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Drivers of evolutionary change in *Podospora anserina*

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2020

ISSN 1651-6214
ISBN 978-91-513-0921-7
urn:nbn:se:uu:diva-407766

Dissertation presented at Uppsala University to be publicly examined in Ekmansalen, Evolutionary Biology Centre (EBC), Norbyvägen 18D, Uppsala, Tuesday, 19 May 2020 at 10:00 for the degree of Doctor of Philosophy (Faculty of Theology). The examination will be conducted in English. Faculty examiner: Professor Bengt Olle Bengtsson (Lund University).

Abstract
Ament-Velásquez, S. L. 2020. Drivers of evolutionary change in *Podospora anserina*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1923. 63 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0921-7.

Genomic diversity is shaped by a myriad of forces acting in different directions. Some genes work in concert with the interests of the organism, often shaped by natural selection, while others follow their own interests. The latter genes are considered "selfish", behaving either neutrally to the host, or causing it harm. In this thesis, I focused on genes that have substantial fitness effects on the fungus *Podospora anserina* and relatives, but whose effects are very contrasting. In **Papers I** and **II**, I explored the evolution of a particular type of selfish genetic elements that cause meiotic drive. Meiotic drivers manipulate the outcome of meiosis to achieve overrepresentation in the progeny, thus increasing their likelihood of invading and propagating in a population. In *P. anserina* there are multiple meiotic drivers but their genetic basis was previously unknown. In **Paper I**, we demonstrated that drive is caused by members of the *Spok* gene family. We discovered two new *Spok* genes, *Spok3* and *Spok4*, which locate in different chromosomes in different strains. In **Paper II**, we showed that *Spok3* and *Spok4* are found on a gigantic (up to 247 Kb long) variant of *Enterprise*, a *Crypton*-like transposable element. *Enterprise* likely mobilize through the action of a putative tyrosine-recombinase that we call *Kirc*. When carrying the *Spoks*, this element has double selfish properties: transposition and meiotic drive. In addition, we found that homologs of the *Spoks* (**Paper I**) and of *Kirc* (**Paper II**) are widespread in fungi but their phylogenies are discordant with that of the species, suggesting that they have undergone horizontal gene transfer. In **Papers III** and **IV**, I turned the focus into genes that have an adaptive function. In fungi, the *het* genes control conspecific self/non-self recognition. Such genes are expected to evolve under frequency-dependent balancing selection. In **Paper III**, we found evidence of balancing selection acting on some *het* genes across the *P. anserina* species complex. Unexpectedly, we also discovered that *het* genes of the HNWD gene family are duplicated in a transposon-like manner, broadening our understanding of their potential fitness effects. Finally, in **Paper IV** we show how *het* genes with pleiotropic effects on sexual recognition lead to the evolution of strong reproductive isolation, and hence speciation. Overall, the results of my thesis highlight the functional intersection between mobile selfish genetic elements and other genes, either selfish or adaptive, and their effects on genome architecture and population structure.

Keywords: Podospora, Meiotic drive, Spore killing, vegetative incompatibility, heterokaryon incompatibility, allorecognition, speciation, Transposable elements, selfish genetic elements, fungi

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ISSN 1651-6214
ISBN 978-91-513-0921-7
urn:nbn:se:uu:diva-407766 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-407766)

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Vogan, A.A.*, **Ament-Velásquez, S.L.***, Granger-Farbos, A., Svedberg, J., Bastiaans, E., Debets, A.J., Coustou, V., Yvanne, H., Clavé, C., Saupe, S.J., Johannesson, H. (2019) Combinations of *Spok* genes create multiple meiotic drivers in *Podospora*. *eLife*, 8: e46454

II Vogan, A.A., **Ament-Velásquez, S.L.**, Bastiaans, E., Wallerman, O., Saupe, S.J., Suh, A., Johannesson, H. (-) The *Enterprise*: A massive transposon carrying *Spok* meiotic drive genes. *Manuscript*

III **Ament-Velásquez, S.L.**, Vogan, A.A., Wallerman, O., Hartmann, F., Gautier, V., Silar, P., Giraud, T., Johannesson, H. (-) The evolution of the allorecognition gene repertoire in the *Podospora* species complex. *Manuscript*

IV **Ament-Velásquez, S.L.**, Vogan, A.A., Granger-Farbos, A., Bastiaans, E., Martinossi-Allibert, I., Saupe, S.J., de Groot, S., Lascoux, M., Debets, A.J.M., Clavé, C., Johannesson, H. (-) Allorecognition genes drive reproductive isolation in *Podospora anserina*. *Manuscript*

* These authors contributed equally

Additional papers

The following papers were published during the course of my doctoral studies, but are not part of this thesis.

Ament-Velásquez, S.L., Breedy, O., Cortés, J., Guzman, H.M.H.M., Wörheide, G., Vargas, S., (2016). Homoplasious colony morphology and mito-nuclear phylogenetic discordance among Eastern Pacific Octocorals. *Molecular Phylogenetics and Evolution*, 98: 373–81

Ament-Velásquez, S.L., Figuet, E., Ballenghien, M., Zattara, E.E., Norenburg, J.L., Fernández-Álvarez, F.A., Bierne, J., Bierne, N., Galtier, N. (2016). Population genomics of sexual and asexual lineages in fissiparous ribbon worms (*Lineus*, Nemertea): Hybridization, polyploidy and the Meselson Effect. *Molecular Ecology*, 25 (14): 3356–69

Contents

Abbreviations

DNA	Deoxyribonucleic acid
bp, kb, Mb	Base pair, Kilo base, Mega base
het	Heterokaryon incompatibility gene
Indel	Insertion / Deletion
TE	Transposable element
LTR	Long terminal region
LD	Linkage disequilibrium
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
VI	Vegetative incompatibility reaction
$\boldsymbol{\pi}$	Nucleotide diversity
$\boldsymbol{\theta_w}$	Watterson's theta
$\mathbf{D_{xy}}$	Average number of pairwise differences

Introduction

In a report meant to introduce the filamentous fungus *Podospora anserina* (Ces. Ex Rabenh.) Niessl as a model organism, the German geneticist Karl Esser wrote "The reader may ask, why are these Europeans using such a strange and, from the first glance, rather complicated organism as Podospora, and, why do they not integrate into the large family of Neurosporologists?" (Esser, 1969). To answer, he then proceeded to list a number of reasons as to why *P. anserina* is great for research. They included things that are important in model organisms, like a short life cycle and the amenability to mutagenesis, but also other properties like the easy isolation of haploid spores, no asexual propagules to contaminate other plates, and interesting biological aspects of its life cycle. With my work, I wish to add to this list by emphasizing the use of *P. anserina* as a model organism for the study of fundamental questions in evolution biology.

In tune with traditional *Podospora* literature, the focus of my research is heavily gene-centric. Specifically, I gravitate around two types of genes: selfish and allorecognition genes. Selfish genetic elements (including protein-coding genes) are parasitic units of DNA sequence that reside within a genome, and whose propagation is disconnected from the organismal fitness. They might replicate neutrally or they might induce deleterious effects on the host, creating a conflict of interests (Burt and Trivers, 2006; Werren et al., 1988). Indeed, selfish genetic elements are notorious for creating intragenomic conflict, a concept extensively discussed below. By contrast, allorecognition genes are direct products of natural selection for the benefit of the organism. Their function is to provide a system of discrimination between the self and the non-self (the greek "allos" means "other" or "another"). Without self/non-self recognition systems, organisms would be effectively blind, vulnerable to exploitation by parasites or incapable of finding suitable mating partners, for example. Despite their differences, both classes of genes have evolve countless times, probably starting at the base of the Tree of Life (Brosius and Tiedge, 1995; Iranzo et al., 2016; Koonin et al., 2017; Rimer et al., 2014; Tsutsui, 2004). Moreover, selfish genetic elements and allorecognition genes are recurrently linked, especially in *Podospora*. This connection sometimes is circumstantial, but often enough it is due to common underlying molecular mechanisms.

The thesis is thus divided in two major topics, with the two first papers focused on selfish genetic elements, and the two last on allorecognition

genes. As we will see, **Paper I** dives directly into the genetics and molecular biology of a specific type of selfish genes known as meiotic drivers. In **Paper II** we reveal how these meiotic drivers can fuse with other selfish genetic elements, creating complex entities capable of having significant effects on genome evolution. In the second part, **Paper III** characterizes the evolution of allorecognition genes in *Podospora* species, uncovering an unexpected selfish twist to their origin. Lastly, **Paper IV** reveals how allorecognition genes with pleiotropic effects on the sexual function can lead to speciation.

As a warning, maybe the "rather complicated" aspects of *Podospora* biology is what prevented others to forget about *Neurospora* or other, more forgiving, model fungi (let alone animals and plants). But as I hope to convey here, once one overcomes the disconcerting shock, *P. anserina* revelations make it all worth it.

Intragenomic conflict and meiotic drive as evolutionary forces

Although we tend to perceive individual organisms as well integrated units, where all the genetic components work harmoniously, a closer look quickly reveals the pervasiveness of conflict between the constitutive parts. Different types of genes, specific chromosomes, or even organelles like the mitochondrion, can all be under selective forces with antagonistic effects (Burt and Trivers, 2006). The concept of "Genomic conflict" has being used as an umbrella term for many different phenomena involving conflict. It can be used to describe conflict between parents and offspring, or between a host and an endosymbiont (Ross et al., 2010). However, researchers often use the term interchangeably with "intragenomic" conflict. While genomic conflict can occur between genes in different individuals (Rice, 2013), intragenomic conflict happens within a single individual (Werren, 2011).

Far from the original notion of a genetic oddity, intragenomic conflict is more and more considered an important contributor to (mal)adaptation and disease (Kazazian and Moran, 2017; Rice, 2013; Úbeda and Wilkins, 2008). The evolution of major features of life, such as sex (Santos et al., 2003), recombination (Archetti, 2003), and genome size (Ågren and Wright, 2015) have all been explained as a consequence of intragenomic conflict. Nonetheless, there is in fact no absolute consensus for the definition of intragenomic conflict.

Some researchers have defined intragenomic conflict in terms of differential transmission modes between gene classes, with selfish genes increasing their own frequency (Lindholm et al., 2016; Werren, 2011). This concept excludes conflict between genes that behave in a Mendelian fashion. Others

concentrate on antagonistic selective forces affecting genes in the same individual (Grafen, 2006; Hurst et al., 1996) regardless of their transmission modes. Gardner & Úbeda (2017) define intragenomic conflict mathematically in terms of the "inclusive fitness" concept, that is, the idea that the evolutionary success (in the sense of increasing frequencies) of a gene is given by the additive fitness effects multiplied by relatedness coefficients of other gene copies (Hamilton, 1970, 1964). In other words, the fitness effects of related copies can contribute to the overall fitness of a gene. In Gardner & Úbeda (2017)'s view, a given pair of genes might have an altruistic, mutualistic, and conflicting behavior depending on the contextual interaction between selection and relatedness. Using this framework, they describe three types of conflict: by situation, origin, and destination.

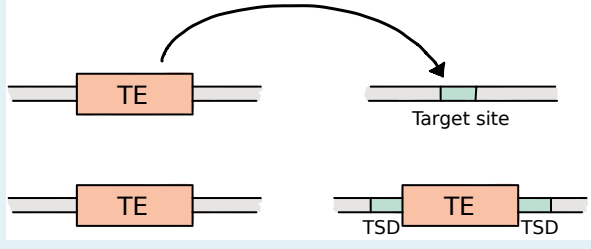
Situation conflict may arise between two genes that have different optima regarding the same trait. This is often the case for sex-chromosomal genes affecting, for example, an extreme value of a phenotype (like horns) that is beneficial for a male but deleterious for a female. On the other hand, two genes might be under *origin conflict* when they disagree about their relatedness to the individual's non-descendant relatives. That is, when there is interaction between two individuals with a common ancestor (e.g. brothers), there might be either benefits or costs for loci within a single individual (e.g. X vs. autosomal chromosomes) based on the probability of sharing genes that are identical by origin with the other individual. This type of conflict is caused by, for example, imprinted genes and genes that are transmitted with the cytoplasm instead of the nucleus. Finally, *Destination conflict* occurs when two genes have different probabilities of being transmitted to direct descendants. Transposable elements (**Box**), biased gene conversion, and B-chromosomes are examples of destination conflict (Gardner and Úbeda, 2017).

In their seminal book "Genes in Conflict", Burt and Trivers (2006) describe at length several cases of genomic conflict that were later reclassified by Gardner & Úbeda (2017). Destination conflict includes the largest variety of examples, but several of them can be grouped together under the common phenomenon of **meiotic drive**. In its broad sense, meiotic drivers are genetic elements (from alleles and genes, to whole chromosomal sets) that circumvent Mendel's law of equal segregation, promoting their own transmission at the expense of their homologues (Lindholm et al., 2016; Sandler and Novitski, 1957; Werren, 2011). In the strict sense, the bias is induced mechanistically during meiosis, with meiotic drivers competing for the inclusion in the gametes. Other genetic mechanisms might alter transmission post-meiotically, in which case they are often termed segregation distorters (SDs). SDs are diverse both in taxonomic span and genetic origin, but they generally act by either toxin-antitoxin or killer-target mechanisms (Bravo Núñez et al., 2018). In the toxin-antitoxin case, all the gametes are exposed to the toxin but only gametes with the SD element can produce the antitoxin (anti-

dote) and hence survive. In the killer-target systems, a killer element encoded by the SD directly attacks the gametes that have a specific "target", which is typically tightly linked to the locus of the SD but in a different haplotype (Bravo Núñez et al., 2018). Further classifications of meiotic drivers can be made based on different criteria, depending on the authors (**Figure 1**). In here, I will follow Lindholm et al. (2016) in their broad use of meiotic drive, which includes SDs.

Box. Transposable elements

Transposable elements are familiar examples of selfish genetic elements, and hence I dedicate less space to introduce them. They are commonly divided in two major categories: Class I and Class II elements (Kapitonov and Jurka, 2008; Wicker et al., 2007). Class I, also known as retrotransposons, mobilize by a copy-and-paste mechanism, meaning that the parental copy is kept in place when new copies are produced. As the name suggests, retrotransposons are characterized by using RNA as transposition intermediate. By contrast, Class II elements, or DNA transposons, move using a cut-and-paste mechanism, where the original copy is excised into a new location via a transposase enzyme. This classification is used for convenience, but several deviations of the original definitions exist (Lynch, 2007; Wicker et al., 2007). In addition, both categories have non-autonomous variants that depend on the enzymatic machinery of fully intact, autonomous ones for propagation (Jurka et al., 2007). Importantly, many elements of both Class I and II integrate into a new target site through a staggered cut, leading to a target site duplication or TSD (Wicker et al., 2007). The presence of TSDs is key for the findings of **Papers II** and **III**.



