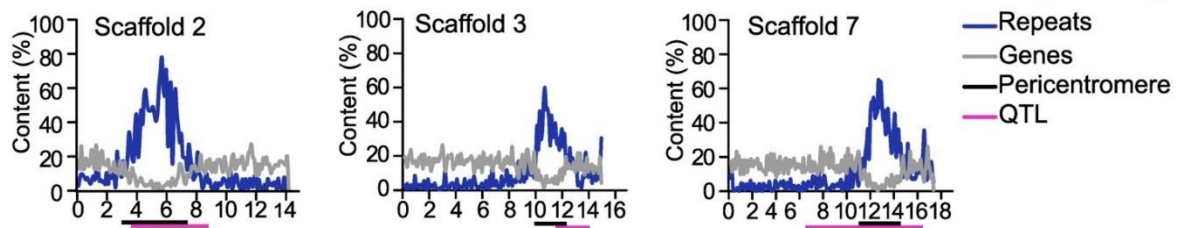
**Figure 7. The three QTLs associated with _C. rubella_ × _C. grandiflora_ hybrid seed lethality are localized in pericentromeric regions.** Plots of gene (grey line) and repeat (blue line) content on three scaffolds containing the identified QTLs. The magenta bars represent QTL regions and the black bars indicate pericentromeric regions as previously defined (Koenig _et al._, 2019). Adapted from **Paper VIII**.

Interestingly from an evolutionary point of view, centromeres are often found as "islands of divergence" (IoD; Christmas _et al._, 2021). IoDs are genomic regions that show high genetic divergence between lineages even in the face of gene flow (they "resist" gene flow), and because of this behaviour they are expected to contain genetic loci responsible for reproductive barriers (Christmas _et al._, 2021). Reduced recombination is another characteristic of IoDs, leading to potential speciation "supergenes". The fact that these "speciation genes" are likely noncoding DNA adds to the accumulating evidence showing that transposons are far from being "junk DNA" and instead play a major role in evolution (Feschotte & Pritham, 2007; Gao _et al._, 2015).

Altogether, in Capsella at least, it thus seems that centromeres act as reproductive barrier loci, and are likely to show high divergence between species. However, the exact nature of the hybrid seed lethality loci remains mysterious: pericentromeric regions are "blackboxes" that are nearly impossible to study with currently available genomic tools due to the high content of repeated sequences. It is therefore not clear whether transposable elements, other repeats, small RNAs or the eventual rare genes present in pericentromeric regions, are the primary causal elements leading to hybrid seed lethality.

## Evolutionary drivers and consequences

### a) Parental conflict

It now appears relatively clear that different lineages show different strength of endosperm genome dosage (EBN hereafter). However, one can wonder why lineages would evolve different EBNs in the first place. A parsimonious hypothesis would be mere stochasticity: with time and genetic divergence, lineages acquire a different set of imprinted genes or a different structure of their epigenetic regulators (TE insertions, variability in epigenetic states…). And indeed, natural variation in imprinting status and causal epigenetic states in the endosperm has been evidenced (Pignatta _et al._, 2014, 2018).

This being said, a group of evidence suggests that species evolve different EBNs due to selective processes. Before going into more details about EBN divergence in the next section, we need to focus on the potential selective processes acting on endosperm parental dosage itself and the related molecular mechanism, genomic imprinting. As explained above, the imprinting of paternal and maternal genomes allows them to have distinct functions in the endosperm, even though they are merged in the same nuclei, via their specific epigenetic landscape and the set of genes they express. This parent-specific function is therefore a trait that selection can potentially act on.

And selection on this trait may be related to the resources accumulated in the endosperm (Haig & Westoby, 1989). Indeed, maternal resources are limited, meaning that each seed needs to share and

compete for them. The amount of resources in a given seed may influence the early growth and establishment of the seedling, thus being essential for early life. In an outcrossing lineage, a maternal plant usually bears seeds produced from different paternal contributors. The inclusive fitness of a given paternal contributor thus depends on the amount of maternal resources that its sired seed(s) can obtain: the more resources, the higher the fitness. Any paternal trait able to promote the amount of resources acquired by its progeny would therefore be under positive selection, and this is where parent-specific genome dosage and genomic imprinting comes into play (Haig & Westoby, 1989). As the paternal genome maintains its identity in the endosperm via genomic imprinting, it has the potential to influence the endosperm development and resource accumulation. Consistent with this idea, we know from interploidy crosses that introducing more paternal copies in the endosperm leads to larger endosperm, or in other words, the paternal genome promotes endosperm growth (Fort *et al.*, 2016). This is also in line with the function of key paternally expressed genes, regulating sugar transport or auxin biosynthesis leading to endosperm growth (Batista *et al.*, 2019a,b). Reframed around the EBN concept, this suggests that positive selection may favour paternal genomes that are able to increase their influence over endosperm development, i.e. with increased endosperm genome dosage (EBN). Selection on increased paternal EBN could happen by increasing the number of paternally expressed genes or their expression level, for example.

On the other hand, the maternal genome shows the opposite effect: it reduces endosperm growth (Fort *et al.*, 2016), and key maternally imprinted genes are expression repressors targeting paternally expressed genes among other genes (Grossniklaus *et al.*, 1998; Batista & Köhler, 2020). This antagonistic role suggests that maternal and paternal selective interests are opposite: the maximum fitness for a paternal contributor would mean lowest fitness for the maternal parent (= sexual conflict; Figure 8a). In the case of endosperm growth and resource allocation, indeed, if one paternal contributor gets more resource for its seeds, it necessarily implies that other seeds will get less resources, and this is likely to be deleterious for the maternal parent (Figure 8b; Haig & Westoby, 1989). Indeed, all seeds are equally related genetically to the maternal parent, therefore "sacrificing" some seeds for the benefit of others provides no selective advantage to the maternal parent, as it only means a smaller brood fit enough to survive. Instead the female fitness is likely to be maximal when all seeds get the same share of resources (Figure 8c). Therefore, positive selection on females is likely to act on an increased maternal genome dosage/EBN, counteracting the paternal genome influence over endosperm growth.

The colliding parent-specific interests, coined "parental conflict", are likely to lead to the antagonistic coevolution and arms race (Figure 8; "chase away" scenario) between maternal and paternal genome dosages (EBN), leading over time to an increased EBN of both parental genome, until an equilibrium is reached (Pizzari & Snook, 2003). This arms race may happen via the set of paternally and maternally expressed genes, however, this remains to be elucidated.
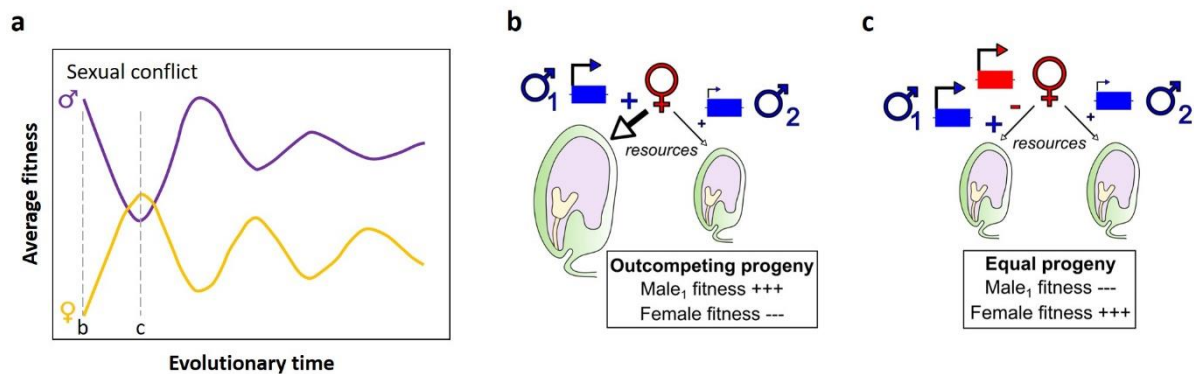
**Figure 8. Parental conflict in plants. a**) evolution of sex-specific fitness across time in case of a sexual conflict. As sex-specific selective interests are antagonistic, when a sex reaches a fitness optimum for the conflicting trait, the fitness of the other sex is at the lowest. This creates a strong selective pressure for the trait to evolve towards a higher fitness for the latter sex. The evolution of the conflicting trait will thus follow a "chase away" scenario, eventually reaching a trade-off between male and female selective interests. Adapted from (Pizzari & Snook, 2003). The time points with letters b and c in the graph are further illustrated in the case of parental conflict over seed resource allocation. **b**) A given paternal contributor able to drive more resources to its own progeny, via an increased endosperm dosage (mediated by paternally expressed genes), will be positively selected. This scenario is deleterious for the female fitness, as the rest of the progeny will be unfit due to not enough resources accumulated. **c**) This may select for the increase in maternal genome dosage via maternally expressed genes, limiting the "selfish" paternal drive and allowing an equal distribution of resources to the whole brood, i.e. the optimum female fitness.

### b)  Transition to selfing and the Weak Inbreeder/Strong Outbreeder hypothesis

Transition from outcrossing to selfing is a widespread evolutionary event across angiosperms (**Paper X**; Sicard & Lenhard, 2011). The reason for this ubiquity is likely related to the reproductive insurance provided by selfing, which becomes particularly important with the rarefaction of pollinators or during the colonization of a new environment (Foxe *et al.*, 2010).