From an evolutionary perspective, meiotic drive can be potentially very important. By their selfish nature, meiotic drivers can reach fixation even if they are deleterious for the individual organism (see **Figure 2**; Crow, 1991; Sandler and Novitski, 1957). If the driver is neutral, deleterious mutations might still hitchhike with them through genetic linkage (Crow, 1991; Lyttle, 1991). Many meiotic drivers are formed by two interacting loci (encoding for example a poison and an antidote) that are maintained together by some form of recombination suppression, which is likely to extend to other genes. Given the fitness impact on the population, drive suppressors might evolve, followed by modifiers capable of increasing drive, in an arm-race dynamic (Burt and Trivers, 2006). Enhancers are most effective when tightly linked to drivers, which may lead to selection for inversions, as can be seen in systems like the *Segregation Distorter* (*Sd*) driver of *Drosophila* flies (Crow, 1991; Temin and Marthas, 1984). Thus, meiotic drive can induce changes in ge-

nome architecture (Lindholm et al., 2016). Moreover, dramatic ecological effects can be seen when meiotic drivers are linked to sex chromosomes, provoking biases in sex ratios that could in principle extinguish populations (Jaenike, 2001).

The potential impact of meiotic drive on the process of speciation has also been emphasized (Crespi and Nosil, 2013; Johnson, 2010). By going to fixation in one population and not the other, and/or by accumulation of different suppressors and modifiers, meiotic drive can lead to Bateson-Dobzhansky-Muller incompatibilities (Bravo Núñez et al., 2018; Crespi and Nosil, 2013). For example, in taxa with asymmetric female meiosis (i.e. only one egg and three polar bodies), meiotic drive could lead to fast evolution of centromeric sequences, and thus to automatic meiotic dysfunctions between diverging populations (Henikoff et al., 2001). Accordingly, Crespi and Nosil (2013) coined the term "conflictual speciation" as oppose to, for example, ecological speciation. Nonetheless, meiotic drive can have the opposite effect as well. For populations connected by some degree of gene flow, a driver might invade all and reach global fixation. In the process, the variation of linked portions of the genome can get swept away, reducing overall divergence across populations (Crespi and Nosil, 2013; Meiklejohn et al., 2018).

Given the indications of a large impact on evolutionary processes, why has meiotic drive been largely ignored in usual evolutionary research? Perhaps the main reason is the lack of data on its prevalence in nature. Although common perception is that it is rare, some forms of meiotic drive have been found in many well-studied organisms like mice, flies, corn, and monkeyflowers [reviewed by Burt and Trivers (2006)]. One hypothesis for being rare is that there could be a strong negative detection bias (Bravo Núñez et al., 2018). For one thing, a meiotic driver often needs to have (or to be linked to) an observable phenotypic trait in order to be detected by a lucky observer. Not surprisingly, the majority of examples come from meiotic drivers biasing sex ratios (Jaenike, 2001). Since classic genetic analyses are often performed using highly fertile individuals, this excludes carriers of drivers that typically have fertility problems to begin with (Bravo Núñez et al., 2018). And importantly, if meiotic drivers really do go to fixation fast (**Figure 2**), they might be difficult to see in action because once fixed they are no longer altering segregation. Some well-studied drivers, like the *t* haplotype in mice and *Sd* in flies, are in fact observed at stable low frequencies in natural populations (Burt and Trivers, 2006). Such situation might arise when the drivers are lethal recessives, or when there are complex dynamics with suppressors and enhancers (Burt and Trivers, 2006; Carvalho and Vaz, 1999). However, it is unknown if stable polymorphism is a special case rather than the rule (Lindholm et al., 2016).

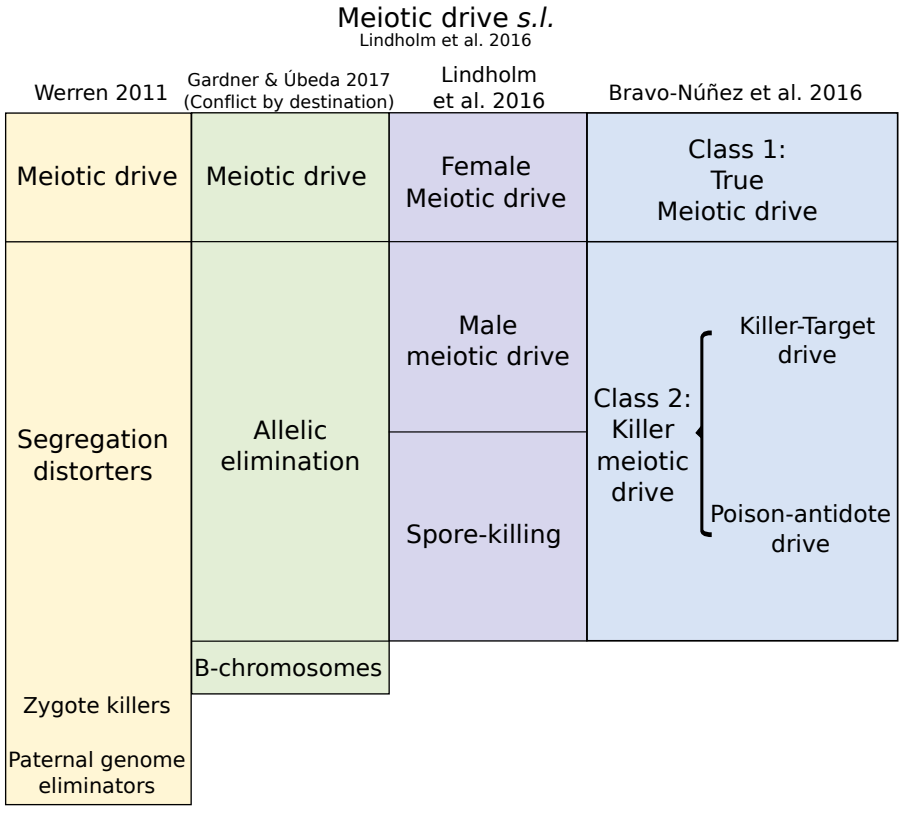


Figure 1. Different terms have been used to define the ability of a genetic element to circumventing Mendel's equal segregation law. Colored boxes correspond to different authors (names on top), whilst the length of the box is proportional to the diversity of genetic elements included. In a broad sense, all these categories can be called meiotic drive (Lindholm et al., 2016). However, a mechanistic view of elements acting strictly during meiosis is what some consider "true" meiotic drive (Bravo Núñez et al., 2018). True meiotic drivers favor their own transmission by ensuring their segregation into the egg, as opposed to the polar bodies, during female gametogenesis (Gardner and Úbeda, 2017; Lindholm et al., 2016; Werren, 2011). Other elements can be broadly classified as segregation distorters (Werren, 2011). Subcategories of the segregation distorters can be based on their mode of action, although they might ignore some special cases like zygote killers and others (Bravo Núñez et al., 2018; Gardner and Úbeda, 2017). Male meiotic drive and spore killing are both gamete killers, with the important difference that in fungi, the surviving haploid cells become the offspring instead of the gametes (Lindholm et al., 2016). Finally, the killer meiotic drivers of Bravo Nuñez et al. (2018) can be divided by their molecular mechanism, with each category (killer-target or poison-antidote) containing examples of both male meiotic drive and spore killing.

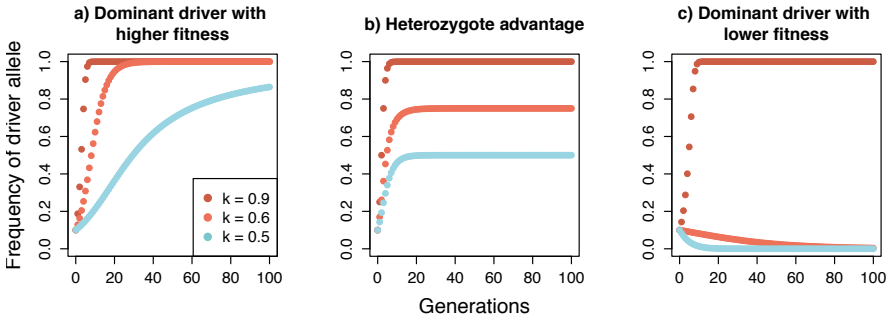


Figure 2. An autosomal meiotic driver goes to fixation when the transmission bias overcomes negative fitness effects. Consider a one-locus, two alleles simple model, where one allele is a meiotic driver (transmission ratio k > 0.5, red). a) If the driver has both higher fitness and the transmission bias advantage, it will quickly go to fixation at a faster rate than a non-driver allele (k = 0.5, blue); b) under a case of balancing selection, where the heterozygote has a higher fitness than both equally fit homozygotes, again the driver goes to fixation instead of reaching a stable polymorphism if the bias is strong enough; b) under a bad scenario for the driver, where it has a lower fitness and it is dominant (so easier to purge by selection), it will still reach fixation as long as the driving advantage is strong enough (in the plot, it goes to extinction for low transmission ratio of k = 0.6). Dynamics based on the equations 1 and 2 of Crow (1991). Notice that the model assumes infinite population size, random mating, non-overlapping generations, and no mutation. The starting frequency of the driver was set to 0.1. Fitness values used were: a) w1 = w2 = 1, w3 = 0.9; b) w1 = w3 = 0.6, w2 = 1; c) w1 = w2 = 0.8, w3 = 1.

An observable case of meiotic drive: spore killing in fungi

Compared to other organisms, fungal meiotic drive is easy to detect. In animals, for example, first you have to notice the presence of a character that has segregation distortion, which might be difficult as mentioned above. Then, careful dissection and genetic studies have to be done on heterozygotes in order to distinguish meiotic drive from other types of incompatibilities (e.g. lethal alleles). In Ascomycetes, however, meiotic drive can be readily observed in crosses between two haploid individuals (Turner, 2001). Meiosis takes place within the fruiting bodies, followed by spore formation. When a meiotic driver is present in one of the parents, the spores that do not carry the meiotic driver ("sensitives") are killed by the spores that do ("killers"). Hence, fungal meiotic drive is known as **spore killing**, which is easily observed by fruiting body dissection.

While similar to male (sperm) meiotic drive in general properties, spore killing is special amongst SDs in that the surviving product of meiosis are not the gametes, but the offspring themselves. This is so because haploidy is the dominant stage of the life cycle in most ascomycetes. As a consequence,

fungal meiotic drive directly results in a fitness cost for the carrier. In animals, the loss of non-driving sperm often has no effect in the total number of offspring that a male may have. However, spore killing alone would result in no absolute increase in the number of driving offspring (just the dead of the sensitives). Lyttle (1991) pointed out that this relative advantage (instead of absolute) makes spore killers a weak type of meiotic drive. Accordingly, theoretical work of Nauta & Hoekstra (1993) suggests that a spore killer can only invade to fixation if it enjoys an additional fitness advantage other than the transmission bias. Moreover, they predict that stable polymorphism can only occur in the presence of resistant (not susceptible to killing but not killers themselves) individuals in the population (Nauta and Hoekstra, 1993).

Examples of spore killers

Although spore killing has been detected in a number of species, including *Fusarium* spp. and *Cochliobolus heterostrophus*, in here I will revise only the best-studied cases.

Spore killing was first described as such by Turner and Perkins (1979) in the genus *Neurospora*. The spore killer *Sk-1* was identified in *Neurospora sitophila*, while two mutually destructive spore killers, *Sk-2* and *Sk-3*, where found in *Neurospora intermedia*. Interestingly, *Sk-1* is common in natural populations, from absent to completely fixed depending on the locality. In contrast, *Sk-2* and *Sk-3* are appallingly rare: only four *Sk-2* and one *Sk-3* strains have ever been found (Turner, 2001). On the other hand, only one strain of *N. sitophila* is considered resistant to *Sk-1*. Conversely, resistant strains for *Sk-2* and *Sk-3* are common in Asia and Oceania, but completely absent from the Americas and Africa (Turner, 2001).

Like many meiotic drivers in animals and plants, *Sk-2* and *Sk-3* are two-loci systems: a killer element (presumably producing some sort of toxin) and a resistance gene (named *rsk*; Hammond et al., 2012). For both *Sk-2* and *Sk-3*, the killer and resistance loci are linked together in a large haplotype including the centromere of chromosome 3. Remarkably, an independent set of inversions for both spore killers suggests paralleled evolution of the two systems as meiotic drivers in *N. intermedia* (Svedberg et al., 2018). In the case of *N. sitophila*'s *Sk-1*, Svedberg et al., (2020) identified a single gene responsible for both killing and resistance (*Spk-1*) that seems to have been introgressed from another species: *Neurospora hispaniola*. Moreover, *N. sitophila* can be divided in three clades: one fixed for *Sk-1*, one fully sensitive, and a clade with a mixture of killers and sensitives (Svedberg et al., 2020). Thus, the genus *Neurospora* shows a great variety of population dynamics and genomic effects amongst related species.

Another well-studied example of spore killing comes from *Schizosaccharomyces pombe*. In this yeast, a diverse family of genes named *wtf* (for with Tf, a transposable element) has been shown to cause spore killing (Hu et al.,

2017; Nuckolls et al., 2017). The *wtf* genes are not present in other studied yeasts (Rhind et al., 2011). Each active gene of this family acts as both a toxin and an antidote by expressing two alternative transcripts of different length. The long transcript functions as an antidote and localizes within the carrier spores. The short transcript can diffuse to spores that do not contain the *wtf* gene, poisoning them (Nuckolls et al., 2017). Most *wtf* paralogs that have been tested against each other were not mutually resistant, meaning that they drive independently (Hu et al., 2017; Nuckolls et al., 2017), but some are able to suppress the drive of other paralogs (Bravo Núñez et al., 2020). Notably, the genome of the analyzed *S. pombe* strains also contains copies of *wtf* that have lost the short (poisonous) transcript, and even more degenerated copies that can be regarded as non-functional pseudogenes. Such pattern is consistent with a full scenario of birth-and-death for the *wtf* killers presumably affecting the evolution of *S. pombe* (Hu et al., 2017; Nuckolls et al., 2017). Moreover, the first indication of spore killing due to these genes was found in hybrids of *S. pombe* and *S. kambucha*, suggesting spore killing might contribute to the reproductive isolation between the two (Hu et al., 2017; Zanders et al., 2014).