As a by-product, the transition to selfing is accompanied with a modification of the selective processes acting on sexual reproduction. Typically, male-male competition for mating is presumably reduced in selfers since the pollen load is virtually from the same paternal contributor, and similarly, sex-specific interests converge as maternal and paternal contributors are the same individual. In other words, sexual selection (see next section) and sexual conflict are expected to be relaxed in a selfing lineage (Brandvain & Haig, 2005; Cutter, 2019). This includes the parental conflict acting on maternal resource allocation, also expected to be relaxed in a selfer lineage. The expectation from this hypothesis, known as Weak Inbreeder/Strong Outbreeder (WISO), is that since selection does not act anymore on parent-specific endosperm dosage (i.e. the EBN) in selfers, selfers would have a lower EBN compared to outcrosser sister lineages (Brandvain & Haig, 2005). This has found numerous evidence in the literature, in several independent taxa including Solanaceae and Brassicaceae (Figure 9; **Papers I, III, VI**; Brandvain & Haig, 2005). This bears important consequences for speciation processes: with divergent EBN between selfers and related outcrosser lineages, hybrid seeds would likely be inviable, reinforcing the reproductive isolation initiated by the transition to selfing. Interestingly, the divergence in EBN leading to hybrid seed lethality seems to arise at a fast pace: for example, I and co-workers have

shown that an extensive hybrid seed lethality can be observed between two closely related species, *C. rubella* (selfer) and *C. grandiflora* (outcrosser), which diverged only a 100k years ago (**Papers III, VII**; Guo *et al.*, 2009). A recent work performed by a PhD student in my group, Ömer İltaş, has shown that hybrid seed defects and differences in EBN can occur even earlier after the transition to selfing (a few 10k of years), between a selfing and an outcrossing lineage within the same species *A. lyrata* (**Paper I**). Thus, it appears that divergence in EBN and hybrid seed lethality may play an important role in the very early stages of speciation, unexpected until now.
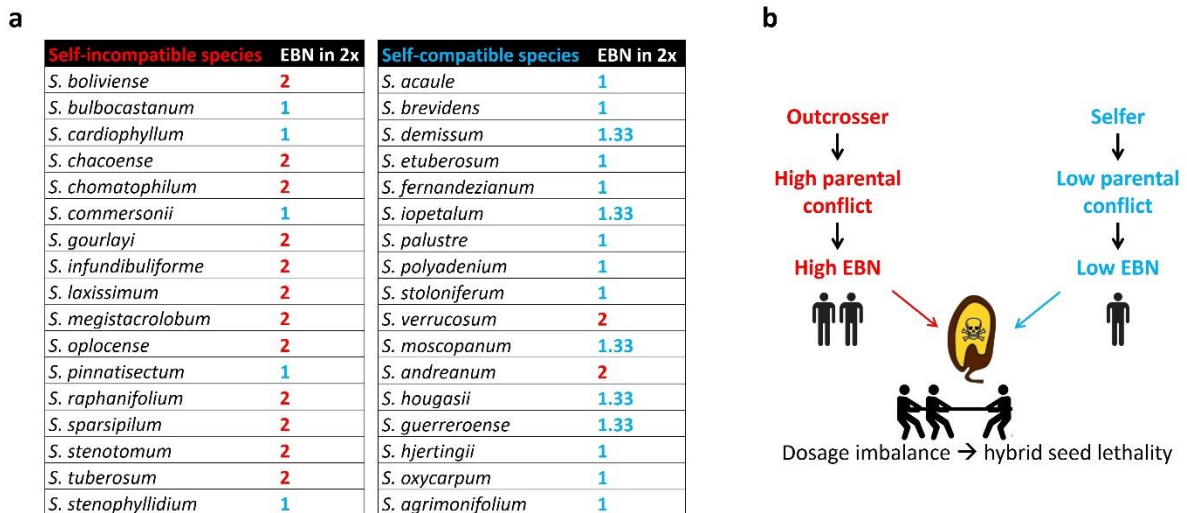
| Self-incompatible species | EBN in 2x |
|---|---|
| *S. boliviense* | 2 |
| *S. bulbocastanum* | 1 |
| *S. cardiophyllum* | 1 |
| *S. chacoense* | 2 |
| *S. chomatophilum* | 2 |
| *S. commersonii* | 1 |
| *S. gourlayi* | 2 |
| *S. infundibuliforme* | 2 |
| *S. laxissimum* | 2 |
| *S. megistacrolobum* | 2 |
| *S. oplocense* | 2 |
| *S. pinnatisectum* | 1 |
| *S. raphanifolium* | 2 |
| *S. sparsipilum* | 2 |
| *S. stenotomum* | 2 |
| *S. tuberosum* | 2 |
| *S. stenophyllidium* | 1 |

| Self-compatible species | EBN in 2x |
|---|---|
| *S. acaule* | 1 |
| *S. brevidens* | 1 |
| *S. demissum* | 1.33 |
| *S. etuberosum* | 1 |
| *S. fernandezianum* | 1 |
| *S. iopetalum* | 1.33 |
| *S. palustre* | 1 |
| *S. polyadenium* | 1 |
| *S. stoloniferum* | 1 |
| *S. verrucosum* | 2 |
| *S. moscopanum* | 1.33 |
| *S. andreanum* | 2 |
| *S. hougasii* | 1.33 |
| *S. guerreroense* | 1.33 |
| *S. hjertingii* | 1 |
| *S. oxycarpum* | 1 |
| *S. agrimonifolium* | 1 |

Dosage imbalance → hybrid seed lethality

**Figure 9. Illustration of the Weak Inbreeder/Strong Outbreeder (WISO) hypothesis. a**) Survey of *Solanum* species, their mating system and their EBN. Red shows an EBN of 2 and blue shows an EBN of 1 or close to 1. Self-incompatible species tend to have a significantly higher EBN compared to self-compatible species. **b**) Principle of the WISO hypothesis. A higher parental conflict in outcrossing species leads to a stronger selective pressure on EBN, leading to its increase, while in selfers, such selective pressure is relaxed, leading to low EBN. The hybridization between the two lineages lead to hybrid seed lethality (endosperm dosage imbalance). Adapted from **Paper VI**.

### c) Beyond WISO: life history affects EBN evolution

Interestingly, hybrid seed lethality and EBN divergence is not only found between a selfer and an outcrosser, but also between selfers and between outcrossers (**Papers III, IV**; Roth *et al.*, 2018; Coughlan *et al.*, 2020; Städler *et al.*, 2021). One explanation involving the selfers expands the WISO hypothesis: EBN evolution probably depends not just on the mating system (selfer/outcrosser), but also on the age of the transition to selfing. One can expect that a "young" selfer (recent transition) would have an EBN close to the outcrosser sister lineage, while for an "old" selfer (ancient transition), the long evolutionary time with relaxed parental conflict would lead to a substantially low EBN. This idea is supported by the relatively low hybrid seed lethality and modest difference in EBN we found between the selfer and outcrosser *A. lyrata* (recent transition to selfing; **Paper I**; Foxe *et al.*, 2010). It is also supported by the large difference in EBN between a young selfer, *C. rubella* (transition to selfing 100 kya; Guo *et al.*, 2009), and an old selfer, *C. orientalis* (transition > 1 Mya; **Paper X**): the old selfer has a much lower EBN compared to the young selfer, consistent with longer time under relaxed parental conflict, and leading to extensive hybrid seed lethality (**Paper III**).

In addition, hybrid seed lethality caused by differences in EBN between outcrossing lineages has been frequently reported (**Paper IV**; Roth *et al.*, 2018; Coughlan *et al.*, 2020). At first sight, it may seem hard

to connect these findings with parental conflict. Nevertheless, I and coworkers found in Arabidopsis spp. that the outcrosser with the lower EBN also showed lower genetic diversity compared to the outcrosser with higher EBN (**Paper IV**). Another work showed that hybrid seed lethality is more extensive between lineages of higher genetic diversity (Coughlan *et al.*, 2020). This led to propose that genetic diversity may be correlated with the efficacy of parental conflict: either the number of genetically different competing fathers increases, increasing the selective pressure and competition for maternal resources, or, more parsimoniously, the efficacy of selection (including selection driven by parental conflict) is higher with higher genetic diversity/efficient population size (**Paper I**; Coughlan *et al.*, 2020). Consequently, the EBN would rise faster in lineages with higher genetic diversity, leading to potential hybrid seed lethality when mating with lineages of lower genetic diversity (and thus lower EBN).

Genetic diversity might be affected by several kinds of demographic events, such as bottlenecks related to colonization of new environments, decreased range of habitats, relict populations from last glaciation… It might also be affected by life history trait such as clonality. The expectation regarding hybrid seed lethality and EBN evolution would here be the same as in the previous paragraph: clonality would reduce genetic diversity, and thus lineages with a higher propension to clonal propagation would evolve lower EBN and show hybrid seed lethality with sister lineages reproducing exclusively by sexual means. This is an idea proposed by my colleague Johanna Leppälä (Umea University), which works like a charm in the Arabidopsis genus: together, we established a ranking of the EBN of the Arabidopsis diploid species, and the "top" EBN species are obligate outcrossers with poor clonality ability (*A. arenosa* and *A. croatica*), followed by outcrossing species with moderate to strong clonal capacities (*A. lyrata*, then *A. halleri*, respectively), and the lower EBN is the selfer *A. thaliana*. This means that *A. arenosa* and *A. croatica* show extensive hybrid seed lethality when crossed to all the other species, while *A. lyrata*, *A. halleri* and *A. thaliana* can be crossed to a certain extent (**Paper IV**; Leppälä et al., in prep). Interestingly, the link between clonality and EBN also works within the same species: in *A. lyrata*, the Scandinavian populations show higher capacity for clonality, and also lower EBN, compared to the Central European populations (Leppälä et al., in prep). These results might be a specificity of the Arabidopsis genus, and further work is therefore required.
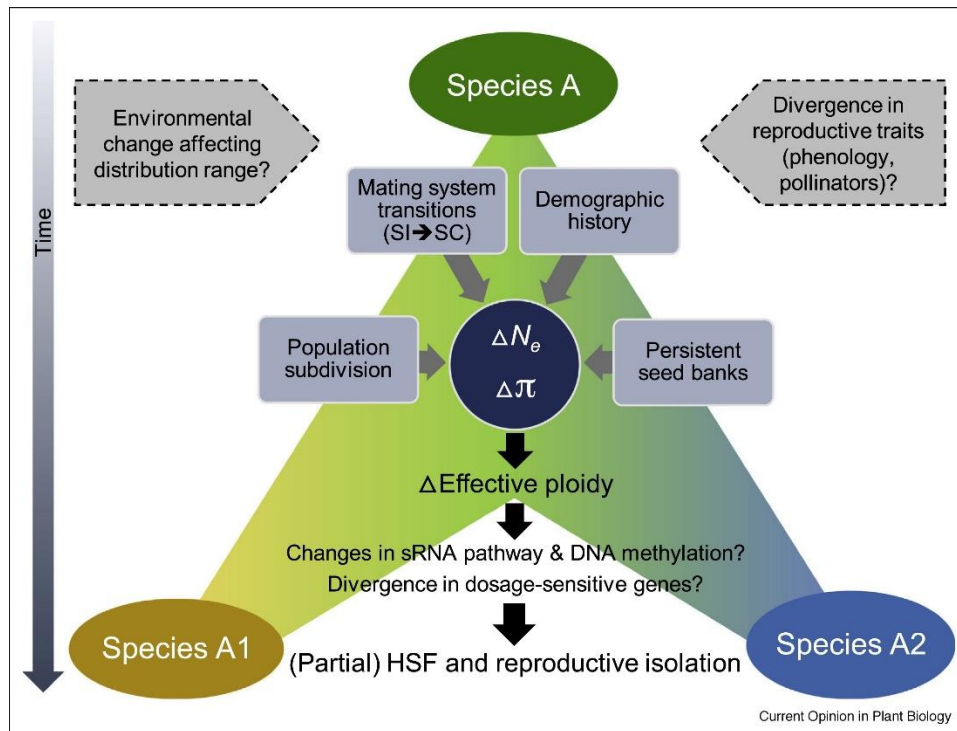
**Figure 10. Link between ecological/evolutionary factors, effective population size ($N_e$) and EBN divergence between lineages**. Lower $N_e$ may lead to higher relatedness among mates, thus lowering levels of parental conflict. Alternatively, Divergence in $N_e$ (roughly estimated by nucleotide diversity π at selectively neutral genome regions) is hypothesized to facilitate divergence in effective ploidy (EBN), which may manifest as altered expression levels at dosage-sensitive genes or other molecular changes. Ecological and life-history features are in light-grey boxes, and major evolutionary factors/processes that impact $N_e$ are in light-blue boxes. HSF, hybrid seed failure; SC, self-compatibility; SI, self-incompatibility. Adapted from (Städler *et al.*, 2021).

Altogether, the evidence so far suggests that the efficacy of parental conflict as a selective pressure depends on several factors such as selfing and the age of its transition, or the effective population size which can be affected by demographic events or life history traits (Figure 10). Consequently, these factors may determine the EBN of a given lineage, and its crossability/reproductive isolation with other lineages, contributing to the speciation process.

### d) Hybrid seed lethality: a redeemed process in speciation research

Hybrid seed lethality arises after the fertilization between individuals from two lineages and is therefore a post-zygotic reproductive barrier. This type of barrier is traditionally considered as playing a minor role in speciation, since it acts last in the reproductive sequence, after all prezygotic barriers, in preventing gene flow between lineages. Under the Bateson-Dobzhansky-Müller model, post-zygotic reproductive barriers are also expected to arise as a secondary product of prior isolation between lineages, not as a primary isolating process (Coyne & Orr, 2004).

While these considerations are valid, they may not fully apply to the case of hybrid seed lethality, which might have been underestimated for its role in speciation. Indeed, as mentioned above, hybrid seed lethality seems to arise very quickly between lineages, suggesting a role in early speciation processes (**Paper I, III, VII**). Also, hybrid seed lethality may explain a common phenomenon: when diploids of two

sister species are genetically well isolated, their polyploid counterparts often readily hybridize with each other in the wild (Figure 11; Schmickl & Yant, 2021). This polyploidy-facilitated hybridization echoes the EBN experiments: inducing (via chemicals) polyploidy of a species raises its EBN, allowing it to produce viable hybrid seeds with species of higher EBN, which were previously uncrossable (Johnston & Hanneman, 1982). These experiments proved useful for breeding purposes, but only recently, our work suggested that this may also happen in the wild (**Paper IV**). In this work, we showed that the differentiated diploid *A. lyrata* and *A. arenosa* do not form viable hybrid seeds due to EBN differences, but when using tetraploid *A. lyrata*, we can bypass hybrid seed lethality, and this mirrors that in the wild, *A. lyrata* and *A. arenosa* show extensive gene flow at the tetraploid level (**Paper IV**; Schmickl & Koch, 2011). Therefore, we proposed that hybrid seed lethality, via polyploidization and its change on EBN, can modulate gene flow between lineages, an idea that became relatively popular and was reviewed in recent articles (Schmickl & Yant, 2021; Städler *et al.*, 2021).
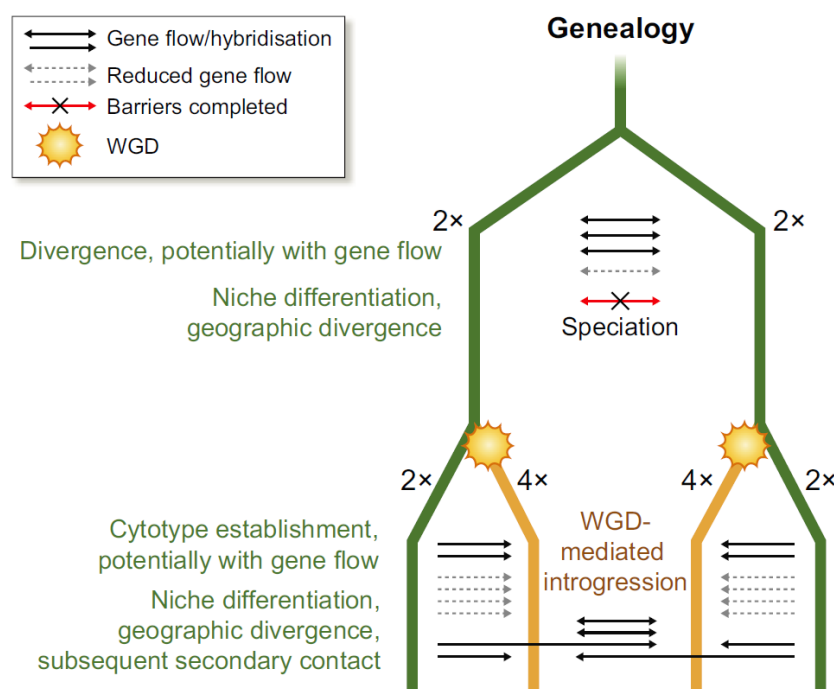


**Figure 11. Weakening of reproductive barriers linked with polyploidization.** The bypass of hybrid seed lethality via polyploidization is one possible reason to explain re-enabled gene flow after prior complete isolation between diploid ancestors. Adapted from (Schmickl & Yant, 2021).

## Remaining questions

The centuries of research on hybrid seed lethality have allowed us to draw a relatively clear picture of the mechanisms at work. Nevertheless, large zones of shadow still remain. One of them concern the physiological reasons behind hybrid seed lethality. These are relatively clear in taxa such as the Brassicaceae, thanks to all the molecular works performed on the model species *A. thaliana*: endosperm cellularization, a crucial development step, is disturbed in hybrid seeds, and this bears direct consequences on the embryo nourishment (**Paper V**). However, for taxa where the endosperm is of the cellular type, such as Solanaceae, the scenario is quite blur: we see endosperm cell size and proliferation being affected, but the causes of this phenomenon and its link with hybrid embryo survival is unknown. Only when we understand better what is happening in such type of endosperm,

we can really evaluate if hybrid seed lethality shares a common basis across all angiosperms, independently of the endosperm type, or whether hybrid seed lethality shows instead features of convergent evolution.

Another aspect of hybrid seed lethality concerns its quantitative basis. It seems pretty clear now that it is the result of a genome dosage (EBN) imbalance in the hybrid endosperm, and this is constant across all angiosperms. We proposed that the rescue of this imbalance, via polyploidization, may bypass hybrid seed lethality and re-establish gene flow in the wild, based on one biological system. However, how frequent is this really in other systems? Also, the EBN imbalance is well backed up by evidence, but it remains to be understood between what elements the imbalance is: is it between parental small RNAs and epigenetic regulators? Which one? And how would this imbalance actually work mechanistically? Or is it rather an imbalance between their downstream targets, imprinted genes? Or both? Are the actors of this imbalance specific to each taxon, or are there common factors to all angiosperms? All these questions remain open.

When it comes to imprinted genes, the parental conflict theory proposes that paternally and maternally expressed genes work antagonistically, the former promoting endosperm resource accumulation while the latter counteract this action. Is this based on a reality of the biological role of imprinted genes? Knowing their putative biological function, as it has been done so far, is not enough. Indeed, the apparent antagonistic role of paternally and maternally expressed genes could simply be the basis of a key-lock system preventing the endosperm to develop without being fertilized, a waste of resources that happens in gymnosperms, but not in angiosperms. Thus, it is important to decipher the gene regulatory networks in which imprinted genes are incorporated, which would allow us to define what functions imprinted genes really play in endosperm development and resource accumulation.

And finally, parental conflict itself and its role on EBN evolution has been questioned (Dilkes & Comai, 2004). Indeed, while parental conflict seems like a convincing story, some of its elements remain elusive. Especially, for any selection to act on paternal or maternal genomes, their dosage, likely underlain by genomic imprinting, needs to be heritable. However, epigenetic marks (establishing genomic imprinting) are relatively unstable, and thus are prone to low heritability, hindering the efficacy of selection on such marks. Genetic variation is another important element for parental conflict, as selective pressure, to act. Can natural variation in parental genome dosage be found within a species? Our work suggests the contrary (**Paper XIII**). However, the lack of variation could indicate the action of selection, which causes reduced variability for the selected trait. This question requires therefore further investigation. Last but not least, the exact same facts regarding endosperm genome dosage and the divergence of this dosage between selfers and outcrossers could find a different narrative: sexual selection (**Paper II**). This will be explained in the further chapter of this thesis.

# Part II – Further investigation: does sexual selection in plants also shape plant reproduction evolution and speciation?
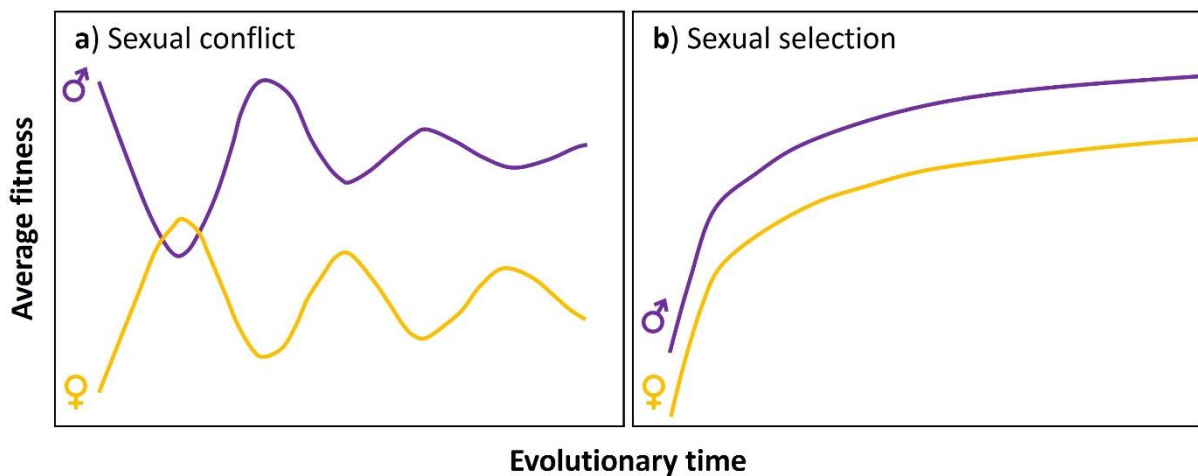


**Figure 12. Sexual conflict vs sexual selection: antagonistic vs synergistic coevolution between male and female function. a**) evolution of sex-specific fitness across time in case of sexual conflict ("chase away" selection; see Figure 8 for full legend). **b**) evolution of sex-specific fitness across time in case of sexual selection ("runaway" selection). Directional selection of certain male traits by females leads to increase fitness of both males and females over time. Adapted from (Pizzari & Snook, 2003).