Similarly to *Neurospora* spp. and *S. pombe*, fungal meiotic drive has been extensively studied in *P. anserina*. This fungus is particular in having at least two independent spore killing systems. The first one is a frankly bizarre system of a single gene called *het-s*. Multiple studies on its molecular mechanisms have shown that *het-s* is a gene with an allorecognition function, defining vegetative compatibility groups (reviewed by Saupe, 2007). At the sequence level, *het-s* has two alleles: *het-s* and *het-S*. Phenotypically, however, the *het-s* allele can have two forms: a prion-forming version termed [het-s], and a non-prion form called [het-s*]. Prion proteins [het-s] can propagate by changing the configuration of "normal" proteins [Het-s*] into more prions, spreading in an infectious manner (Saupe, 2007). In a heterozygous cross with a female [het-s] and male [het-S], it was shown that the prion triggers the death of spores with a *het-S* genotype in a process that is dependent on the temperature (Dalstra et al., 2005). The prion is transmitted via the cytoplasm, hence the maternally-biased meiotic drive. However, despite its selfish behavior, the allorecognition function of *het-s* is probably adaptive, as it limits the spread of a deleterious senescence plasmid (Debets et al., 2012).

The other type of *P. anserina* spore killing that is well described at the molecular level depends on a novel family of genes named *Spok* (for spore killing). Much like the *wtf* genes of *S. pombe*, a single *Spok* gene can act as both the toxin and the antitoxin (Grognet et al., 2014b). In a cross of a reference strain of *P. anserina* termed S and another strain called T from the related species *Podospora comata*, Grognet et al. (2014b) described spore killing due to a gene in T's chromosome 5: *Spok1*. When deleting *Spok1* from the strain T, they discovered that the S strain has its own killer gene in

the same chromosome but in a different location: *Spok2*. Interestingly, the *Spok* genes can interact: *Spok1* is resistant and dominant over *Spok2*. Grognet et al. (2014b) further show that the *Spok* family is widespread across ascomycetes, but it is unclear if they act as meiotic drivers in other species. Since deleting the *Spok* genes has no obvious phenotypic effects, there is no evidence of an alternative, benign function (Grognet et al., 2014b).

While the *het-s* and *Spok1/2* systems are relatively well understood at the molecular level, there are other observations of spore killing in different *P. anserina* strains that remain unexplained. For example, although not interpreted as such in the moment, other *P. anserina* crosses were shown to have segregation distortion as early as 1967 (Padieu and Bernet, 1967; Turner and Perkins, 1991), but with different distortion patterns. And even more remarkably, in the early 2000s a population from the Netherlands was shown to be polymorphic for different spore killer types called *Psk* (for *Podospora* spore killing) that interact in a hierarchical way (van der Gaag et al., 2000). The genetic basis of the *Psks* was not clarified, however. In addition, a correlation was observed between vegetative incompatibility (different from *het-s*) and the *Psk* phenotype, suggesting they might be connected either physically or mechanistically (van der Gaag et al., 2003). Furthermore, for all its potential as a model system, there is very little knowledge on the evolutionary history of *P. anserina* or its relatives. Most of these gaps in knowledge are addressed extensively in the **Papers** of this thesis. Yet, in order to understand their implications, first it is necessary to revise several aspects of the *Podospora* natural history.

The protagonists: *Podospora anserina* and allied taxa

The biology of *P. anserina* was extensively studied during the past century, particularly from the 40s to 70s (Silar, 2013 and references therein). Much is known on its fruiting body and ascospores development (Beckett and Wilson, 1968; Raju and Perkins, 1994) and allorecognition systems (Esser, 2016; Labarère, 1978), making *P. anserina* an important model for filamentous fungi biology. Like species of *Neurospora* and *Sordaria*, other model fungi in Sordariales, *P. anserina* has a relatively small genome size (~35 Mbp; Espagne et al., 2008b) and it is very amenable for lab work (Silar, 2013). *P. anserina* is, however, remarkable in other aspects. For example, after one to three weeks of being grown in the lab, a *P. anserina* strain typically enters a process of ageing (senescence) and eventually dies, a phenomenon infrequent in the fungal kingdom. It is believed that the short life span of *P. anserina* is related to its coprophilous lifestyle, which selects for rapid sexual reproduction but not for mycelium maintenance (Geydan et al., 2012).

Only a limited number of French laboratory strains collected in the 30's have been the focus of all major studies, with mainly a couple of strains acting as laboratory standards (eg. the strain S used for the reference genome; Espagne et al., 2008). During the 1990s and early 2000s, the Laboratory of Genetics of Wageningen University & Research started a large collection of strains sampled locally, allowing for multiple population studies (Bastiaans et al., 2016; Debets et al., 2012; Hermanns et al., 1995; van der Gaag et al., 2000, 1998; van Diepeningen et al., 2010). Additionally, a few strains have been collected successfully in Usingen, Germany (Hamann and Osiewacz, 2004). Other strains collected elsewhere were originally identified as *P. anserina*, *P. pauciseta* or *P. comata*, but recent phylogenetic analysis revealed up to seven distinct lineages (Boucher et al., 2017). Therefore, the full geographical distribution of all species in the complex is unknown and it can only be approximated by the identified strains (**Figure 3**).

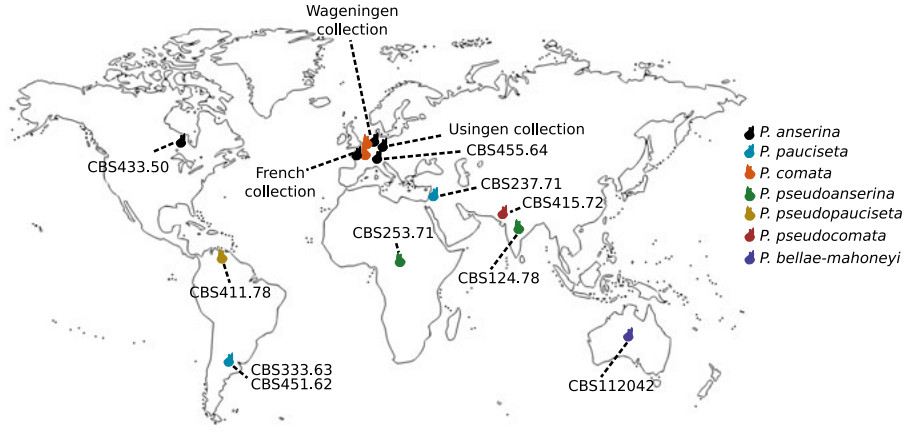


Figure 3. The geographical sampling of the *Podospora anserina* complex. The taxonomic affiliation of different strains is based on Boucher et al. (2017) and our own phylogenetic analyses (**Paper IV**). Most taxa have only one strain collected worldwide, available at the Westerdijk Fungal Biodiversity Institute Collection (CBS numbers). The *P. anserina* population sampling comes from the French (several laboratories), Wageningen (Laboratory of Genetics in Wageningen University & Research), and Usingen (Goethe University) collections.

In **Paper IV** we explore the relationships between the lineages within the *P. anserina* species complex. Apart from these taxa, however, other species under the name *Podospora* may or may not be related to *P. anserina*[1]. Indeed, the genus *Podospora*, like most of the genera in the family Lasiosphaeriaceae, is extremely polyphyletic (Kruys et al., 2015). There is a gen-

[1]A recent publication assigned the *P. anserina* complex to the genus *Triangularia* (Wang et al., 2019). However, I have chosen to use the classic name in order remain congruent with thousand of articles and decades of research using the name *Podospora*.

eral lack of support on the phylogenetic relationships within Lasiosphaeriaceae, but the family can be roughly divided into five clades: I, II (including Sordariaceae), III, IV and Chaetomiaceae. The *P. anserina* complex is clearly nested within the clade Lasiosphaeriaceae IV, but it remains unclear which taxa are their closer relatives (Kruys et al., 2015), making evolutionary inferences on trait evolution problematic.

A peculiar life cycle

P. anserina can be found growing on dung of a variety of herbivore animals, including rabbit, sheep, cow, and especially horse (**Figure 4**). Its fruiting bodies (perithecia) appear late in the succession of the coprophilous fungal community, after around 10 days (personal observation). Strains typically live around three weeks in laboratory conditions (van der Gaag et al., 1998), which might be a reasonable estimate of how long the mycelium is viable in nature. When ripe, the perithecia shoot the spores forcefully into surrounding vegetation that gets subsequently eaten by an herbivore. The spores then go through the digestive track of the animal until deposited once more in dung, where they germinate and reproduce sexually – either by selfing or outcrossing. This interpretation of the life cycle is supported by the fact that germination rate of spores in the lab is substantially higher under an acid treatment of ammonium acetate (Silar, 2013), a condition resembling the herbivore digestive track. Moreover, unlike many other fungi, *P. anserina* has no asexual spores (Silar, 2013), suggesting that it relies heavily on the sexual spores (known as ascospores) for propagation.

Reproductive biology of fungi and of *P. anserina*

Traditionally, sexual breeding systems in fungi are divided into heterothallism and homothallism. Heterothallic individuals can only mate with another individual of different mating type. Homothallic individuals, in contrast, have no discrimination system in place and can either fertilize themselves or any other individual in the population (Billiard et al., 2011). In ascomycete fungi, mating types are genetically defined by a single locus with two variants that are highly divergent and often contain completely different genes. Since the two variants at the mating type locus (MAT) are not alleles, they are instead called idiomorphs (Butler, 2007; Metzenberg and Glass, 1990). Hence, heterothallic strains may have one of two idiomorphs, which are prevented from recombining during meiosis (Idnurm et al., 2015). Homothallism can be achieved in a number of ways, but often the two idiomorphs are placed in the same haploid genome by, for example, unequal crossing over (Gioti et al., 2012; Yun et al., 1999).

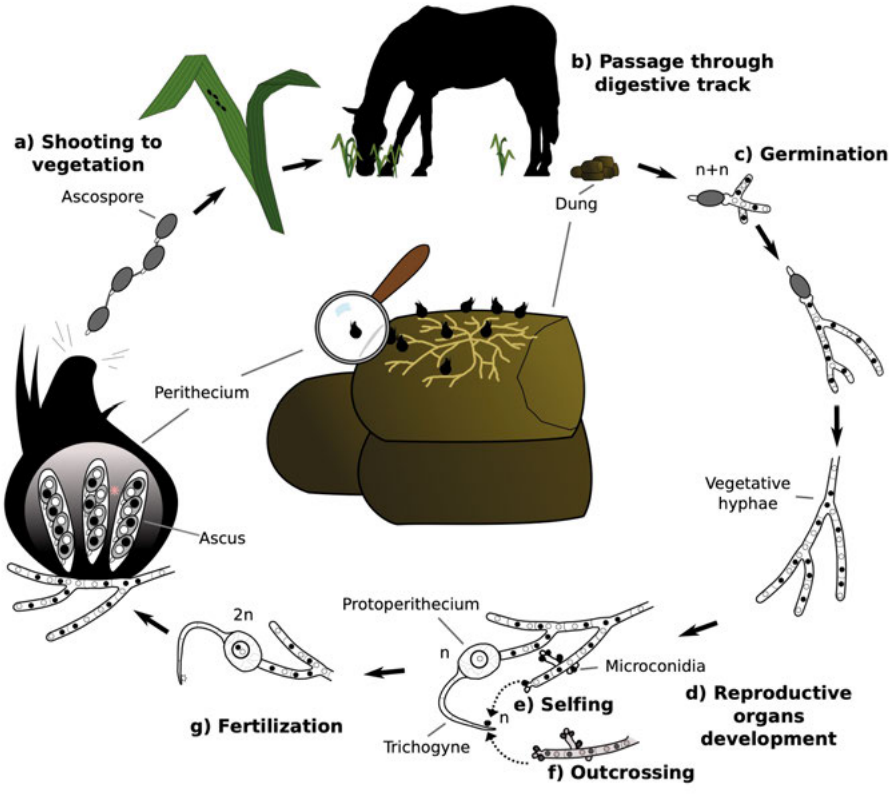


Figure 4. The life cycle of *Podospora anserina*. As other coprophilous fungi, *P. anserina* develops fruiting bodies (perithecia) that shoot spores into the surrounding vegetation (**a**). There, the spores get eating by a herbivore, such as a horse or a rabbit. Going through the digestive tract of the animal activates the spores (**b**), which germinate later in the dung (**c**). The resulting dikaryotic (n+n) mycelium grows until starvation, at which point it differentiates reproductive organs. Each *P. anserina* individual is hermaproditic and produces anisogamic gametes (n): smaller male gametes (microconida or spermatia) and large complex female structures (protoperithecia). The protoperithecium has a large projection termed trichogyne, which reacts to a compatible microconidum that might come from the same individual (**e**) or from a different one (**f**). The nucleus of the microconidium travels through the tricogyne to the protoperithecium's nucleus. After fertilization (**g**), an ascogenous hypha differentiates into a sac-like structure or ascus where meiosis takes place, producing spores. Diagram based on Saupe (2007) and Pinan-Lucarré (Pinan-Lucarré et al., 2007). See **Supplementary Figure 9** of **Paper III** for an extended version.

A special case has evolved in species like *P. anserina*. Although functionally heterothallic, *P. anserina* is considered to be "pseudohomothallic" (or secondarily homothallic) because it attains self-fertility by co-packaging nuclei of the two different mating types (called *mat-* and *mat+*) into the same spore (Ames, 1934). As a result, the sexual spores of *P. anserina* germinate into a mycelium with two types of haploid nuclei, a condition known as heterokaryosis or dikaryosis. Indeed, this fungus is a dikaryon (n+n) most of its life

cycle (**Figure 4**), in contrast to related ascomycetes that are typically haploid (n). After meiosis, heterothallic taxa like *N. crassa* undergo a round of mitosis before forming the spore walls, developing eight haploid spores in a sac-like structure called ascus. In the case of *P. anserina*, after the mitosis, pairs of non-sister nuclei are assorted into a single spore. Hence, a normal *P. anserina* has typically four spores per ascus (Ames, 1934).

The segregation of the mating type in dikaryotic spores is genetically controlled by a single reciprocal crossover between the MAT locus and the centromere, combined with non-overlapping second division spindles (Rizet and Engelmann, 1949). The MAT locus undergoes second division segregation (SDS), ensuring a final arrangement of nuclei with both mating types (**Figure 5a**). SDS happens ~98% of the time, with the remaining ~2% resulting in first division segregation (FDS) of the mating type and therefore, in self-sterile spores of a single mating type (**Figure 5b**). In very rare cases [0-5% of the asci, depending on the strain; van der Gaag (2005)], the co-packaging of one or more spores fails, resulting in small, monokaryotic (haploid) spores (**Figure 5c**). In nature, the individuals growing out from these spores will need to meet another individual in order to reproduce sexually, acting like normal heterothallic fungi. In the lab, the monokaryons are extremely handy for genetic research.

The fact that lab strains are mostly isogenic and stable under heterokaryosis has lead some authors to suggest that *P. anserina* is a predominantly selfing species (Esser, 1969; Grognet et al., 2014a). Others have argued instead that at least monokaryotic spores need to meet other strains to reproduce in nature, opening the door for some outcrossing (van der Gaag, 2005). On the other hand, the spores stay together via a sticky appendix after being shot (Grognet and Silar, 2015) suggesting that even monokaryotic spores might just end up mating with siblings (P. Silar, personal communication). However, there is no reason to believe that the sticky appendix survives the gut of herbivores and that siblings remain together in the dung. In **Paper III** we use genetic and simple ecological data to demonstrate that this fungus encounters different individuals in a given piece of dung and that it outcrosses at least occasionally in nature.

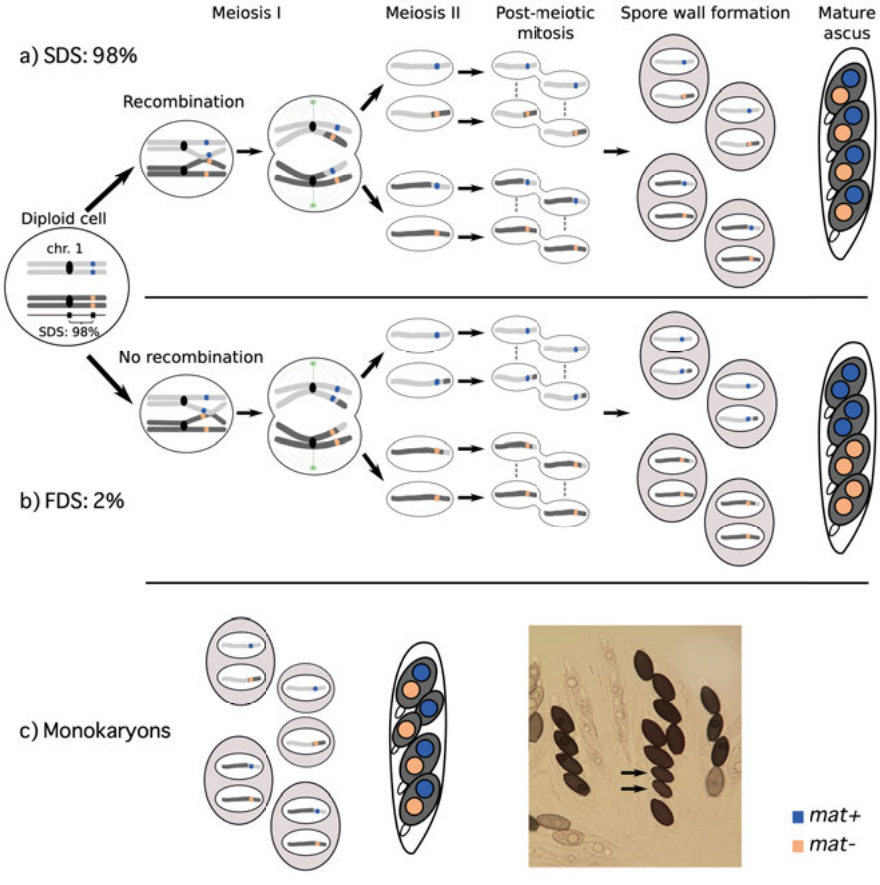


Figure 5. Segregation of the mating-type locus in *Podospora anserina*. Like in most related ascomycetes, after fusion of haploid nuclei, the diploid cell immediately undergoes meiosis, followed by a mitosis event. The spindles of the post-meiotic mitosis are perpendicular to the spindles during meiosis (not shown). Then, two non-sister chromatids (in independent haploid nuclei) get co-packaged into a single spore. The segregation of the mating-type locus (MAT) in chromosome 1 is tightly controlled. Around 98% of the time there is a recombination event between the centromere and MAT, leading to a second division segregation (SDS) pattern (a). The resulting spores have the two mating types (*mat+* and *mat-*) in different haploid nuclei, making the germinating individuals self-fertile. Rarely, there is no recombination event between the centromere and the MAT, producing a first division segregation (FDS) pattern and self-sterile spores (b). Sporadically, the co-packaging fails and the nuclei get packed individually (c). The haploid spores or monokaryons are smaller and also self-sterile. In c) the diagram of chromatid arrangement in the spores is presented to the left, and a picture of real asci with monokaryotic spores (arrows) to the right. Modified from Vogan et al. (2019), which is protected by a CC-BY-4.0 license (https://creativecommons.org/licenses/by/4.0/).

To fuse or not to fuse: the heterokaryon incompatibility system

Growth in filamentous fungi is accomplished by extending their cells or hyphae, by branching, and by fusing with other cells (Glass and Kaneko, 2003; Harris, 2006). Fusion can happen either between hypha of the same mycelium, or with hypha from a different individual. Like other Eukaryotes, fungi have a genetically-controlled allorecognition system in order to distinguish self (or close kin) from non-self (Buss, 1982; Nydam and DeTomaso, 2011). Because the fusion of two genetically different individuals leads to a heterokaryotic condition, the genes involved in self/non-self recognition are named heterokaryon incompatibility (*het*) genes[2] (Glass and Kaneko, 2003). In order to fuse successfully, two individuals must be compatible at all *het* loci, which often (but not always) means having exactly the same alleles. If they are incompatible at one or more *het* loci, the fused cells are compartmentalized and a reaction of programmed cell death is triggered (see **Figure 6**; Esser, 2016; Pinan-Lucarré et al., 2007). Generally, this results in a barrage, a macroscopic line of dead cells dividing the two fungal individuals, which indicates a vegetative incompatibility reaction (Esser, 2016).

Amongst filamentous fungi, *P. anserina* has one of the best-studied repertoire of *het* genes (reviewed in Esser, 2016; Pinan-Lucarré et al., 2007). Up to nine *het* loci have been genetically characterized with classical genetics (Pinan-Lucarré et al., 2007). The underlying genes have been identified for six of them, and in **Paper IV** we describe one more (**Figure 7**). Based on their interactions, the *het* loci can be divided in allelic and non-allelic systems (Bernet, 1967; Esser, 2016). Allelic systems include loci that behave like a single gene with two alleles. The spore killer *het-s* is a good example of that: if strains with different *het-s* alleles fuse, then a barrage will form between them (Saupe, 2007). By contrast, the non-allelic systems include unlinked genes that interact epistatically. For example, certain alleles of *het-c* will interact with specific alleles of either *het-d* or *het-e*, triggering barrage formation (Bastiaans et al., 2014; Espagne et al., 2002). At this point, only *het-b* and *het-q* await identification (**Figure 7**).

[2] They are also known as *vic* genes, after vegetative incompatibility, or as *vcg* loci, for vegetative compatibility group. However, I will be using the name *het* throughout the thesis for consistency with the *Podospora* and *Neurospora* literature.

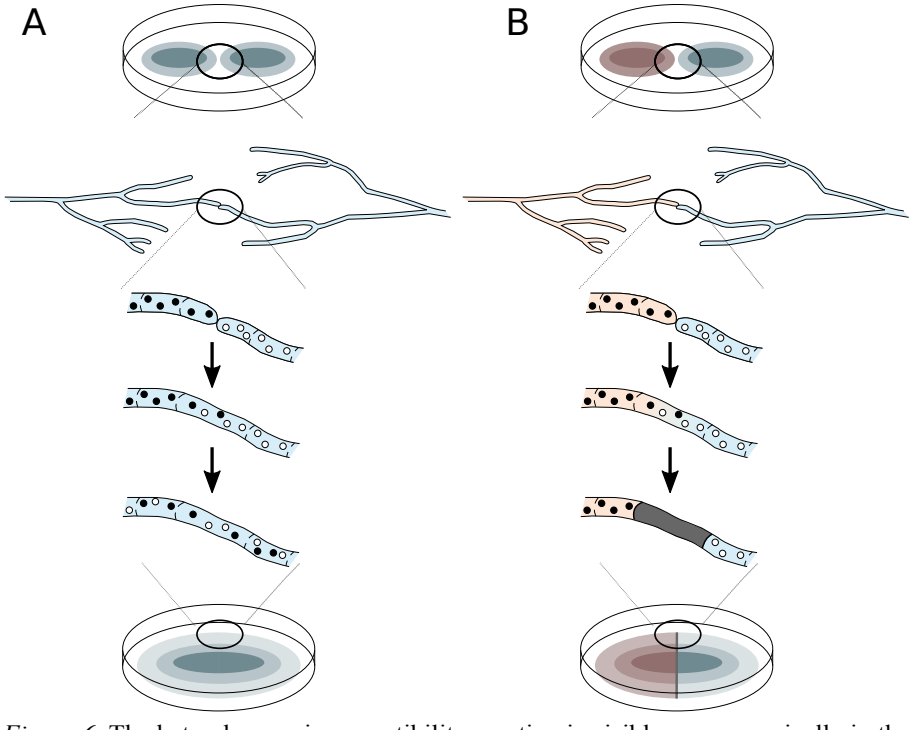


Figure 6. The heterokaryon incompatibility reaction is visible macroscopically in the form of a barrage line. When two individuals meet, the cells in contact fuse and exchange contents. If the two individuals are compatible at all *het* loci, the two mycelia merge completely into one (**A**). However, if the individuals are incompatible, the fused cell undergoes programmed cell death and compartmentalization to prevent further mixing (**B**). Macroscopically, this is observed between two colonies as a clear divisive line named barrage.

Similar characterization of the *het* genes in the relatively distant genera *Neurospora* and *Cryphonectria* revealed that each lineage has its own set of *het* loci (Choi et al., 2012; Heller et al., 2018; Powell et al., 2007; Wu et al., 1998; Zhang et al., 2014), hinting at recurrent selective pressures to develop such system. Experimental data suggest that fusion is advantageous within an individual colony (e.g. to distribute resources), but it is deleterious when it happens between different individuals (Aanen et al., 2008; Bastiaans et al., 2015). Moreover, it has been shown that the *het* reaction can prevent or diminish the horizontal spread of harmful fungal plasmids (Debets et al., 1994) and mycoviruses (Biella et al., 2002). It can also function against the spread of cheating nuclei, which can evolve when the relatedness of nuclei within a single mycelium is low (Bastiaans et al., 2016). This is so because, as the number of *het* loci increases, the likelihood of being compatible at all *het* genes in a sexually reproducing population is very low. In other words, the *het* genes serve as proxies for genetic identity, and their allorecognition properties seem truly adaptive.

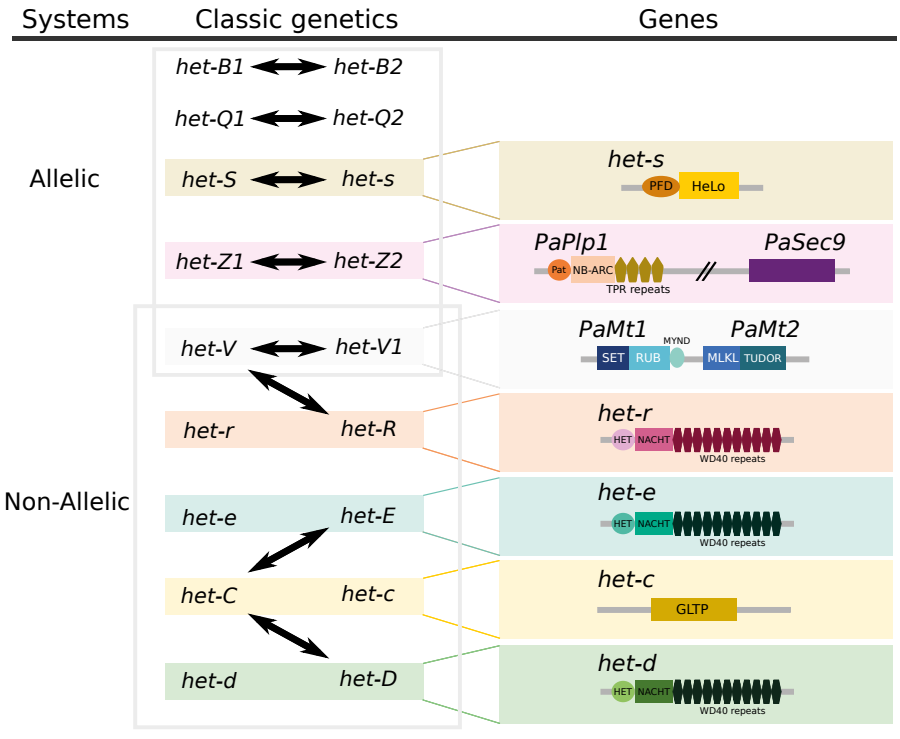


Figure 7. The genetic basis of the heterokaryon incompatibility system in *P. anserina*. During the 60s and 70s, nine *het* loci were identified in a collection of French *P. anserina* strains using classical genetics methods (left). They were classified as allelic and non-allelic based on their vegetative incompatibility interactions (arrows). Most genes are biallelic (eg. *het-z* has two alleles, *het-Z1* and *het-Z2*) but *het-c*, *het-d* and *het-e* are multiallelic. The alleles of these genes are simplified as the reactive alleles in capitals (eg. *het-D*), and the non-reactive alleles in lower case (*het-d*) (Bernet, 1967). Only one locus, *het-v*, is involved in both allelic and non-allelic interactions. In the past couple of decades, the genes underlying each *het* locus were characterized molecularly (right). The *het-v* genes are identified and described in **Paper IV**. Notice that *het-z* and *het-v* loci are each defined by two genes linked in a haplotype. Protein domain architecture of each gene is based on relevant literature (Daskalov et al., 2015; Espagne et al., 2002; Heller et al., 2018; Paoletti et al., 2007). PFD: prion forming domain; HeLo: Prion-inhibition and propagation domain; Pat: Patatin-like phospholipase domain; NB-ARC: nucleotide-binding adaptor shared by Apaf-1, resistant proteins, and CED-4; TPR: tetratricopeptide repeats; SET: SET lysine methyltransferase domain; RUB: Rubisco LSMT substrate binding domain; MYND: MYND zinc binding domain; MLKL: MLKL pseudo-kinase; TUDOR: Tudor domain; HET: heterokaryon incompatibility domain; NACHT: NTPase domain; WD40: WD40 repeats; GLTP: Glycolipid transfer protein.

Since distinct alleles are key to the function of allorecognition genes in general, their evolution is tightly linked to the maintenance of polymorphism in natural populations (Nydam and DeTomaso, 2011). Examples from different animals and plants allorecognition systems show evidence of balancing se-

lection (Nydam and DeTomaso, 2011). Similarly, fungal *het* genes often have balanced alleles frequencies and their phylogenies exhibit trans-species polymorphism, indicating current and long-term balancing selection (Bastiaans et al., 2014; Milgroom et al., 2018; Zhao et al., 2015).