The first part of this thesis delt with sexual (parental) conflict, where the fitness optima of the male and female functions are antagonistic to each other. Consequently, sex-specific traits may coevolve in a "chase away" scenario, such as in the case of paternal/maternal endosperm genome dosage under parental conflict (Figure 12a). However, sex-specific traits are not always conflictual. Male and female traits may evolve independently, or even follow a synergistic evolutionary trajectory. For example, under sexual selection, female choice may select for specific male traits, and this may lead to a coevolution between female preference and male trait ("runaway" selection), improving the reproductive success of both the male and female functions over time (Figure 12b). The second section of this thesis reflects about sexual selection in plants, which is the ongoing research performed by my team (https://lab-allience.natur.cuni.cz/plantreproevo/), and which started in 2018, after I established the Plant Repro Evo lab in Charles University.

## Sexual selection in plants: really?

The concept of sexual selection aims to explain the evolution of sexual traits due to their positive impact on reproductive success, despite their occasional deleterious effect on survival (Darwin, 1871). It proposes that since mating is in general limited, especially for males (Bateman, 1948), this creates *de facto* male-male competition for mating (intrasexual selection), and on the other hand, choosing the most suitable partner from this competition may provide an advantage to the female function (intersexual selection). Several explanations can be found for the ubiquitous observation that the choosing sex is female (Kokko *et al.*, 2006), and among other explanations, the female sex is often the major resource (and sometimes care) provider to the progeny and therefore choosing the most suitable male progenitor may become crucial to avoid any waste of resources (Trivers, 1972).

These concepts have been applied to plants only relatively recently (Janzen, 1977; Willson, 1979). One probable reason for this late interest is the fact that most plant species are hermaphrodite, which makes it harder to think in terms of male or female individuals' reproductive success (Moore & Pannell, 2011). Nonetheless, it is now clear that the male and female functions of hermaphroditic plants evolve as separate entities with their own organs, genes and selective interests. In this thesis, I will therefore refer to sexual "functions" rather than female/male individuals. Another likely reason for the long lack of interests in plant sexual selection is the absence of obvious features such as courting behaviour. In addition, there are steps in plant reproduction where female choice simply cannot act (pre-mating events, see below). This led to reframe the definition of sexual selection to include plants (Willson, 1979): it is also based on male-male competition (intersexual selection) as mentioned before, but when it comes to female choice, it may or may not exist, but at least the female function may get a fitness advantage by mating with males outcompeting others. Typically, this applies with the "sexy-son" effect, where successfully mating males will transmit their traits to their sons, which will also benefit the female's reproductive success (Fisher, 1915).

### a) Pre-mating sexual selection

In plants, "mating" can be defined as the deposition of pollen on the stigma. Before mating, as mentioned above, female choice cannot act for obvious reasons. However, at this stage of reproduction, male-male competition for mating is likely to be intense in plants. Indeed, plants usually produce way more male than female gametes: the pollen:ovule ratio is always skewed towards pollen, even though this ratio varies from a few dozen to several tens of thousands depending on the species (Gong & Huang, 2014). In animals, where the situation is similar, it was thus proposed that while the maximum female reproductive success is reached after a few mating events and is rather limited by resources, the male reproductive success is always limited by the number of mating events (Figure 13a; Bateman, 1948). Consistently in plants, from all the pollen produced by a given community of plant species, it was estimated that only 2.9% reaches a conspecific stigma (Gong & Huang, 2014). This suggests that mating in plants is an extreme bottleneck for male reproductive success (Minnaar *et al.*, 2019).
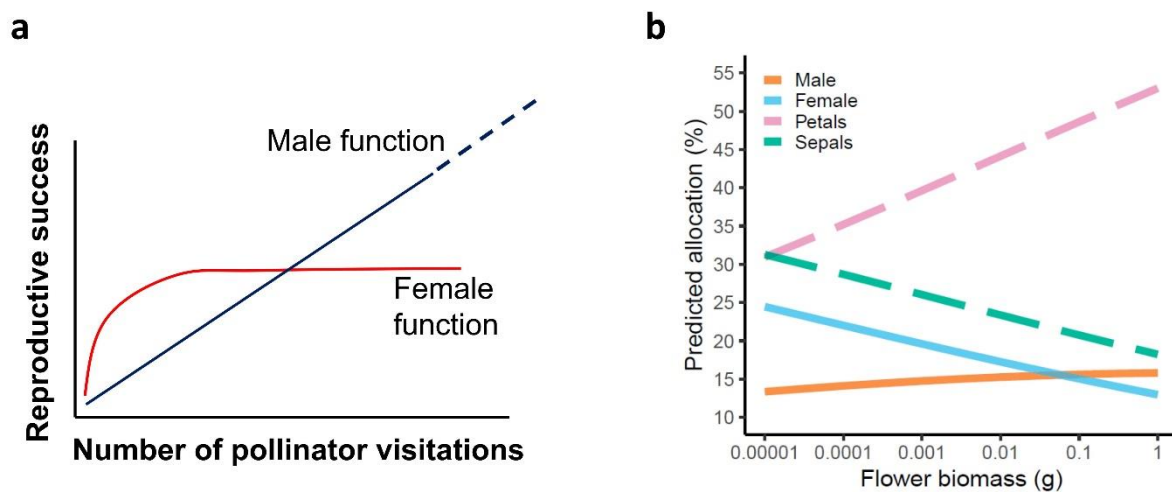


**Figure 13. Pre-mating sexual selection in plants, or the "Fleurs-du-mâle" hypothesis (Queller, 1997). a)** Bateman's principle (1948) applied to plant (i.e. mating event = pollinator visitation). **b)** Across angiosperms, larger flowers show increased allocation in male function, consistent with the

idea that floral attractant traits evolve largely under male interests ("Fleurs-du-mâle" hypothesis; Queller, 1997). Adapted from Paterno et al., 2020.

Therefore, any heritable trait increasing male mating success is likely to be under strong selection. This led to propose that even in hermaphroditic species, the evolution of floral traits increasing pollinator visitation, such as floral display or scent, is mostly driven by male selective interests, a hypothesis coined as "Fleurs-du-mâle" (Queller, 1997) - a pun on Baudelaire's "Les Fleurs du Mal" and "male flowers". A recent survey of floral morphology across angiosperms supports this idea, showing that larger flower are usually so due to increased investment in male-related organs (Figure 13b; Paterno *et al.*, 2020). The body of pollinators is another potential "arena" where pollen grains compete for placement, eventually leading to the evolution of different pollen placement strategies and pollen morphologies (Minnaar *et al.*, 2019; Lynn *et al.*, 2020). These relatively new ideas nevertheless remain to be tested for the most part.

### b) Post-mating sexual selection

In most species, after pollen transfer to the stigma, there is still an excess of pollen grains compared to the number of ovules (Figure 14a; **Paper XIV**; Gong & Huang, 2014). This lays the groundwork for another step of male-male competition. Also, as pollen grains germinate and grow tubes in interaction with female tissues, this also means that female choice can exert a role via molecular crosstalks (Figure 14b; Tonnabel *et al.*, 2021). In this section, I will provide more details on post-mating sexual selection traits that my team is interested in.

Male traits likely to improve competitive abilities are pollen germination and tube growth rates (Snow & Spira, 1991; Austerlitz *et al.*, 2012; Mazer *et al.*, 2018): indeed the faster a pollen can germinate and grow, the more likely it is to fertilize ovules before others. Nevertheless, it was shown that these traits are not fully intrinsic male performances (Marshall, 1988, 1998). Indeed, while paternity success (ability to sire seeds in a mixed pollen experiment) appears to be relatively heritable, the physiological state of the maternal plant affects the siring success differences between males (Marshall, 1988, 1998).

Several works have suggested another male trait to be under sexual selection: the ability to interfere with the germination or growth of competing pollen grains (Marshall *et al.*, 1996; Varis *et al.*, 2009; Swanson *et al.*, 2016). This however remains unclear, as the opposite, i.e. positive pollen-pollen interaction promoting pollen germination/tube growth, has been evidenced (Parantainen & Pasonen, 2004; Zhang *et al.*, 2010). The latter scenario may sound more intuitive: if pollen grains do have the ability to inhibit each other, this may lead to overall low reproductive fitness for both males and females, and this would be negatively selected. On the other hand, both scenarios (positive and negative pollen-pollen interaction) may co-exist and depend on the molecules underlying pollen-pollen interactions: while these are mostly unknown, works have suggested that phytohormones such as GA are involved (Zhang *et al.*, 2010). Phytohormones are well-known to have opposite effects on the physiology depending on their concentration (Pasternak *et al.*, 2019), and it is tempting to speculate that in low concentrations (low pollen load), they would promote pollen tube growth, ensuring reproductive success despite a low number of pollen grains deposited on the stigma, while in high concentrations (high pollen load), the negative effect of phytohormones would reveal itself. The underlying molecules and their concentration-dependent effect remain to be elucidated. Also, the nature of pollen-pollen interactions may be selected by life history traits of a given lineage, such as reproductive strategies favouring male-male competition (e.g. high pollen load on the stigma vs low