Beyond vegetative incompatibility, however, there is evidence suggesting that *het* genes can have additional functions also subject to balancing selection, such as immunity. This suspicion comes from striking parallelisms between vegetative incompatibility and the immune system of plants and animals. First, detail accounts of the vegetative incompatibility reaction show communalities with inflammatory immune responses, such as autophagy and secreted proteins resembling peptides with antibiotic activity (Pinan-Lucarré et al. 2003; Paoletti & Saupe, 2009). Second, transcriptional responses of *P. anserina* to bacterial exposure overlap with the transcriptional profile of its own vegetative incompatibility reaction (Lamacchia et al. 2016). And finally, some allorecognition genes in *P. anserina*, *Neurospora crassa*, and *Cryphonectria parasitica* share a specific domain architecture typical of Nucleotide Oligomerization Domain (NOD)-like receptors, or NLRs (Chevanne et al., 2010; Dyrka et al., 2014; Heller et al., 2018; Uehling et al., 2017). In plants and animals, NLRs have evolved independently as key elements of the innate immune system, and many of them lead to the activation of programmed cell death (Ausubel, 2005; Meunier and Broz, 2017), just like the *het* genes. Thus, it was suggested that at least some *het* genes might have a general role in pathogen recognition (or xenorecognition), and that the barrage formation is a byproduct or exaptation of the same molecular mechanisms (Paoletti and Saupe, 2009).

NLRs: a recurrent theme in the structure of intracellular guardians

NLRs are a diverse group of intracellular receptors that go by different names depending on the taxon or the focus of the study. Instead of "NOD-like receptors", they might be known as "nucleotide-binding site and leucine-rich repeats" genes, a name that is shortened as NB-LRRs for plants (Caplan et al., 2008), or again as NLR for animals (Yuen et al., 2014). When the focus is on the class of proteins and their functions, NLRs might be classified as STAND proteins, which stands for "signal transduction ATPases with numerous domains" (Leipe et al., 2004). In the end, they all have in common a specific tripartite domain organization: a N-terminal effector domain, a central nucleotide-binding oligomerization domain, and C-terminal domain composed of superstructure-forming repeats (**Figure 8A**).

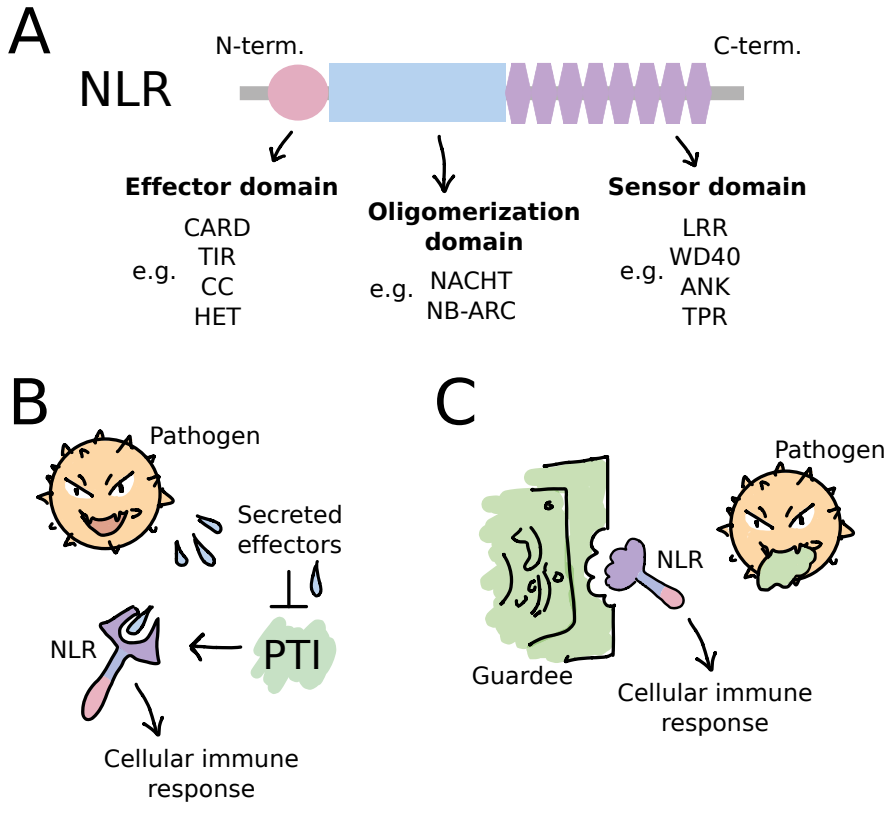


Figure 8. The NLRs structure and function in the innate immune system of plants and animals. An NLR is defined by the presence of three domains with specific role (**A**). However, the NLRs are not a monophyletic assembly, so multiple and unrelated domains can be recruited to perform the same function. When a pathogen attacks, it might release effector proteins meant to suppress the pathogen-triggered immunity response (PTI), the first line of immunity defense (**B**). The sensor domain of a given NLR can detect the presence of the pathogen effector and lead to further cellular immune responses. Alternatively, the NLR might guard the integrity of a cellular component (the "guardee"), and trigger the cellular immune response if that is compromised (**C**).

Generally, the NLR domain structure allows these receptors to act as cellular guardians, either by directly detecting the non-self (e.g. pathogens) or by surveilling the integrity of the "self" (**Figure 8B-C**). For example, a given pathogen might carry or release molecules that the NLR detects through its repetitive C-terminus (the "sensor" domain). Binding of the pathogen molecule changes the conformation of the protein, activating the central domains, which triggers oligomerization of the NLR proteins. That in turns activates the signaling effector N-terminus, ultimately leading to the activation of a general immune response (Daskalov et al., 2015; Yuen et al., 2014). What is remarkable about this protein architecture is that it has evolved countless

times by recruiting many different domains of unrelated origin (Ausubel, 2005; Dyrka et al., 2014; Gao et al., 2018). So for instance, the oligomerization domain might be of the NACHT or of the NB-ARC type, while the repeats might be the LRR, WD40 or TPR types, to name a few (**Figure 8A**).

As a result of allorecognition research, *P. anserina* has the best-characterized repertoire of fungal NLRs. These include *het-r*, *het-d*, and *het-e*, which belong to the same gene family (Paoletti et al., 2007), as well as one of the genes of *het-z* (Heller et al., 2018) (**Figure 7**). In addition, an NLR called *nwd2* in known to interact with the *het-s* gene through its prion-forming domain (Daskalov et al., 2015). Curiously, classical genetics on the *P. anserina* NLR-*het* genes showed that they are often linked to sexual incompatibilities (Esser, 2016). If these genes induce deleterious sterility, why are they not removed by natural selection? Could other forces influence their maintenance, in addition to balancing selection? Moreover, could the compound effects of balancing selection and pleiotropy on the sexual function affect population structure? We explored these questions in **Papers III** and **IV**.

Research aims

The overarching aim of my PhD project was to characterize the properties and evolution of single genes that have large effects on key phenotypic traits of *Podospora* fungi, with an emphasis on processes occurring within and between early-diverged species. In particular, I focused on selfish genetic elements that have strong deleterious effects on fertility (spore killers), as well as on genes that control essential self/non-self recognition capabilities (*het* loci). The specific aims were to:

1. Identify the genetic basis of spore killing in the Wageningen Collection of *P. anserina* strains, a unique case of population sampling with temporal data (**Paper I**). Linked to this objective was to determine the relationship, or lack thereof, between spore killing and vegetative incompatibility.
2. Understand the mechanisms behind the genesis of new spore killers (**Paper I** and **II**).
3. Characterize the means of genomic mobility of the "*Spok* block", a genetic unit that carries spore killer genes, while exploring its effects on genome architecture and fitness (**Paper II**).
4. Develop a phylogenetic framework for the study of the *P. anserina* species complex (**Paper III**).
5. Determine whether known *P. anserina het* genes exhibit signatures of balancing selection in closely related species, as an indication of conserved function (**Paper III**).
6. Establish the consequences that *het* genes with pleiotropic effects on sexual compatibility may have on population structure (**Paper IV**).

Summary of results

The genetic basis and evolution of meiotic drive in *Podospora anserina* (Papers I and II)

Meiotic drivers are selfish genetic elements capable of breaking Mendel's law of equal segregation, increasing its own frequency on the products of meiosis at the expense of the other allele (Lindholm et al., 2016). Through this selfish advantage, meiotic drivers can quickly spread and invade populations, even if they have deleterious fitness effects (Crow, 1991). In fungi, a meiotic driver is known as a spore killer because it induces the dead of the meiotic products (spores) that do not carry it. Using classical genetic methods, previous work uncovered multiple spore killers segregating in a single population of the fungus *Podospora anserina* sampled in Wageningen, the Netherlands (van der Gaag et al., 2000). These *Podospora* spore killers (*Psks*) are capable of killing each other in hierarchical ways, and behave as a single locus, but their genetic basis was previously unknown.

In **Paper I** we used a combination of next generation sequencing technologies, classical genetics, and knock-ins to dissect the genes responsible for the *Psk* phenotypes. We found that meiotic drive is exerted by members of the *Spok* (for spore killing) family, whose members code for a single protein with both toxin and antitoxin capabilities (Grognet et al., 2014b). We confirmed the presence of the previously described *Spok2* gene in the chromosome 5 of most strains. Unlike *Spok2*, however, we found that two new members of the family, *Spok3* and *Spok4*, are located in different chromosomal locations depending on the *Psk*. Moreover, these two genes (alone or together) are always located inside a large (74 – 247 kb) genomic region that we named the *Spok* block. In **Paper II**, we determined that the *Spok* block is, in fact, a special variant of a gigantic *Crypton*-like DNA transposable element that we called *Enterprise*. The *Enterprise* element is defined by the presence of the *spore killing related crypton* (*Kirc*) gene, containing a putative tyrosine-recombinase domain, and by target site duplications. The *Spok* block in particular is an *Enterprise* that carries *Spok* genes as well as multiple other open reading frames and smaller transposable elements likely accumulated from the host genome. The transposition of the *Spok* block at different genomic locations creates new *Psk* types, which are able to drive against other *Psk* due to the peculiarities of meiosis in the *Podospora* life

cycle. Hence, the *Spok* block is a genetic element with dual selfish properties: transposition and meiotic drive.

Mechanistically, the *Spok* genes (within or outside the *Spok* block) in *P. anserina* do not interact epistatically, and thus can kill each other (**Paper I**). This in contrast with the previously described *Spok1* gene, only found in the species *P. comata* (Grognet et al., 2014b; Paper II). *Spok1* is resistant to all *Spok* genes in *P. anserina*, and it is able to kill *Spok2* and *Spok3*, but not *Spok4*. This is a puzzling finding, because there is very little divergence between all SPOK proteins. It is possible, then, that new *Spok* genes can emerge easily from few changes. Notoriously, we found evidence of gene conversion amongst all *Spok* homologs, opening the door for the creation of chimeric *Spok* variants with different killing capabilities (**Paper I**). On the other hand, functional annotation and point mutations on the SPOK3 protein revealed that a predicted kinase domain is important for the resistance function, while a predicted nuclease domain is necessary for the killing function. These same mutants confirmed that disrupting the nuclease domain can easily lead to resistant alleles (**Paper I**). A resistant allele would be selected for because it would effectively suppress the advantage of the driver (Crow, 1991). It is surprising then, that no resistant alleles have been found in the Wageningen collection.

At the population level, we found that the *Spok* genes within the *Spok* block (*Spok3* and *Spok4*) are in low frequencies, in contrast to *Spok2* that is closer to fixation (**Paper I**; see also **Paper III**). We speculated that the *Spok* block might be (more) deleterious in some way, limiting the spread by meiotic drive. In accordance to this hypothesis, we found evidence of a maternal fitness cost associated to the *Spok* block presence, regardless of spore killing (**Paper II**). Moreover, this effect is contingent to the genomic position where the *Spok* block is inserted. It is possible then that the *Spok* block profits from the *Spok* transmission advantage, while the *Spok* genes spread further by creating new copies in the population. However, in the long term, perhaps the deleterious effects of the *Spok* block eventually backfire for the transported *Spok* genes.

From a broader perspective, we found that there are multiple homologs of both the *Spoks* (**Paper I**) and *Kirc* (**Paper II**) across ascomycetes, including the plant pathogen *Fusarium*. Importantly, their phylogeny often disagrees with the species phylogeny, which suggests possible events of horizontal gene transfer, although multiple loses cannot be excluded. Likewise, it does not necessarily mean that they travel together. Indeed, we found cases of *Enterprise*-like elements without *Spok* genes outside *Podospora*. We conclude that there is evidence of selfish behavior for both elements in other fungal groups.

In addition to our main findings, the **Paper I** in particular has the added value of serving as a mini review on the history of meiotic drive research in *Podospora*, reconciliating previous puzzling findings with the behavior of

the *Spok* family. Also, little gems are hidden in the Appendixes and Supplementary Figures. For example, once the *Spok* genes and *Spok* block were confirmed to cause drive on their own, it became clear that previous correlations between the *Psk* phenotypes and vegetative incompatibility groups was the result of simple genetic linkage. A backcrossing analysis confirmed this. In addition, many of our genomic analyses in Paper I and II provided a first look into the landscape of repetitive elements in *Podospora* species. Hopefully, this work provides ample resources for future research.

The intersection between allorecognition genes and other recognition systems in *Podospora* (Papers III and IV)

All organisms have a genetic system in place to distinguish self from non-self (Nydam and DeTomaso, 2011; Rimer et al., 2014). Such systems are necessary for fundamental life events like mating partner choice, vegetative growth by cell fusion, and pathogen attack (Bastiaans et al., 2015; Richman and Kohn, 1996; Tsutsui, 2004). In all these instances, the challenge of recognition is resolved by using highly polymorphic loci that function as indicators of genetic identity, and whose frequencies are maintained by balancing selection forces. The reliance on polymorphism makes self/non-self recognition systems mechanistically similar, providing the potential for a transition from one function to the next. For example, the major histocompatibility complex system in vertebrates has an essential role in the acquired immune system, while simultaneously affecting mate choice and graft rejection (Apanius et al., 1997; Eizaguirre et al., 2009). Thus, the study of allorecognition genes directly informs us on the evolution of key organismal traits.

In filamentous fungi, genes controlling self/non-self recognition at the vegetative stage are called *het* (for *het*erokaryon incompatibility) loci (Saupe, 2000). Vegetative fusion between two different individuals can happen if they are compatible at all of their *het* loci. Otherwise, a vegetative incompatibility reaction follows where the fused cells undergo programmed cell death (Glass and Dementhon, 2006). The model species *Podospora anserina* has one of the best characterized repertoires of *het* genes, several of which belong to a family of Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) called the HNWD genes (Paoletti et al., 2007). However, despite the thorough knowledge on their molecular biology, little is know about their evolution.

In **Paper III** we examine the presence, absence and phylogenetic patterns of known *het* genes across the *P. anserina* species complex. Making use of chromosome-level genome assemblies, we characterize the phylogenetic

relationships between these closely related *Podospora* species and determine that some *het* genes, like *het-z* and *het*-s, show evidence of long-term balancing selection. In particular, we present evidence that the prion-forming state of *het-s* predates the diversification of all species. However, we find no trans-species polymorphism in *het-c*, despite population data showing balanced allele frequencies in *P. anserina* (Bastiaans et al., 2014 and **Paper IV**). In contrast to the relative conservation of other *het* genes, the members of the HNWD family have multiple duplications and deletions across the phylogeny. Moreover, while most species have around four or five HNWD paralogs, the species *Podospora pseudocomata* has at least 20. We discovered that the HNWD paralogs are flanked by target site duplication sequences with the specific motif TGTTC, implicating a DNA transposon-like mechanism in the genesis of new copies. We failed, however, to uncover any other transposon feature such as terminal inverted repeats.

The discovery of a dynamic turnover of HNWD genes is particularly interesting in light of many parallelisms between these *het* genes and elements of the innate immune system in plants and animals. Previous work has lead to the hypothesis that fungal NLRs might have a general pathogen recognition function (Paoletti and Saupe, 2009; Uehling et al., 2017). Thus, a transposon-like mechanism can further fuel the diversification of this self/non-self recognition system, as it has happened before in jawed vertebrates (Kapitonov and Jurka, 2005).

In **Paper IV** we focus on a different intersection between self/non-self recognition systems. Classical genetic studies showed that many *het* genes in *P. anserina* have pleiotropic effects on the sexual function (Bernet, 1967; Labarère, 1978). We hypothesized that vegetative incompatibility systems that have pleiotropic effects on sexual recognition might lead to the equivalence between allo-groups and reproductively isolated groups. Thus, we sequenced and characterized the genetic diversity distribution of 106 strains of *P. anserina* sampled around Wageningen, the Netherlands, from 1991 to 2016. While the Wageningen Collection has extremely low genetic diversity (π = 0.00049), many known *het* genes exhibit very high polymorphism and signatures of balancing selection, as expected from their allorecognition function. In addition, linkage disequilibrium decay and field surveys confirm that this species is not a strict selfer, implying that the *het* pleiotropic sexual effects might be of significance. Clustering analysis of published mating success data of about half the samples revealed two differentiated groups, which are perfectly recapitulated by an equivalent clustering analysis the genetic variation of chromosome 5. In other words, we found that the Wageningen Collection is composed of two mating groups that are genetically differentiated.

Next we determined that the most likely candidate driving mating group isolation is the epistatic pair *het-r* (chromosome 2) and *het-v* (chromosome 5), which are both bi-allelic loci. As the exact location of *het-v* was un-

known, we used positional and complementation cloning, as well as site-directed mutagenesis, to characterize the molecular basis of this *het* locus. Our results confirmed that the allelic identity of *het-v* perfectly defines the mating groups and that deletion of this locus restores fertility. We further genotyped the Wageningen collection and discovered that the alleles of *het-r* and *het-v* are in nearly perfect linkage in nature, despite segregating in different chromosomes. Furthermore, field surveys demonstrated that the two mating groups are found together in their substrate, indicating that reproductive isolation is maintained despite coexistence. Finally, we showed how individual-based simulations of the *het-r/v* system are able to recapitulate the formation of two mating groups based on the interaction between balancing selection, sexual incompatibilities, and selfing rates.

Our work on the *het-r/v* system exemplifies how self/non-self recognition loci can influence the evolution of reproductive isolation. Notoriously, the *het-r* gene is a member of the HNWD gene family, and hence an NLR. Previous research in plants has shown that a mismatch between NLR immunity genes of different populations can trigger Betson-Dobzhansky-Muller incompatibilities, leading to a lethal phenotype called hybrid necrosis (Bomblies, 2009; Bomblies et al., 2007). Theoretical work suggest that hybrid necrosis NLRs under the influence of pathogen-driven balancing selection can lead to speciation (Ispolatov and Doebeli, 2009), much like what our results show for the *het-r/v* system (**Paper IV**). Moreover, the fast evolution of the HNWD gene family across the *P. anserina* species complex might create incompatibilities analogous to the hybrid necrosis NLRs, serving as additional sources of reproductive isolation between these closely related species (**Paper III**). Hence, both plant and fungal NLRs can contribute to the evolution of reproductive isolation, which is even more remarkable considering that NLRs in these two groups likely evolved independently (Ausubel, 2005; Dyrka et al., 2014).

Conclusions and future prospects

When I started my PhD back in 2015, the standard concept of non-female meiotic drivers (i.e., of segregation distorters) came from plant and animal models, where the toxin and the antitoxin functions are performed by different loci linked in big haplotypes via inversions (Burt and Trivers, 2006). Since then, multiple papers on the diverse *wtf* genes in *Schizosaccharomyces pombe* (Bravo Núñez et al., 2020; Eickbush et al., 2019; Hu et al., 2017; Nuckolls et al., 2017), *Spk-1* in *Neurospora sitophila* (Svedberg et al., 2020), and our own newly described *Spok* genes in *Podospora* (Vogan et al., 2019) have shown that single-gene meiotic drivers might in fact be very common. These are all fungal examples, but their discovery might reflect a methodological advantage rather than a biological peculiarity, as single-gene meiotic drivers also exist in *Drosophila* flies (Phadnis and Orr, 2009). Possibly, single-gene drivers might entail less deleterious effects if they are not associated with large non-recombining regions, and hence drive to fixation with more ease. Following this logic, one might speculate that the size and content of the *Spok* block might have a hampering effect on the spread of *Spok3* and *Spok4*. In comparison, the "block-less" *Spok2* (and perhaps even *Spok1*) seems to be in much higher frequency. However, the discovery of transposition–like mobility of the *Spok* block suggests that other factors could come into play to determine the final equilibrium frequency of drivers in *Podospora*.

One might wonder just how much is a genome affected by the mobility of a large element like *Enterprise* and all the "passengers" that might get progressively on board, especially in compact, gene-rich genomes like the fungal ones. We found deleterious fertility effects associated to the *Spok* block in *P. anserina*, suggesting that the insertions, or their positions, likely have fitness consequences. Moreover, remnants of the *Spok* block ends are frequently found in transposon-rich areas. Perhaps insertions in such "TE islands" are less likely to disturb host genes, recombination hotspots, or tridimensional arrangements of chromatin, avoiding purging by natural selection. However, all of the observed insertions that create the *Psk* phenotypes in the Wageningen population are found in between genes, away from any TE. Alternatively, if after a deleterious insertion there is selection to remove most of the *Enterprise*'s body, the carcass might become a neutral place for smaller TEs to colonize and proliferate. Notably, TE islands are common in other groups (e.g. *Aspergillus*; Fedorova et al., 2008), and a similar grave-

yard argument has been done for the insertion of another large TE in plant pathogens from the family Pleosporaceae (McDonald et al., 2019). In addition, the centromeres of Sordariomycetes like *Podospora* and *Neurospora* are strongly defined by low GC content and numerous TEs (Svedberg et al., 2018; Vogan et al., 2019), much like TE islands. Thus, the insertion of large mobile elements could shape the landscape of TE islands across the genome and perhaps even interfere with centromere evolution. The genomic resources that we created for the *P. anserina* species complex provide an ideal place to start exploring these conjectures.

Given its intricacies, it is natural to ask how much of an oddball is the case of meiotic drive in *Podospora*. Clearly, the *Spok* homologs and *Enterprise*-like elements are widespread in filamentous ascomycetes. Thus, it is key to determine next if homologs of these elements behave selfishly in distant taxa. In addition, the mutational and diversification dynamics of the *Spok* genes resemble closely those of the *wtf* genes in *S. pombe*. The split between the lineages that contain *Podospora* and *Schizosaccharomyces* dates back to the origin of all of Ascomycota (around 590 Mya; Lutzoni et al., 2018). Hence, convergent evolution of similar single-gene systems could happen in any taxa in between, and broad conclusions drawn from both systems might be highly generalizable.

The discovery of HNWD TE-like mobility invites a multitude of questions on their function and fitness effects. For example, are the HNWD genes autonomous elements or do they depend on other TE to get mobilized? If the latter case is true, is the same TE mobilizing other genes? What is the faith of a new HNWD copy entering a population, given a possible confrontation between balancing selection and the deleterious effects of producing self-incompatible progeny? And finally, how much are HNWD insertions contributing to reproductive isolation between different populations and species?

Our work on the *het-r/het-v* incompatibility system begins to address the potential effects of allorecognition genes in the evolution of reproductive isolation. Interestingly, the *het-r* gene has remained immobile since the diversification of the entire species complex. Still, further characterization is needed to see if this gene functions differently in other species, given the delicate properties of the sensor domain in NLRs. In comparison, the evolution of *het-v* remains completely unexplored.

The striking coincidences between the fungal NLRs and those causing hybrid necrosis in plants imply that their involvement on the speciation process is far from exclusive to fungi. Moreover, our simulation work suggests that the necessary ingredients (pleiotropic allorecognition genes on the sexual function, balancing selection, and selfing) are likely to occur in all major Eukaryotic groups. Finally, it is noteworthy that allorecognition-based reproductive barrier can evolve without concomitant morphological changes, which, if common, might help explain the evolution of cryptic species.

As an extension of these general properties, the lessons learned from natural systems like *Podospora* are highly informative for the development of artificial meiotic drivers, better known as "gene drives[3]". Gene drives could in principle help spread desirable traits, or control and even collapse pest populations, such as Malaria mosquitos, which is potentially dangerous and has ethical implications (Brossard et al., 2019; Resnik, 2017). By observing the behavior of meiotic drivers in natural populations, appropriate strategies and policies can be developed. Single-gene drivers based on CRISPR/Cas-9 technologies are currently very popular candidates for field applications (Champer et al., 2016; Schmidt et al., 2020), and hence natural segregation distorters like the *Spoks* are better points of comparison than more traditional two-loci systems. And while most gene-drive discussion focus on animal pests (e.g. mosquitos, flies, and mice), fungal pathogens of crops have dramatic socioeconomic effects (Bebber and Gurr, 2015). A good example is the *Fusarium* genus, whose pathogenic members happen to have both *Spok* and *Kirc* homologs that could be targeted as a management alternative.

Just as the *Spok* genes are a good system to understand the consequences of gene drives, the characterization of *het* genes and their evolution can be applied to manipulate the spread of pathogenic elements in fungal populations. For example, in the plant pathogen *Cryphonectria parasitica* the molecular identification of its *het* gene repertoire allowed for the creation of a strain that is universally compatible. This engineered strain can fuse with any other wildtype strain, and hence facilitate the transmission of viruses that reduce its pathogenicity (Zhang and Nuss, 2016). The *Podospora* species are not pathogenic, but simply understanding the evolution of their allorecognition system can be informative. If the fungal NLRs have indeed a role in pathogen recognition, they might become a target for the control of crop and animal (including humans) pathogens.

In conclusion, *P. anserina* has served beautifully as a model organism for the study of evolutionary biology and applications, and likely will continue to do so in the future. That is, it will "live long and prosper".

[3] There seems to be a difference between fields on the use of "driver" vs. "drives", the former being common in natural systems, and the latter on artificial ones.

Svensk sammanfattning

Biologisk mångfald återfinns på flera nivåer i naturen – i allt från hela ekosystem ner till enstaka gener. Mångfald inom en arts arvsmassa kallas genetisk variation och är en förutsättning för att populationer skall kunna utvecklas och anpassas i en föränderlig miljö. De individer som är bäst anpassade till miljön lyckas bäst med fortplantningen, och kommer att föra vidare sina anlag över generationer vilket leder till att dessa anlag bli vanligare inom populationen. Många gener i ett genom har samma agenda som individen, dvs dess variant som förs vidare i störst frekvens är även den som bidrar till bäst anpassning hos individen. Det finns dock även gener som har en egen agenda, och bryter mot regler för sin egen vinnings skull. De kallas "själviska gener" eftersom de bryter mot Mendels lagar om nedärvning och återfinns i mer än hälften av avkomman, trots att de inte bidrar till att öka livskraften hos individen. Ibland kan de till och med orsaka skada, vilket leder till en så kallad genetisk konflikt.

I min avhandling fokuserar jag på gener som har betydande effekter på livskraften hos svampen *Podospora anserina* och dess närmaste släktingar. I artiklarna I och II utforskade jag identiteten och evolutionen av en viss typ av själviska gener som kallas ”gendrivare”. Gendrivare manipulerar något steg i meiosen för att uppnå överrepresentation i avkomman, vilket ökar sannolikheten att de invaderar och sprider sig i en population. I *P. anserina* har forskare länge vetat att det finns gendrivare, som orsakar spordöd hos de sporer som inte ärver dem och därigenom minskar individens livkraft. Deras genetiska identitet var dock tidigare okänd. I papper I demonstrerade vi att gendriv orsakas av medlemmar i en genfamilj som heter *Spok*-genfamiljen. Vi upptäckte två nya *Spok*-gener, *Spok3* och *Spok4*, som är lokaliserade på olika kromosomer i olika individer, och utförde molekylärbiologiska analyser för att verifiera att de är de som leder till sporernas död. I papper II visade vi att *Spok3* och *Spok4* är lokaliserade inom en gigantisk (upp till 247 Kb lång) variant av Enterprise: ett Crypton-liknande transposabelt element. Vi visar indikationer på att Enterprise mobiliserar sig i genomet med hjälp av ett tyrosin-rekombinas som vi kallar Kirc. När Enterprise bär Spok gener har detta element dubbla själviska egenskaper: transposition och meiotiskt gendriv, och kan därmed ses som ett hypersjälviskt element. Dessutom fann vi att homologer från Spok familjen (Paper I) och Kirc (Paper II) är vanligt förekommande i ett brett spektrum av olika svampar, men deras fylogenier överensstämmer inte med arternas

släktskap. Detta resultat antyder att de har genomgått horisontell genöverföring, dvs spridits mellan arter som inte är närbesläktade.

I artiklarna III och IV fokuserade jag på gener som har en funktion som är viktig för individens livskraft och fortlevnad, de så kallade *het*-generna. I svampar kontrollerar *het*-generna hur en individ känner igen sig själv från andra individer. I varje svampgenom finns ett antal *het*-gener och endast om två mycel har exakt samma allel vid varje gen känner de igen varandra som samma individ. *Het*-gener förväntas befinna sig under frekvensberoende selektion, i och med att ovanliga alleler gör igenkänningen mer effektiv och därför har en evolutionär fördel framför vanliga alleler. I papper III hittade vi belägg för att frekvensberoende selektion verkar på vissa *het* gener i *Podosporas* artkomplex. En oväntad upptäckt var att *het*-gener inom HNWD-genfamiljen dupliceras på ett transposonliknande sätt, vilket breddar vår förståelse för deras potentiella effekter på individens livskraft. Slutligen visar vi i papper IV hur *het*-gener med pleiotropiska effekter på igenkänning av lämplig sexuell partner kan leder till utvecklingen av reproduktiv isolering och därmed artbildning. Datorsimuleringar visar att detta kan ske då det finns både pleiotropiska interaktioner, frekvensberoende selektion och en hög grad av självbefruktning.