number of ovules; Figure 14a). This would explain that in some cases, pollen-pollen interactions are negative and in other instances, they are positive (Marshall *et al.*, 1996; Parantainen & Pasonen, 2004; Varis *et al.*, 2009; Zhang *et al.*, 2010). An expectation from this idea is that depending on reproductive strategies, the set or amount of molecules produced by pollen grains would vary.
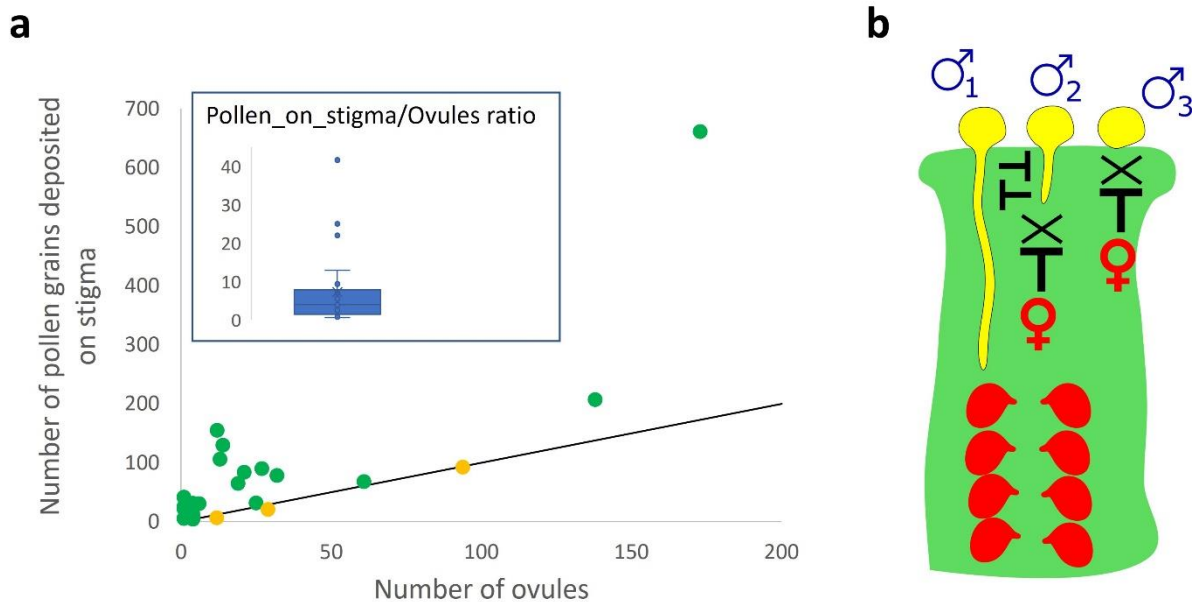


**Figure 14. Post-mating sexual selection in plants. a**) The number of pollen grains deposited on the stigma most of the time still exceeds the number of ovules (adapted from Gong & Huang, 2014). Each dot represents one species. The black line shows a 1:1 ratio between pollen grains and ovule numbers, meaning virtually no competition for mating between pollen grains. Green dots show species with more pollen deposited than available ovules, and orange dots show the opposite trend. The insert shows the ratio between deposited pollen and ovule number from the same data. **b**) potential means for male-male competition (intrasexual selection) and female mate choice (intersexual selection). Male-male competition may happen via pollen germination and tube growth performance or hinderance of other pollen grains (shown by block bars). Female mate choice may act on the stigma (pollen rejection) or style (pollen tube growth hinderance). Adapted from İltaş et al. (in prep).

On the female side, traits related to mate choice may be under sexual selection. As mentioned above, the germination and tube growth of pollen grains deposited on the stigma depends on the molecular crosstalk with female tissues (Tonnabel *et al.*, 2021). The pollen requires molecules provided by the female tissues, such as calcium, to germinate and grow, leveraging a potential first step for females to choose mating partners via the delivery or not of such molecules. In addition, the female guides the pollen tube towards the ovules via the exchange of signalling peptides (Zheng *et al.*, 2018). This leaves a potential step for female choice via the misguidance of pollen tubes. Nevertheless, while the underlying signalling peptides are responsible for the misguidance of the pollen tube from a foreign species (Escobar-Restrepo *et al.*, 2007), it remains to be shown that this can be a molecular way for female choice of conspecific mating partners.

In addition, another female trait proposed to act as a means for female choice is style length (Ramesha *et al.*, 2011). The style is a narrow stalk that connects the stigma and the ovary. Therefore, the pollen tubes need to grow through it in order to fertilize the ovules. Style length shows great variation within

and between plant species (Ramesha *et al.*, 2011; Gotelli *et al.*, 2017), ranging from non-existent to several centimetres. This suggests variation of female choice on which sexual selection can act. Indeed, a longer style may reveal slight differences in pollen tube growth or extend the "arena" where pollen-pollen interference (see above) occurs. Also, the longer the style, the more opportunities for the male-female molecular crosstalk explained above (Tonnabel *et al.*, 2021), meaning additional chances for female choice. These ideas are summarized in a review led by Ö. İltaş, a PhD student working in my team, where we propose that style length selects for faster pollen tube growth, and in a runaway selection mechanism (Figure 12b), style length and pollen tube growth, as well as the genes responsible for these traits, are likely to coevolve (**Paper XIV**).

## Sexual selection driving plant reproduction evolution

Whether sexual selection exists or not in plants is nowadays quite resolved: it does exist. But an equally important question is whether sexual selection has a minute or rather substantial role in shaping the diversity of sexual traits we observe in plants. A difficulty, when studying the impact of sexual selection on plant reproduction evolution, is to distinguish traits merely involved in general reproductive processes and traits that fit the concept of sexual selection (i.e. traits selected under male-male competition and/or female mate choice). The pistil-pollen negative interactions leading to interspecific pollen rejection or misguidance (Escobar-Restrepo *et al.*, 2007) is a typical example: why this phenomenon could be the result of female mate choice, it could also simply be due to the neutral divergence of the male-female recognition process between two species.

A feature of plants that may help in unravelling *bona fide* sexual selection traits is the myriad of different reproductive strategies existing across clades and their expected impact on sexual selection. In particular, while most plant species are outcrossing (needing a mating partner to produce an offspring), the transition to selfing (mating with oneself) has occurred recurrently and independently across angiosperms (Sicard & Lenhard, 2011). As mentioned in the previous section of this thesis, in a selfing lineage, not only sexual conflict, but also sexual selection is expected to be relaxed compared to its sister outcrossing lineage (Cutter, 2019). Indeed, with a selfing plants, all pollen grains are produced from the same male, which is also the female, likely removing any evolutionary advantage for the male function to compete for mating, and for the female function to select mating partner. The divergence of sexual traits between an outcrosser and a sister selfer lineage can thus be imputed to the effect of (divergent) sexual selection. Our team uses the selfer-outcrosser contrast as a tool to identify traits under sexual selection. We especially focus on members of the genetically characterized Arabidopsis genus, namely *A. lyrata*, which is mostly outcrossing but also includes a selfing lineage (Foxe *et al.*, 2010), *A. arenosa*, exclusively outcrossing, and *A. thaliana*, exclusively selfing. This allows us to have a range of divergence between lineages, and also a range of ages of the selfing transition (10,000 yr for *A. lyrata* vs a few 100,000 yr for *A. thaliana*; Bechsgaard *et al.*, 2006; Foxe *et al.*, 2010).

### a) Pollen intrinsic performance

As mentioned above, pollen germination and tube growth rate is an obvious trait influencing male reproductive success under intrasexual competition (Mazer *et al.*, 2018). It is however unclear to which extent this trait is intrinsic or determined by the interactions with the female partner (Marshall, 1988, 1998). One way to solve this issue is to perform *in vitro* pollen germination, *de facto* removing the influence of the female.

This is what we did: we germinated pollen from selfing and outcrossing *A. lyrata*, and measured pollen performance of these two lineages (İltaş et al., in prep). Interestingly, we found that the outcrosser pollen germinates better than the selfer within the timeframe we used (16 hours; Figure 15), but we found no difference in pollen tube growth. The lack of difference could be due to the recent transition to selfing (10 kyr) and not enough evolutionary time for the divergence in this trait to emerge between selfer and outcrosser *A. lyrata*. However, we found the exact same result when comparing *A. arenosa* and the "old" selfer *A. thaliana*: better germination for the outcrosser *A. arenosa*, but no difference in pollen tube growth (data not shown; İltaş et al., in prep). Faster pollen tube growth in outcrossers compared to selfers has been found *in vivo*, i.e. with pollen on female tissues (Mazer *et al.*, 2018). Thus, the consistent lack of difference we observe *in vitro* between selfers and outcrossers led us to propose that pollen tube growth performance is the product of male and female interaction. Previous studies have found that paternity success is a robust male trait (Marshall, 1988, 1998), and as we see that pollen germination seems to be an intrinsic male trait, it is tempting to speculate that paternity success might be largely determined by pollen germination ability rather than tube growth. We are now performing *in vivo* pollen germination, so that we can evaluate the role of the female organs on pollen tube growth performance.

*In vitro* experiments also allow us to measure accurately the effect of pollen concentration (and thus of the concentration of secreted molecules) on pollen performance to infer the nature of pollen-pollen interactions, i.e. positive or negative. Our expectations would be that in an outcrossing lineage, such interaction would be negative, as pollen interference is a trait potentially improving competitiveness. On the other hand, we would expect the opposite scenario in a selfing lineages, i.e. a collaborative behavior (positive pollen-pollen interactions). Indeed, we do see that in the outcrosser *A. lyrata* and *A. arenosa*, higher pollen concentration is associated with lower pollen germination and pollen tube growth (Figure 15, example of *A. lyrata*), suggesting negative pollen-pollen interactions. The preliminary data for *A. thaliana* seem to suggest the complete opposite trend, i.e. better germination and pollen tube growth with higher pollen concentrations, or in other words positive pollen-pollen interaction (data not shown). While this is consistent with our expectations, we found a somewhat different result in the selfer *A. lyrata*: we found no clear relationship between pollen concentration and pollen performance (Figure 15). This could actually be related to the recent transition to selfing in this species, not leaving enough time to evolve towards a collaborative pollen-pollen interaction as in the case of *A. thaliana*, but at least, the results suggest that the interfering molecules are not produced anymore by the selfer *A. lyrata* pollen.
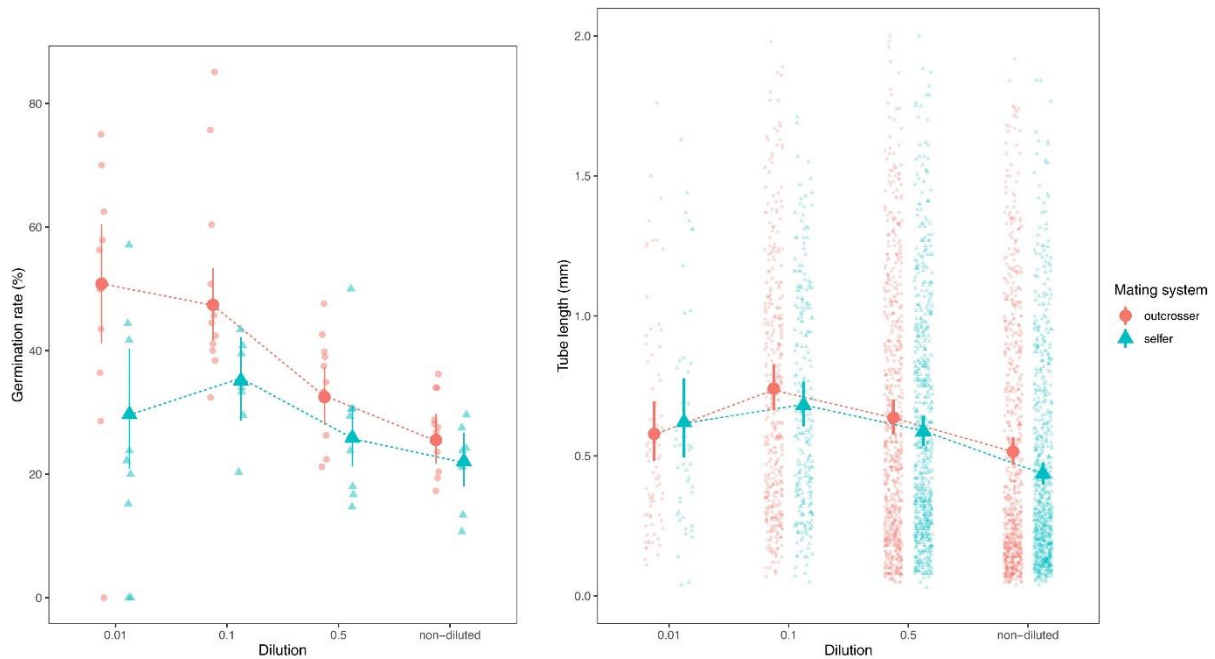
**Figure 15. Pollen performance is lower in selfers compared to outcrossers.** Pollen germination (left) and tube growth (right) according to the *in vitro* concentration of pollen grain (expressed as dilution factor from original pollen solution). Orange: outcrossers; blue: selfers. There is a significant difference ($P < 0.05$) between outcrossers and selfers for pollen germination only.