Sammantaget belyser resultaten av min avhandling den funktionella interaktionen mellan olika typer av självviska gener och andra gener, och hur deras nedärvningsmönster och betydelse för individernas livskraft kan ge effekter på både genomarkitektur och populationsstruktur.

Resumen en español

En todos los niveles de la naturaleza encontramos biodiversidad, desde en ecosistemas enteros hasta en genes individuales. La diversidad dentro del conjunto genético de una especie se llama variación genética y es un requisito previo para que las poblaciones puedan desarrollarse y adaptarse en un entorno cambiante. Aquellos individuos que estén mejor adaptados al medio ambiente tendrán más éxito para reproducirse, y transmitirán su descendencia durante sucesivas generaciones, lo que hará que la descendencia con esas características se vuelva más común en la población. La mayoría de genes en un genoma siguen las mismas reglas que un individuo, es decir, la variante que se transmite con una frecuencia más alta también es la que contribuye a la mejor adaptación del individuo. Sin embargo, también hay genes que tienen su propio plan y quebrantan las reglas para su propio beneficio. Se les llama "genes egoístas", porque incumplen las leyes de herencia de Mendel y se encuentran en más de la mitad de la descendencia, a pesar de que no contribuyen a aumentar la adecuación biológica del individuo. A veces incluso pueden ser perjudiciales, lo que conduce a un llamado conflicto genético. En mi tesis, me centro en los genes que tienen efectos significativos en la adecuación del hongo *Podospora anserina* y sus parientes más cercanos.

En los **artículos I** y **II**, exploré la identidad y la evolución de un tipo de gen egoísta que produce el llamado impulso meiótico. Los impulsores meióticos manipulan algunos pasos durante la meiosis con el fin de lograr una representación excesiva en la descendencia, aumentando así la probabilidad de que invadan y se propaguen en una población. En *P. anserina* se conocen, desde hace mucho tiempo, impulsores meióticos que causan la muerte de las esporas que no los heredan, comprometiendo así la adecuación del individuo. Sin embargo, su identidad genética permanecía desconocida. En el **artículo I**, demostramos que el impulso meiótico es causado por miembros de una familia de genes llamada *Spok*. Descubrimos dos nuevos genes, *Spok3* y *Spok4*, que se encuentran en diferentes cromosomas en diferentes cepas, y realizamos análisis biológicos moleculares para verificar que son los causantes de la muerte de las esporas. En el **artículo II** mostramos que *Spok3* y *Spok4* están ubicados dentro de un elemento genético gigante (de hasta 247 Kb de largo) llamado *Enterprise*, identificado como un elemento transponible similar a aquellos presentes en el grupo *Crypton*. Encontramos evidencia de que *Enterprise* puede trasladarse en el genoma por medio de una tirosina recombinasa, a la que hemos llamado *Kirc*. Cuando *En-*

terprise contiene genes *Spok*, este elemento adquiere propiedades egoístas duales: transposición e impulso meiótico y, por lo tanto, puede verse como un elemento híper-egoísta. Además, encontramos que homólogos de la familia *Spok* (**Artículo I**) y *Kirc* (**Artículo II**) son ampliamente comunes en diferentes hongos, pero sus filogenias están en discordancia con la que se observa a nivel de especie. Este resultado sugiere que se ha producido una transferencia horizontal de genes, es decir, se han propagado entre especies que no están cercanamente relacionadas entre sí.

En los **artículos III** y **IV**, me centré en los genes que tienen una función importante para la vitalidad y supervivencia del individuo, los llamados genes *het*. En hongos, los genes *het* controlan cómo diferentes individuos se reconocen entre sí. En cada genoma fúngico hay varios genes *het* y cuando dos micelios tienen exactamente el mismo alelo en cada gen, se reconocen entre sí como el mismo individuo. Se espera que los genes *het* sufran selección balanceadora dependiente de frecuencia, ya que los alelos inusuales hacen que el reconocimiento sea más eficiente y, por lo tanto, tienen una ventaja evolutiva sobre los alelos comunes. En el **artículo III**, encontramos evidencia de que, efectivamente, la selección balanceadora actúa sobre algunos genes *het* en el complejo de especies de *Podospora*. Un hallazgo inesperado fue que los genes *het* en la familia de genes HNWD se duplican de forma similar a un transposón, ampliando así nuestra comprensión de sus posibles efectos sobre la viabilidad del individuo. Finalmente, en el **artículo IV**, revelamos cómo los genes *het* con efectos pleiotrópicos en el reconocimiento de una pareja sexual adecuada, pueden conducir a un aislamiento reproductivo y, por lo tanto, a la formación de nuevas especies. Simulaciones en computadora muestran que esto puede suceder cuando existen interacciones pleiotrópicas, selección balanceadora y un alto grado de autofecundación.

En conjunto, los resultados de mi tesis resaltan la interacción funcional entre genes egoístas y otros genes, y cómo sus patrones de herencia y su importancia para la adecuación de los individuos pueden tener efectos tanto en la arquitectura del genoma como en la estructura poblacional.

Acknowledgments

First of all I would like to thank my supervisor, Hanna Johannesson. From the very beginning, I was happy to be in the Johannesson lab because of the super caring and supportive atmosphere. And fun too! Even though you were so busy, you always made an effort to check on me, and to listen to my problems. I always felt free to express myself, from laughter to tears, which I think is very special and a need for me as a student. And you always trusted me completely, thank you! I can tell you genuinely care for our happiness as people, not only as scientists. I hope you know how highly we all think of you in the group; that is why we like to stay for so long hehe. Allow yourself to be proud about that (even if it's not very Swedish).

It feels a bit weird to thank you, Aaron, because this thesis is kind of yours too. We often joked that this was your second PhD, and for good reason. It's like I would take a step, and you would take the next. I am certainly happy that you took over the wet lab part, which I dislike. And many times I felt like I would have never solved the *Spok* drama without you. We almost murdered each other during the writing periods, especially with the *Spok* paper, but I think we managed to survive it. Clearly, you like to take your (enormous) creativity to speculative at extremes sometimes. And clearly, I often get too absorbed by my obsession with precision and perfection. But for the most part, we balanced each other. More importantly, I think we are equals in our levels of passion and excitement. We both really, really, really like science, right? Can we feel proud of our *Podospora* work? I think so. Do you think Bernet and Esser would be pleased? Maybe they would just look at us and whisper "Duh! We knew that". Anyway, we did our best. And as Hanna often says, *Podospora* is like a treasure box (or did she mean a Pandora box???).

I am much in debt with my unofficial co-supervisor in Wageningen, Fons Debets. I learned a ton about genetics and fungi from you during my short time in Wageningen, and during all of our Podospora meetings. I hope you are happy with our findings, that relied so heavily and all the amazing work done by you and your group during the past couple of decades. On the same note, I am extremely grateful to Eric Bastiaans. During your postdoc time with us you tried so hard to teach us about the complicated Podospora, and you had a lot of patience with me then and later in Wageningen. Sometimes your Dutch straightforwardness would get to me (although perhaps you didn't notice), but it was always in the end to help me grow. I was also glad

to meet Jianhua, and I hope the new family is happy and healthy! To complete the Wageningen experience, I am also thankful to Suzette de Groot for your enthusiastic, careful, and friendly help isolating the new Podospora strains (and for discussing Rick and Morty with me). Thank you too to Marijke Slakhorst for all your help teaching me the *Podospora* isolation methods back in 2016.

I cannot even begin to express how grateful I (and Hanna and Aaron) am to all the team in Bordeaux University. Sven Saupe, you were also like my unofficial co-supervisor, but in France, and I cannot think of a cooler and nicer person to collaborate with. I think you might be the only person in the world that can get me interested in molecular biology. Thank you so much for your insight and patience working with us. I know it was a bit traumatic at first, but I hope you are happy with the results. I am also super grateful specially to Corinne Clavé and Alexandra Granger-Farbos, and also Virginie Coustou and Hélène Yvanne. I think you are the real superstars, doing all the amazing molecular biology work. Our work was a million times better thanks to you! Thanks also to Mathieu Paoletti for the discussions and insights.

I remember happily the brief time Dave Jacobson spent with us in Uppsala early in my PhD, teaching many members of the lab how to deal with *Neurospora* spp. For my project, Dave became a critical eye from the hardcore fungal biology perspective. That was super formative and interesting to me! Thank you very much, Dave. Thank you also for taking care of us during the Asilomar conference of 2017.

I am also very thankful to Martin Lascoux, my official co-supervisor. I always have fun listening to your adventures and opinions, and I was very grateful for your advice with pop gen and writing. Thank you so much to Sylvain Glémin for giving so much of your time and energy to my questions. I also learned a lot from you because you are so open and willing to help students, and from the experience of having you as my half-time opponent. I am also grateful to Per Unneberg for introducing me to Snakemake and for helping me out whenever I would get stuck with it.

I am eternally grateful to Douglas Scoffield, my bioinformatics mentor. I learned so much just by watching you navigating in your computer, and I hope you know that I valued a lot all the piece of advice that you ever gave me. At the end of my master, you told me that I had aptitude for bioinformatics and I took those words very much at heart. So here I am, years later.

Everyone in the Johannesson lab, thank you so much also for creating such a cool, safe and fun environment to work on. I really appreciate the smart and funny remarks of (Big) Jesper and Markus; you guys are really astounding and talented. Thanks also to Markus for helping out with the Swedish abstract. Diem, I am constantly amazed of how organized and kind you are! I really hope you get a job were your invaluable skills are really cherished. Thank you so much for all the million things you did for me and

the others, like reading and commenting our texts and presentations, getting dumplings, taking care of orchids, and just any other single little detail that really made our lives easier. In the same way, thank you so much to Cecile, Sara, Kristina, and Petr for your good energy and insights. I remember dearly the times when we were so many in the group that we were almost filling the meeting room. Also, thank you so much to Magdalena (Magda) Grudzinska-Sterno for your help in the lab, producing a lot of the *Podospora* data, and helping the group in general. Thank you to Ivain for your creative input, the fun little drawings exchange, and all the laughter. I know I said it many times, but your theoretical models were really a dream come true for Hanna, Aaron, and myself.

Thank you so much, also, to the lichen girls. Veera, you were always very kind to me. I hope you find that sweet balance between quality of life and work that you (and we all) deserve. Ioanaaaa!! Your presence in EBC for me always felt more like having family around. You always made me feel safe and welcomed, like I am home. Nothing like seeing you struggle to avoid spoiling Game of Thrones or Outlander haha. I think you have a very special power of creativity, I admire that deeply. And your witch powers, you knew I would come back! I hope you get to do all the cool things you want to do, and that you keep kicking ass at karate.

I am SUPER grateful to my officemates: Sarina, Brendan, Raquel, and Anneli. I know you all must have suffered a lot with Aaron and I discussing at full power our crazy *Podospora* stuff at the office. You all were super gracious about it and never once complained. Thank you so much for your patience. Dear Sarina, your friendship was extremely therapeutic to me. I admire you deeply as a person for all of your qualities and drive to improve yourself, and as a professional because you have great talents with people. You were always willing to listen to me at my worst, and put a lot of attention to everything I had to say. I had so much fun with you and with Julius, and I wish you both the best in the future! Likewise, thank you Brendan sooo much for not making fun of me whenever you would catch me singing at the office :P, for sharing the pain with conda and snakemake, for the amazing desserts, the eternal flux of random wisdom, for helping me with English, and for your overall nice attitude. Let's keep having karaoke nights! I want to also thank Raquel extensively. After all these years I am convinced that you are a deeply kind and sensitive person, absolutely willing to sacrifice yourself for others. I hope you find the strength to love yourself and to see all of the good points that others see in you. Take care of yourself. Also, I want to say that I was very happy of having somebody around with similar sense of humor and interests, especially with anime, chonky cattos, and all around Internet culture. And Anneli. I had less chances to interact with you, but every single time I thought you were a super cool person. It was fun to talk about drawing, tablets, and mustard-color cloths. And thank you so much for sharing your chocolate when I needed it desperately!

Manolis, Gizem and Bianca, thank you soooo much for the fun and good energy with teaching the Animal Physiology course. It was an enriching experience for me (even if I was complaining all the time) but the best part by far was that I got to spend time with you guys. Same for Cesar, we only taught together one semester but it was great to get to know you a bit more. Likewise, thanks a lot to Sanea for the brief but funny times teaching together, for sharing personal things with me even though you didn't know me much, for all the advice with genome annotation, and of course, for the great and acid sense of humor.

I want to thank also the administrative staff of EBC for all the invaluable help the provided through the years, specially Karin, Iva, Sandra, and Agneta. Also for the occasional fika cakes you would bring and share after lunch.

SystBio! I really loved working in the department of Linneus. Thank you to all the staff, PIs and PhD students. Thank you to Martin Ryberg for tolerating my constant complains about the teaching activities, and for allowing for a fun atmosphere in the department. I love your t-shirts. Thank you Nahid for taking care of us and always making sure that I was healthy and okay. I am grateful to Jenni for the laughter and the fika, but especially for tolerating Aaron and I discussing (or even fighting) in your office.

Thank you so much to Fabien Burki for all the advice and insight with the phylogenetic analyses. And for taking the time to check on me when we were planning on working on the big phylogenomics project, although that failed in the end haha. I also enjoyed our struggles together in Swedish class. Muchas gracias a Iker Irisarri to take time helping me with phylogenetics questions, for the useful journal clubs, and the good sense humor.

I want to thank Juma and Stella for the good moments that we shared, however brief. I had a lot of fun joking around with Juma, I think you have a certain color to your personality: very bright and warm. I was always happy to hear Stella talking about my past love, *Usnea*. Thank you for keeping me in mind and introducing me to P. Clerc when he came to visit Uppsala. I only regret not having spent more time to get to know you guys better.

Thanks a bunch to Alex Suh and his group. Alex, I am eternally grateful for your willingness to help and teach others, and for showing curiosity about every living creature, however bizarre. More than that, I consider you somebody precious and kind, who has gone through a lot of hardships but has managed to maintain personal integrity and positive attitude. I hope I get the chance to keep learning from you and your amazing TE, networking, and science skills. I am also very grateful to Valentina for your TE-advise and for listening to me ranting during the last weeks of writing the thesis. Likewise, thanks a bunch to Julie! We don't know each other much, but you quickly opened to me about your own struggles and took my side during really bad times. I really appreciated the wonderful chocolate! Thank you

also to Marco, Octavio, and everybody else in the Suh lab for all the help, the TE Jamborees, and the cool energy.