Our team is also interested in the effect of whole genome duplication on sexual selection. Indeed, theoretical works propose that polyploidy leads to less efficient selection, due to allele masking and longer evolutionary times to reach fixation for beneficial alleles (Monnahan & Brandvain, 2020). This would be also true for sexual selection, leading to a slower evolution of traits under sexual selection. We tested this hypothesis on intrinsic pollen performance, comparing diploids and natural tetraploids of *A. arenosa* (İltaş et al., in prep). Consistently, we found that pollen germination, likely under sexual selection as shown by our results presented above, was higher in diploids compared to tetraploids (Figure 16). This difference cannot be explained by meiotic issues leading to pollen inviability in the tetraploids, as we know that *A. arenosa* tetraploids already evolved stable meiosis, and pollen viability rates are similar to the diploids (**Paper XII**; Yant *et al.*, 2013). In parallel, we didn't find any difference in pollen tube growth, suggesting again that this trait requires the female influence to reveal its divergence, if any, between lineages. Also, both diploids and tetraploids didn't differ for the effect of pollen concentration on pollen performance, i.e. the more concentrated pollen, the less good performance (Figure 16). This suggests that, even though sexual selection may be less efficient in tetraploids than diploids, it still acts on pollen interference as trait involved in male-male competition.
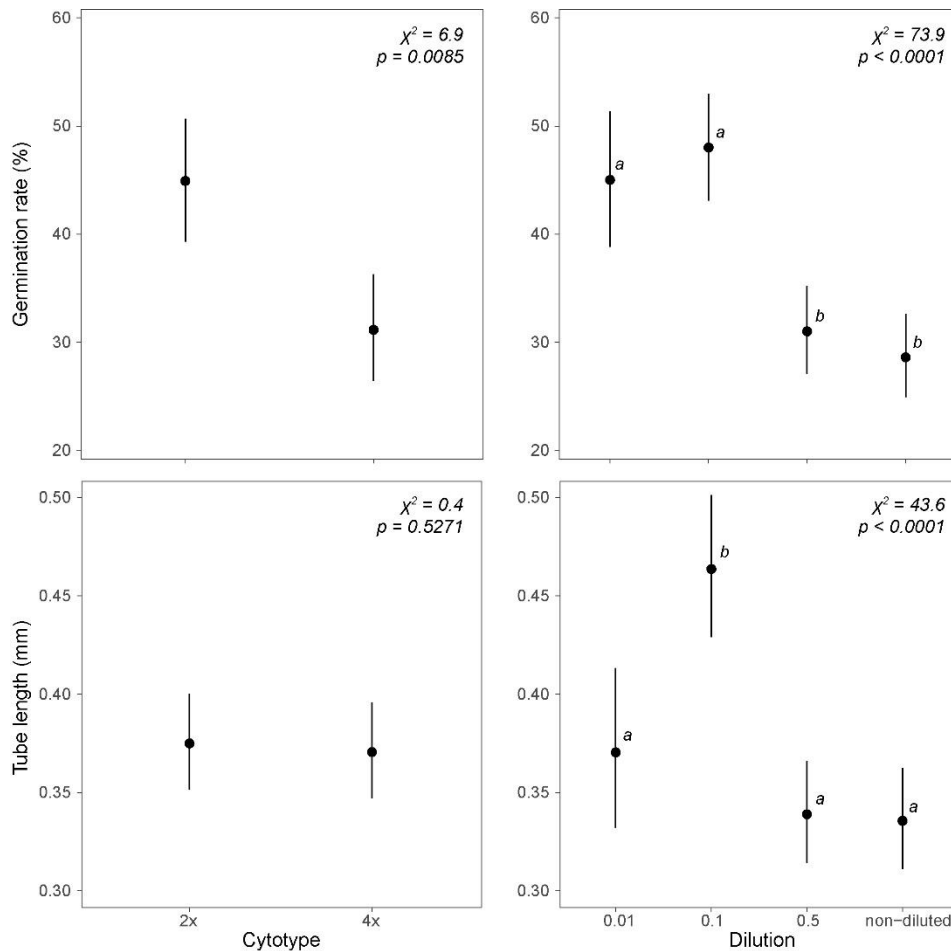
**Figure 16. Pollen performance is lower in tetraploids compared to diploids.** Pollen germination (top) and tube growth (bottom) according to cytotype (left) or *in vitro* concentration of pollen grain (right; expressed as dilution factor from original pollen solution).

### b) Female choice

In hermaphrodites, the evolution of optimal male and female trait values is likely constrained due to the presence of both male and female organs in the same flower. It is actually one proposed reason for the emergence of dioecy (physical separation of sexes): evolving male and female individuals allow the evolution of each sexual function to its optimum (Freeman *et al.*, 1997). Separated sexes thus can allow us to observe the effect of sex-specific selection, and especially sexual selection, to its maximum. The species *Stellaria graminea*, studied by the collaborator Marek Slovak (Dept of Botany, Charles University), is gynodioecious, meaning that female and hermaphrodite individuals coexist. Thanks to the physical separation of the female sex, we can thus monitor the effect of sexual selection acting on this sex. We found that female flowers have smaller petals, consistent with the "Fleurs-du-mâle" hypothesis (see above), but also have longer styles compared to hermaphrodites (**Paper IX**). This is consistent with the expectation that under sexual selection, females are selected for longer styles, increasing the female mate choice ability. We are now planning to perform *in vivo* pollen germination experiment, to test whether pollen tube growth is reduced on female vs hermaphroditic styles (selection for "choosier" females).

It has been proposed that female choice also acts after fertilization, i.e. during seed development, via selective seed abortion from unsuitable male progenitors (Willson & Burley, 1983; Marshall & Ellstrand, 1988). In this line of idea, I proposed to link the observations interpreted as parental conflict

(see first part of this thesis) with a narrative related to sexual selection (**Paper II**). The parental conflict hypothesis relies on the assumption that if a given seed outcompetes others for maternal resources and thus gets more (and the others less), this is deleterious for the female fitness (Haig & Westoby, 1989). And this is the basis to explain that the male and female genome dosages increase over evolutionary times, as an antagonistic coevolution scenario (Figure 8). However, I proposed that in some ecological scenarios, such as reduced environmental resources or high competition between plant species, having an equal distribution of maternal resources to every seed may not be the optimal strategy (Figure 17). With such an equal strategy, due to harsh environmental conditions, none of the seeds may be fit enough to survive. Instead, providing more resources to certain seeds, via the paternal genome influence, to the detriment of other "weaker" paternal contributors, would assure that the favoured seeds would get establish in the harsh environment. The repressive role of the maternal genome in the endosperm would therefore act as a filter for the most performing/demanding paternal genomes, as in "good gene"-like sexual selection (**Paper II**). This alternative narrative to parental conflict remains however to be tested, which is not easy. For example, one way would be to see whether mating with a mix of paternal contributors with different genome dosage (and thus unequal distribution of maternal resources) would lead to increased or decreased inclusive fitness of the female parent in an optimum vs harsh environment.
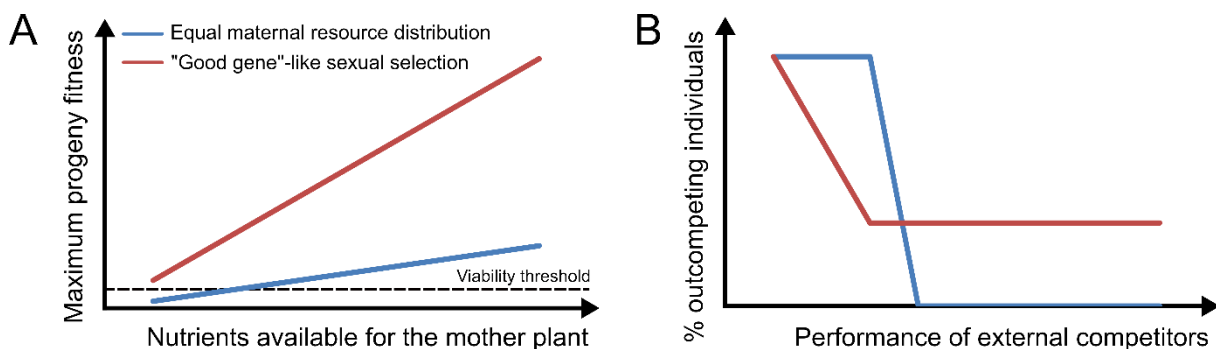


**Figure 17. Female resource allocation response to male drive and consequences for progeny fitness.** (A) Relationship between the amount of available nutrients in the maternal environment and progeny fitness. Here, progeny fitness is assumed to be directly correlated to the amount of resources available in the seed. The blue line represents the situation where maternal resources are divided equally between seeds. In situations of scarce nutrients, equal distribution among seeds may mean that not enough resources are allocated to guarantee the viability of any seed. In this case, maximum progeny fitness = average progeny fitness. The red line represents the case when only a few seeds get most of the maternal resources. In this case, maximal fitness largely exceeds average fitness. (B) Competitive ability of progeny depending on female resource allocation strategy. In the equal distribution scenario (blue), all seeds will have an average competitive performance during early life stage, being easily outcompeted by others. In the "good gene"-like sexual selection scenario, few seeds have a high competitive ability, being able to resist most competitors. Adapted from **Paper II**.

### c) Evolutionary genomics of sexual selection

Now that we have a better overview on traits under sexual selection, we are starting to characterize the role of sexual selection on genome evolution. The comparison of the genome of selfers and outcrossers is one way that we are pursuing. Notably, we can expect that footprints of selection on sexual genes would be stronger in the genome of outcrossers compared to selfers. A recent study

however did not find differences in the evolution of sexual genes comparing self-compatible and self-incompatible Solanum species (Moyle *et al.*, 2021). More studies are required in this direction, but it therefore seems that the impact of sexual selection might be more complicated than expected. We are currently working on similar data comparing selfer and outcrosser *A. lyrata*, from data recently published (Willi *et al.*, 2018).

In parallel, we have started to characterize the transcriptome of sexual tissues in selfing and outcrossing *A. lyrata*. The expectation we have is that differentially expressed genes between the two lineages may be under sexual selection and explain the differences of pollen performance or female mate choice between these lineages. We then plan to further characterize the function of such genes in order to establish a mechanistic explanation on how divergent sexual selection affects sexual genes. The first step of this process was to establish a bioinformatic pipeline to identify sexual genes, i.e. expressed preferentially in sexual tissues. This pipeline is now set (see Figure 18 for pistil-specific genes as an example).
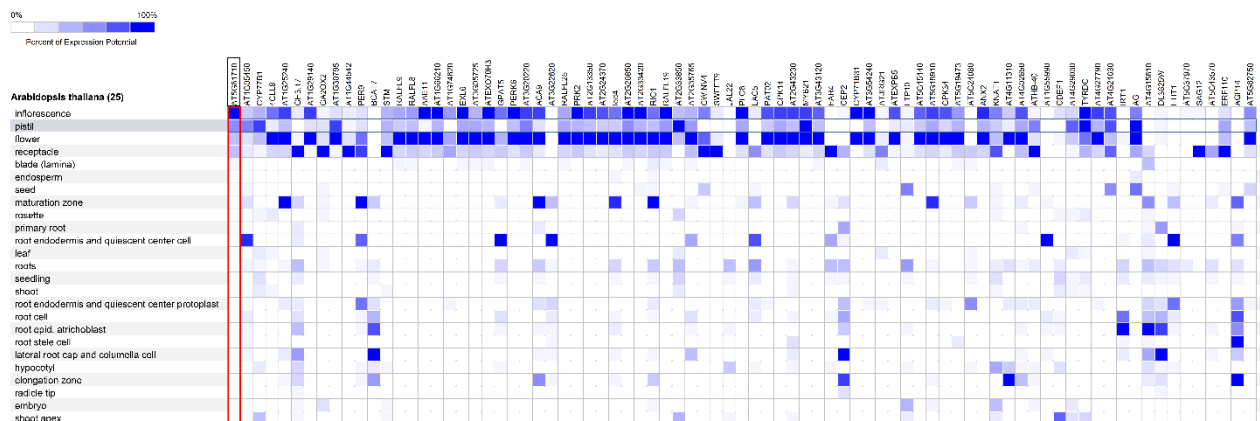


**Figure 18. Example of the tissue-specificity detection pipeline.** Heatmap showing across-tissues expression of *A. thaliana* homologs of *A. lyrata* genes identified as pistil-specific. The data show enrichment of genes preferentially expressed in pistil, inflorescence, flower and receptacle (first four rows). Figure created using Genevestigator.

In addition, sexual selection may affect genome structure evolution via gene clustering: indeed, if several sex-specific genes, such as genes involved in male performance, are genetically linked, the selection of several beneficial allele would be facilitated, leading to male or female genomic regions without the need for separated sexes and sex chromosomes (Patten *et al.*, 2013). This expectation, that finds evidence for loci responsible for sexual conflict, remains however to be fully addressed, which is part of future plans of my research team.

Finally, runaway selection may lead to the coevolution of male and female traits (Figure 12b). This implies a gene-gene coevolution: certain male alleles would be segregating with specific female alleles in natural populations. If we can detect the association between such alleles, we will be able to understand better how the male-female interactions and coevolution works in a mechanistic manner, which is a work about to start in my team (January 2022).

## Sexual selection driving plant speciation

The role of sexual selection in the emergence of new species in animals is a school case of evolutionary biology (Darwin, 1871; Seehausen *et al.*, 1997). A mechanistic explanation is that male trait and female preference coevolve in two lineages independently, eventually leading to a mismatch between male trait of one lineage and female preference of the other lineage, establishing mating incompatibility (reproductive isolation) and eventually leading to two phenotypically and genetically distinct species. In plants, this may apply to post-mating reproductive isolation, where female choice can act. Pre-mating sexual selection in plants may also lead to speciation via the selection on the male function to attract pollinators (Queller, 1997; see above). In this respect, pollinator shift (Cardona *et al.*, 2020) can be considered as a reproductive barrier driven by male-male competition for mating. While these expectations are relatively intuitive, whether sexual selection plays a major or rather anecdotal role in establishing reproductive barriers, and ultimately speciation in plants, remains to be elucidated (**Paper XI**). In the next paragraphs, I evaluate the potential role of sexual selection in specific speciation processes in plants.

### a) Alien pollen rejection and tube misguidance

As explained above, pollen germinates on the stigma, grows along the style and targets ovules, all being influenced by male-female molecular interactions occurring from the stigma down to the ovule (Zheng *et al.*, 2018; Tonnabel *et al.*, 2021). Female choice can thus act at any of these steps: pollen germination, pollen tube growth, and pollen tube guidance. If sexual selection establishes reproductive barriers via a mismatch between female preference and male trait, one can expect 1/ alien pollen rejection on the stigma (no germination), 2/ arrest or slowdown of alien pollen tube growth, or 3/ alien pollen tube misguidance.

Pollen germination on the stigma happens only after being hydrated, and hydration is provided by the female tissues to the condition that the pollen is accepted, after a molecular crosstalk between pollen and stigmatic cells (Tonnabel *et al.*, 2021). A rejected pollen will not be hydrated, and thus will not germinate. Such a scenario happens for example in species with sporophytic self-incompatibility, where self-pollen is rejected (Hiscock & Tabah, 2003). It also happens between species of Arabidopsis, where alien pollen gets rejected, establishing a reproductive barrier (Li *et al.*, 2018). Reduced alien pollen germination on stigma was also found between two accessions of the same species *A. thaliana* (Swanson *et al.*, 2016). These barriers could be the result of sexual selection, however, as mentioned above, they may as well be explained by neutral divergence of the pollen-stigma recognition process between the lineages. Nevertheless, one evidence supports sexual selection as the underlying driver: alien pollen rejection was found to be unilateral, i.e. the stigma of a self-incompatible species is more likely to reject alien pollen, while the stigma of a selfing species is less stringent and accept alien pollen more readily (Li *et al.*, 2018). As sexual selection is expected to be relaxed in a selfing lineage (see previous sections), this can be interpreted as selection on stronger female mate choice in outcrossing lineages compared to selfers, leading to alien pollen rejection in a unilateral way. One could however argue that alien pollen rejection by self-incompatible species may be a by-product of the sporophytic self-incompatibility system, which is non-functional in selfing species (Hiscock & Tabah, 2003). Interestingly nevertheless, our preliminary data suggest the presence of unilateral pollen rejection between *A. lyrata* and *A. arenosa*, both of them being self-incompatible (Figure 19; Haghighatnia et al., in prep), ruling out a difference in the sporophytic self-incompatibility system functionality as a reason for the unilateral pattern. *A. arenosa* stigma appears to be more likely to reject *A. lyrata* pollen than vice versa, suggesting stronger female choice ability in the former species, i.e. stronger sexual selection acting. As mentioned in previous sections, *A. lyrata* shows lower genetic diversity than *A. arenosa*, suggesting that sexual selection is less efficient in *A. lyrata*, either due to general lower selection efficacy (lower $N_e$), or due to lower competition between genetically different males. These

hypotheses remain however to be investigated further. In particular, we plan to sequence the transcriptome of the unilateral *A. arenosa – A. lyrata* pollen rejection in order to better understand the mechanisms at work (Haghighatnia et al., in prep).
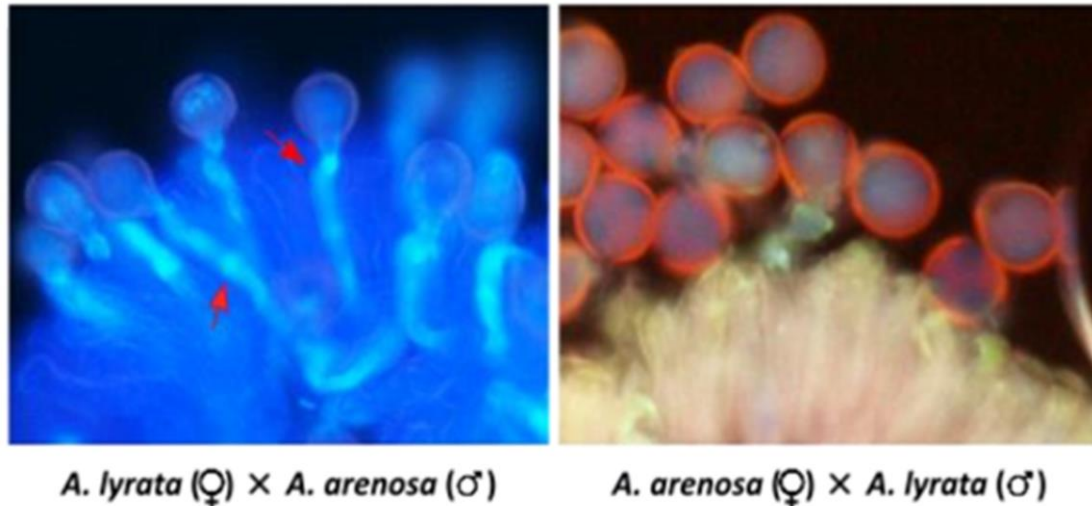


**Figure 19. Unilateral pollen rejection between *A. lyrata* and *A. arenosa*.** Pollen tubes were observed only in the direction of *A. lyrata* ♀ × *A. arenosa* ♂, as shown by red arrows.