Thank you to past and present friends from the Evolution floor. I had fun and interesting times with Willian, Homa, Matthias, Paulina, and Sergio that I remember with a smile. Book clubs, Cesky Krumlov trips, dinners, karaoke nights, and plenty of discussions. I really miss those days. Also, thanks to Ghazal for your wonderful affection. I really like when you pamper me haha. To me, you are like an ASMRtist but in real life: relaxing and sweet. Thank you also to Berrit for helping me (and others) out all the time; you are a fantastic person and I hope life gives you more puppies and happiness!

I truly appreciate as well all the fun I had with the people from the Human Evolution program, especially Mario, Gwenna, TJ, Luciana, and Alex C. I see Mario and Gwenna like a mini cohort of mine, if that make sense. It's like we started at similar times and we struggled for similar reasons a few times. Thanks for your friendship and help. TJ!! How cool you are! You and I are so introverted at times, and we are always in our own worlds, so it's so hard to ever see you, man. But I really enjoyed the few dinners we had together, and the anime nights we had with Bere… Next is Luciana. Oh Luciana. I think when I first met you, you seemed a bit unreachable to me, and that is why it took us so long to interact. But now that we have, I consider you an extremely kind, sensitive and beautiful person (and so handsome!!!). You went completely out of your way to help me a lot in more than one occasion and I am forever grateful. I hope I get more chances to get to know you even more. And Alex… I met you even before starting our PhDs, so we have some history. You always caught my attention. Like, "I want to be friends with that girl". I saw you evolve in emotional and physical ways, and I am ultimately just so proud of you. Thank you for the conversations, the fikas, the trips, and specially, the times at the dance floor. If there is somebody that I want to dance with until I break my shoes and lose my voice… it has to be you.

Thanks to Jordi for helping me out in the very last panic days of submission!

Having the Uppsala MEMEs around was really valuable to me, because it connects me to a very important part of my life and education. So thank you to Sergio, Paulina, Homa, Willian, Bere, Moos, Foteini, Mahvash and Caro! You all supported me in my struggles one way or another. On that note, thank you to Jacob Höglund. Maybe you don't remember, but you were my interviewer for the MEME program (along with Alice) and I'm pretty sure it was thanks to you that I was accepted, which ultimately led me to Uppsala. I'm most grateful for the opportunity. Thank you also to other MEMEs elsewhere, in particular Daniel, Marina, Miya and Romain. I was very happy to visit you when I had the chance, and you were the most amazing hosts and friends. From eating Czech food, experiencing the chameleon in Groningen, or watching marine birds in the North of France, I had very wonderful expe-

riences thanks to you. I also want to thank the other MEMEs, Chelsea, Nicky, Flora, Damian and Kasia (you're basically a MEME), for all of your support during the hard times in Zurich.

Even though the PhD thesis is ultimately an academic exercise, I think emotional support is just as important as scientific input. Still, from all of my dear friends, the one that had the biggest role in both aspects (scientifically and emotionally) is no doubt Sergio. You had superhuman tolerance with me, listening to all of my complains and rants. You always helped me by discussing ideas, by sharing scripts, and papers. I was very impressed (a pesar de que estaba tan somnolienta jeje) when, during a long train trip we had, you actually tried to write an R script on a paper notebook (!) for my analysis. During the masters and first half of my PhD you were also the one pushing me outside my house so I would have human interaction. But at the same time you were always very understanding of my introverted side, and managed to find a good balance with me. Needless to say, I was very forlorn after you left to Germany. Y bueno, aunque seguro no lo tomo bien en el momento, muchas gracias por decirme mis verdades (calling out my bullshit). No lo digo seguido, pero la verdad es que te quiero mucho :). Gracias por todo.

To Bere in particular … I can't even articulate how grateful I am. Although I know you were not very happy in Sweden, to me the time we lived together in Botvidsgatan was perhaps the best time during my PhD. I was very happy with you. I never felt uncomfortable being myself; together we tried to reinforce our slightly weak Mexican identity; we watched anime sometimes, we shared stories of our lives, and we comforted each other during difficult days. It was very sad for me when you left (but I knew it was for the best!), and I still miss you! However, later I found so much joy in watching you live your best life in France. I'm so proud of you and so happy seeing you with your newfound luck and energy. And without exaggerating, I think that week I spent with you in Paris, with the Japan Expo and the Monsta X and NCT 127 concerts, is really in the top 10 of my life. I have so much fun sharing monbebe stuff with you and I hope in the future we keep sharing amazing experiences following the YOLO philosophy. ¡Te quiero mucho!

My dearest Miya. You were also there for me (virtually) all the time. That's a big deal. Very few people can nurture a long-distance friendship, so I'm very grateful that we managed! Thank you for checking on my constantly, for inviting me over to visit you countless times (even if I only went once), for cooking for me when you came here, and for sharing memes, kawaii stuff, and Jaehyun videos. And for helping out with the thesis abstract. I hope we both can get over our own issues and mature further into happiness.

Marinaaaaaaa. My favorite brasileira. I had few chances to enter your busy life during the past years, but when I did it was super fun. I remember especially when I visited you in Groningen during my first "field-work" in

the Netherlands, and when you came visiting us here in Uppsala. I'm confident you'll achieve anything you want. I admire you and I can't wait to visit you and your rainforest in Brazil.

Of course, many thanks to Foteini! I have so many good memories with you, like dancing, fikas, conversations, and especially the trip to Greece. I am very grateful to you for taking care of us even though you were so busy. I only regret not having spent more time with you, especially during your own hard times. I miss you! And by extension, I also miss you too, Moos! I am very thankful to you for checking on me in the last writing months; that was very cool of you. Thank you for all the conversations about science, movies, and food! Likewise, I really want to thank André for all the time spent with me during 2018. The conversations we had were absolutely key for my mental health, and for reevaluating many aspects of myself. Thanks to you I experienced laughter in a way that reminded me to my infancy. I hope life gets kinder to you in the future, you deserve it.

I am also super, super grateful to Mahvash (aka. "Muffin"). Thank you for all those times that you took care of me, listened to my endless rants, and allowed me to sit down and cry at your office couch. I had a lot of fun with you and Mercè at our dinners and fikas, shopping for each other, or going to the movies. I think you and I had a cultural understanding, with some shared similarities as foreigners from warm, colorful countries. And on the personality side, I know I can always count on you to understand my introverted behaviors. Thank you for always cheering me up, for tolerating my musical obsessions, and for feeding me in times of need. I wish you all the happiness and chocolate muffins in the world.

Muchas, muchas gracias a Mercè. Just like with Mahvash, thank you for being there all the time, checking on me and cheering me up. I admire your passion and determination. And your energy! I know I always say I have a hard time understanding the Spanish from Spain (jejeje), but I was glad to have an ally in my language and pronunciation confusions. You were also my ally in all things fungal and queer. We fight sometimes, but I hope we can keep on maturing together. Also, thanks a bunch for helping out with the Spanish abstract and its "genes calientes". Que el futuro te traiga cosas maravillosas :).

Towards the end of my PhD time I sort of stumble into wonderful people that help me in very gracious ways, several times as a surprise. First, thank you to Karin Steffen for checking on me so enthusiastically and for transmitting your good energy and attitude. Next is Katerina: although I failed to say it well the first time, I still think you are an exceptionally kind person. Thank you soooo much for organizing that little kayaking trip, and for feeding me in times of need just because you felt like it. Amazing. Likewise, muchas gracias a Ariadne por alimentarme con vitamina T (tortillas, tlacoyos) y por hacerme compañía en el gym. Generalmente me choca ejercitarme con otras personas, pero contigo siempre me sentí muy cómoda :). Y Sarahí, mi otra

aliada mexicana, aunque a ti te conocí desde incluso antes del doctorado, creo que nos volvimos mucho más cercanas en este último año. Hay algo en ti que me inspira familiaridad, me haces sentir en casa, y por tus circunstancias de vida yo sé que entiendes muchas de mis dificultades, como crisis de identidad, dramas familiares, y los corajes migratorios. Espero que sepas que te admiro muchísimo y te agradezco infinitamente todas las veces me invitaste a comer y a cotorrear en tu casa. Mi intención es seguir pendiente de tu vida y aprender lo más que pueda de ti :P.

Thank you also to Madee and Anne-Marie for being great flat mates during the short, or not so short times we had together. It's pretty cool that you both are such nice people, and at the same time such great scientists :). And on that note, thank you also to Philipp for all the nice times at parties, the time in Turku, and other occasions.

This last year I had the super unexpected surprise of finding an extraordinary new friendship in my new-ish flatmate Maria. I think you and I clicked instantaneously during our first Skype talk, and ever since I felt like I was automatically blessed with the opportunity of finally getting to know a Scandinavian person for real (even if not Swedish). You have a rare talent to make me feel relaxed around you. And even more, I feel like you take care for me in a way that only family can do. I am so grateful for that. And for your delicious bread, cookies, and Danish bullar. Amazingly, with you it came the bonus of also getting to know Magnus. It's crazy that you and Magnus, from all people, were the ones that made me feel truly welcomed into the North when I got my permanent residence permit. I hope I can keep you in my life for as long as possible.

I am very grateful to Agnes, Artis, Jonas and Freya for giving Maria and me the chance to share your house. I feel like I was lucky to find a comfortable place with everything I could possibly need, and to share it with responsible and good people.

Although we talk every seven months or something, I am also super thankful to my dearest BFF Marisa (aka. Lutita). Still, cada vez que hablamos es como que nada ha cambiado, y siempre termino riéndome como loca. Y bueno, you know how I feel about you. Espero que podamos estar más presentes la una con la otra en los años por venir.

Y mi querida Coral, mi hermana de otra madre. También te agradezco a ti todo el apoyo que me diste desde lejos. Nos vimos contadas veces desde que me fui de México, pero aunque sea por poquito, pasar la tarde en el aeropuerto contigo es lo máximo para mí. ¡Te quiero!

También muchas gracias a Ángel, mi hermano foca, por toda la compañía virtual. Tiki tiki tum tum.

I want to thank Alfonso ("Alfonoso") Lizárraga for all the support and (virtual) company all these years, and especially at the beginning of my PhD. A deep connection despite the time and distance. *Se tira por la ventana*

Y por supuesto, quiero agradecer extensamente a toda mi familia. Mi familia en Francia me rescató en más de una ocasión. Muchísimas gracias a mis tíos Obey e Isabelle, y a mis queridas primas Cami y Juanita por acogerme en su casa e incluirme en las navidades parisinas. Me encanta pasar tiempo con ustedes, y platicar de comida, música y política, aunque yo no entienda nada jaja. Gracias a ustedes pude relajarme en momentos claves de la maestría y el doctorado. ¡Los quiero mucho!

Mi abuela Lydia es una de las personas más importantes de mi vida y aunque no entienda nada de lo que hago, siempre expresó muy abiertamente que (al menos para ella) yo soy "el orgullo Ament". Te quiero mucho, abuela. Gracias por tu apoyo. Mis otros tíos y tía también expresaron su apoyo en algún momento durante mis actividades académicas, pero especialmente mi tío Jorge y mi tío Carlos estuvieron presentes a través de Facebook jeje.

Muchas gracias también a mi familia colombiana, especialmente a David Torres por estar al pendiente de mí y por visitarme en Uppsala. También a mis queridas tías Adriana y Leti, y a mis primos Alejo y Laurita por la energía chévere y todo el cariño.

Gracias a Guille por tener tu eterna energía extravertida y positiva. Me da siempre mucho remordimiento haberme perdido tu infancia y ahora adolescencia, pero espero que comprendas que mi felicidad está en perseguir mis sueños locos. Ojalá podamos conectar más en el futuro.

Finalmente, a las personas más importantes, mi mamá y papá, les agradezco la vida. Ambos estuvieron siempre allí, muy presentes y muy cariñosos. Los extraño todos los días. Muchas veces me pregunté si venir tan lejos era un error, simplemente por no tener la oportunidad de verles la cara. Pero por otra parte, siempre sentí que ambos estaban muy contentos y satisfechos de mis logros, con la certeza de que yo estoy mejor aquí haciendo lo que me gusta, en un ambiente que me acomoda. Además, francamente este estilo de vida está inspirado en ustedes. De mi papá viene mi gusto por la naturaleza (¡y en especial por los hongos!), y mi mamá me heredó esa vena viajera y gitana. Gracias mami por tu cariño, tu humor fastidioso, tu creatividad y tu inteligencia. Mi ética de trabajo y concepto de calidad vienen totalmente de ti. Verás que le puse muchas ganas a todas las figuras de la tesis. Gracias también por aguantarme mis malhumores y por consentirme cuando regresaba a México. Gracias papi por siempre hacer el esfuerzo de llamarme, de platicar, y especialmente en el último año del doctorado, de comprenderme. Se me va la onda, pero cada que hablamos me acuerdo de mis orígenes, y pues de tal palo, tal astilla. Todavía me acuerdo de los libros sobre hongos comestibles que tenías en el librero. Mira, ahora yo escribí el mío, aunque *Podospora* no se coma. Es decir, ésta es mi contribución a la humanidad para entender a los hongos un poquitito más. Y qué chistosos son, ¿verdad?

Cover explanation

The cover is a bit extravagant because I chose to make a little tribute to my younger self, who wanted to be a comic artist. I was inspired by the Japanese oni (ogres or demons) to represent the four *Spok* homologs, following the color scheme of Paper I. The darker red oni is *Spok1*, the orange oni is *Spok2*, the green oni is *Spok3,* and the lighter red oni is *Spok4.* They are all decorated with something *Podospora*-related, like fruiting bodies, asci, or spores. *Spok1* gets to do the iconic hand gesture, because he was the first *Spok*. *Spok1* and *Spok4* look alike in color and clothes, but *Spok4* also looks like *Spok3* in some features. For example, *Spok3* and *Spok4* are both part of the crew of the *Enterprise*. So, which one is more closely related to *Spok4*? They all have different weapons, and are capable of hurting each other. Except for *Spok1*, which can defeat everyone (although it won't harm *Spok4*), and nobody knows what his weapon is. *Spok2* is the happiest, because he's so successful. And *Spok3* is just … a bit crazy. Finally, the background is a three-way alignment of the genome assemblies of *Podospora anserina*, *P. comata* and *P. pauciseta*, the species that have these four *Spok* onis, I mean, genes.

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Acta Universitatis Upsaliensis

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Editor: The Dean of the Faculty of Science and Technology

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