Another step where female choice can act as hybridization barrier is during pollen tube growth across the style (Tonnabel *et al.*, 2021). Indeed, it was found that pollen tube growth is slower (Swanson *et al.*, 2016) or even arrests (Broz & Bedinger, 2021) if the pollen belongs to a different lineage than the one of the female sporophyte. Here again, female sporophyte from self-incompatible species are more likely than self-compatible species to prevent alien pollen tube growth (Broz & Bedinger, 2021), leading to a unilateral hybridization barrier, which is consistent with the expectation of divergent sexual selection between the two species. We are currently investigating if pollen tube growth, and not just germination, is altered after interspecific mating between Arabidopsis species.

Finally, alien pollen tube may grow properly, but may fail to target ovules due to an incompatibility between female signal peptides and pollen receptors, acting as a reproductive barrier (Escobar-Restrepo *et al.*, 2007; Zheng *et al.*, 2018; Broz & Bedinger, 2021). Current evidence cannot rule out whether such a reproductive barrier involves sexual selection or simply a neutral sequence divergence between female signal peptides and pollen receptors, and therefore additional work is required.

### b) Interlineage pollen-pollen interference

It has been reported that alien pollen, when competing with pollen of the same species as the female sporophyte, is disfavored (Rieseberg *et al.*, 1995; Swanson *et al.*, 2016). Two processes are likely to be at work: 1/ the female tissues disfavour the alien pollen in any way (see above) and 2/ there is an interspecific pollen-pollen(-female) interaction leading to the alien pollen being outcompeted. This was evidenced to happen between pollens of two different accessions of *A. thaliana* (Swanson *et al.*, 2016). As mentioned in previous sections, negative pollen-pollen interactions is a potential male trait

under sexual selection, since it may provide a competitive advantage (Marshall *et al.*, 1996; Varis *et al.*, 2009).
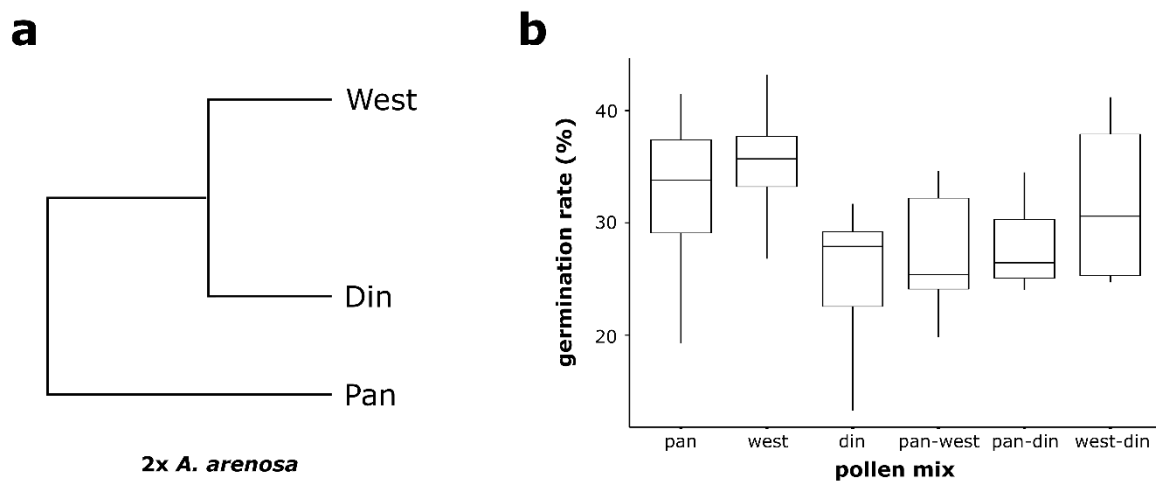


**Figure 20. Interlineage pollen-pollen interference. a**) schematic representation of the genetic relationship between the diploid lineages of *A. arenosa*. **b**) in vitro pollen germination rate with pure vs mixed pollen. West: West Carpathian; Din: Dinaric; Pan: Pannonian. Adapted from Haghighatnia et al. (in prep).

The observed interlineage pollen-pollen interference may be in line with expectations from sexual selection: for a given pollen grain, the same as it may be advantageous to promote closely related pollen (kin cooperation), it may be advantageous to interfere with pollen carrying a distant set of alleles. We thus could expect a genetic distance-dependent nature of pollen-pollen interactions: the more genetically distant the pollen grains, the more negative interactions between them, and the extreme case would be pollen from different species. We tested this hypothesis by performing *in vitro* pollen mix experiments between phylogenetic lineages of *A. arenosa*. We used 3 lineages, so called "West Carpathian", "Dinaric" and "Pannonian" defined by colleagues (Kolář *et al.*, 2016) and with the genetic relationship schematically represented in Figure 20a. We found that when pollen of the most distant lineage, Pannonian, was mixed with the two others, germination rates were lower than expected by an average between the two parents. On the other hand, mixing the closely related lineages West Carpathian and Dinaric, the mixed pollen germinated at an average rate between the two parents. Interestingly, no clear trend could be found for pollen tube growth (data not shown). These results are nevertheless preliminary, and statistical analyses are ongoing. If they are confirmed, they suggest that pollen-pollen interference affects pollen germination, and follows the expectation of sexual selection, with a positive relationship between genetic distance and pollen-pollen interference.

### c) Speciation at large scales

A current pressing question in evolutionary biology is whether microevolutionary processes such as the emergence of hybridization barriers translate into macroevolutionary processes, i.e. changes in speciation/extinction rates at large evolutionary scales (Harvey *et al.*, 2019). This applies to sexual selection as well: are the reproductive barriers driven by sexual selection impacting the diversity of

species we can observe at large scales, such as at the plant family level? Answering this question would allow us not only to evaluate the importance of sexual selection for plant speciation, but also to provide a mechanistic link between micro- and macroevolutionary processes.

Together with M. Koch (Heidelberg University, Germany), A. Machac (Center for Theoretical Studies, Charles University) and R. Schmickl (Dept of Botany, Charles University), M. Haghighatnia, a PhD student in my team, and I are currently addressing this question by inferring trait-dependent diversification rates in the Brassicaceae family. The underlying hypothesis is that if sexual selection significantly affects speciation at large scales, then in clades with more intense sexual selection, we should observe faster rates of speciation. This requires a proxy trait to measure the intensity of sexual selection, and we are currently focusing on 3 traits:

- **The total pollen:ovule ratio**: we consider this trait as a proxy for the intensity of pre-mating sexual selection (male-male competition) as postulated by Bateman's principle and the "Fleurs-du-mâle" hypothesis (Bateman, 1948; Queller, 1997). If we see an impact of this trait on diversification rates, we expect it to be linked with pre-mating speciation processes such as pollinator shifts.
- **The ratio between ovules and pollen deposited on stigma**: we consider this trait as a proxy for the intensity of post-mating sexual selection (pollen-pollen competition), as with higher pollen load on the stigma, the stronger the competition between pollen tubes to access ovules. If we see an impact of this trait on diversification rates, we expect it to be linked with post-mating speciation processes such as reproductive barrier via pollen-pollen interference.
- **Style length**: we consider this trait as a proxy for the intensity of intersexual selection (female mate choice), as the longer the style, the more stringent the female choice. If we see an impact of this trait on diversification rates, we expect it to be linked with post-mating speciation processes such as alien pollen rejection.

While the phylogeny of the Brassicaceae family is already well established by our collaborators M. Koch and R. Schmickl, M. Haghighatnia is currently generating the phenotypic data for these 3 traits. So far, all these traits show large variation across species, showing a promising groundwork to unravel sexual selection-dependent diversification across the Brassicaceae family.

## Conclusions & future works

**Are sexual selection and sexual conflict playing a relevant role in the evolution of plant species and their reproductive processes?** This is the guiding question of this thesis. Parental conflict appears as a major evolutionary factor impacting endosperm genome evolution, and as a by-product, hybrid seed incompatibility. The importance of sexual selection in plant evolution remains however less clear. Pre-mating sexual selection (male-male competition for pollen transfer), on one hand, seems to have a major role in floral trait evolution, as predicted under the "Fleurs-du-mâle" hypothesis and recently evidenced (Queller, 1997; Paterno *et al.*, 2020). On the other hand, post-mating sexual selection processes appear less impactful on plant evolution, perhaps due to their more cryptic nature for a human eye. Case studies have certainly shown that male-male competition and female choice can occur and affect reproductive traits in plants, but a broader, more holistic view on these processes and their role in plant reproduction evolution and speciation is required to reach firm conclusions. This will be the priority of my research team in the upcoming years, notably by working at different scales, from genes & populations up to large evolutionary scales.

In particular, the molecular and genetic basis for traits under sexual selection remain poorly understood. Thanks to the -omics era, we now can (and plan to) investigate, for example, the potential molecules secreted from pollen, leading to pollen-pollen interference or vice versa, to pollen-pollen collaboration via metabolomics. We can also better understand the general role of imprinted genes in endosperm development, and the interaction between paternally and maternally expressed genes, may it be antagonistic or collaborative, via the study of gene regulatory networks (GRNs). If we know what GRNs paternally and maternally expressed genes are included in, and their hierarchy in such GRNs, we will be able to evaluate the actual importance of imprinted genes and their link (or absence thereof) with parental conflict or sexual selection. In collaboration with Daniel Lang (HelmholtzZentrum München, Germany), this will be one of the tasks of a postdoc to be recruited in January 2022. Finally, population genomics tools are being developed to detect gene-gene coevolution in natural populations (Märkle *et al.*, 2021). Thanks to these methodological advances, we plan to unravel the coevolution between male and female genes in the wild, which will allow us to draw a mechanistic picture of sexual selection processes in plants, and also to evaluate the actual impact of sexual selection on genome evolution.

Finally, sexual selection acts on traits involved in reproduction: it is therefore in the front line among the processes able to drive the emergence of reproductive barriers and ultimately speciation. Whether it has such a prominent role in plants therefore deserves to be addressed. To convincingly answer this question, we cannot only focus at the microevolutionary scale, deciphering the mechanisms and genes leading to specific hybridization barriers. We also need to expand horizons and work at the macroevolutionary scale, i.e. study the pace at which species emerge, and test the role of sexual selection in this process. Only then, bridging the gap between two evolutionary scales, we may answer the crucial question: does sexual selection have a significant impact on plant evolution, and if so, how?