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Catching the Spore killers

Genomic conflict and genome evolution in Neurospora

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Abstract
Svedberg, J. 2017. Catching the Spore killers. Genomic conflict and genome evolution in *Neurospora*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1561. 51 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0074-0.

A genome is shaped by many different forces. Recombination can for instance both create and maintain genetic diversity, but the need to locally reduce recombination rates will also leave specific signatures. Genetic elements can act selfishly and spreading at the expense of the rest of the genome can leave marks of their activity, as can mechanisms that suppresses them, in a phenomenon known as genomic conflict. In this thesis, I have studied the forces driving genome evolution, using modern genome sequencing techniques and with a special focus on a class of selfish genetic elements known as Spore killers found in the fungus *Neurospora.* First, we show novel findings on large-scale suppression of recombination by non-structural means in the *N. tetrasperma* genomes. In contrary, in the genomic region harbouring the spore killer elements *Sk-2* and *Sk-3* of *N. intermedia*, a dense set of inversions that are interspersed with transposable elements have accumulated. The inversions are unique for each killer type, showing that they have a long separated evolutionary history and likely have established themselves independently. For the *Sk-2* haplotype, where we have polymorphism data, we see signs of relaxed selection, which is consistent with the hypothesis that recombination suppression reduces the efficacy of selection in this region. These results show the strong effects the divergent selective forces of genomic conflicts can have on chromosome architecture. Furthermore, we investigate the hypothesis that spore killing can drive reproductive isolation, by comparing the fertility of crosses between *N. metzenbergii* and either killer or non-killer *N. intermedia* strains. We show that crosses with spore killer strains have lower fertility, which cannot be explained by the killing itself, but is potentially caused by an incompatibility gene captured in the non-recombining region. Finally, we identified the genetic element responsible for causing spore killing in the *Sk-1* spore killer strains found in *N. sitophila*. Unlike the *Sk-2* and *Sk-3* elements, *Sk-1* is not connected to a large, non-recombining region, but is caused by a single locus, and we also find indications that this locus was introgressed from *N. hispaniola*.

Keywords: Genomics, genomic conflict, genome evolution, meiotic drive, spore killer, suppression of recombination, inversions, fungi, Neurospora

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Sometimes science is more art than science, Morty. A lot of people don't get that.
Rick Sanchez

List of Papers

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

I **Svedberg, J.**, Hosseini, S., Chen, J., Mozgova, I., Hennig, L., Manitchotpisit, P., Hammond, T. M., Lascoux, M., Johannesson, H. (-) The effect of genomic conflict on genome evolution in *Neurospora intermedia*. *Manuscript.*

II **Svedberg, J.**, Vogan, A., Rhoades, N., Sarmarajeewa, D., Hammond, T. M., Lascoux, M., Johannesson, H. (-) A single gene causes meiotic drive in *Neurospora sitophila*. *Manuscript.*

III Vogan, A., **Svedberg, J.**, Grudzinska-Sterno, M., Johannesson, H. (-) Meiotic drive is associated with reproductive isolation in Neurospora. *Manuscript.*

IV Sun, Y.*, **Svedberg, J.***, Hiltunen, M., Corcoran, P., Johannesson, H. (2017) Large-scale suppression of recombination predates genomic rearrangements in *Neurospora tetrasperma*. *Nature Communications* (in press).

* These authors contributed equally to the study.

Abbreviations

bp	basepairs
kbp	1000 basepairs
sk	spore killer
GFP	Green Flourescent Protein
SNP	Single Nucleotide Polymorphism
SMRT	Single Molecule Real-Time
ChIP-seq	Chromatin Imunoprecitation sequencing

Contents

Introduction

When studying the evolution of genomes, we try to infer evolutionary trajectories from present day genomic data and to deduce which forces could have shaped a genome, both as a whole, and locally on the chromosomes. By comparing species, we can also see how both evolutionary history and life history influenced the genome. But this is not an easy task, as genomes are highly complex molecular systems that, perhaps more than anything else, the main determinant of their own evolution.

Every genome consists of mutations that throughout time have appeared randomly, and through various evolutionary processes, accumulated into what we can observe today. The fate of any such mutation can be affected by countless factors, but just like the survival of an organism is dependent on the environment it interacts with, the persistence of any mutation is shaped by the genomic environment it interacts with. To illustrate how the different forces affect the fate of mutations we can start by considering what happens at a single locus. First, a new mutation might simply disappear from the population if it is not passed to the next generation, and this is independent of whether the mutation is neutral or adaptive. For a neutral mutation, the probability that it is not passed to the next generation is 37% (Gillespie, 2004)! The fate of a positively selected mutation is hardly better: its probability of fixation is solely $2s$, where s is its selective advantage (Haldane, 1927). If the mutation does not vanish right away then its fate generally depends on the background in which it occurred. Indeed, selection does generally not work directly on mutations, but through complex molecular interactions between many parts of the genome, which generates phenotypes that can be selected upon, and the "fitness effect" of a specific mutation is only a sensible concept in a specific genetic background.

The fate of a mutation also depends on selection on other parts of the genome, even if they do not functionally interact. Being closely linked to a strongly deleterious mutation may lead to extinction no matter what fitness effect a mutation itself result in, and positive selection on linked or unlinked genes can also lead to either fixation or extinction (Charlesworth et al., 1993). Furthermore, the activity of selfish genes, which can act in their own favour and at the expense of other components of the genome, may also affect the fate of a mutation (Burt and Trivers, 2006), as will suppressive mechanisms intended to counteract such selfish behaviour (Johnson, 2007).

Population level processes can also be an important factor. The strength of stochastic effects varies with population size, and depending on the fitness effects of the mutation, this can both increase or decrease the risk of extinction of a mutation (Lynch, 2007). In a sexual and outcrossing population, recombination both reduces the effects of linked selection and exposes the mutation to many different genetic background, thereby increasing the chance of finding an allele combination that confers high fitness (Otto and Lenormand, 2002). At the same time, recombination can unlink a gene from an especially beneficial background, and if this happens often enough the mutation may disappear (Ortiz-Barrientos et al., 2016). Furthermore, other population level processes, such as mating system, sexual selection and life history traits can also be of influence (Ellegren and Galtier, 2016).

These are some of the basic principles of genome evolution and in my own research, I use fungi as a model for studying some of them. Fungi have a number of advantages that make them very well suited for studies on genome evolution, such as generally small genomes and being tractable to manipulations in the lab. In the model fungus *Neurospora* I have investigated two processes that can affect genome evolution. The first is linkage and its causes, and the second is genomic conflict. The latter is the main subject of this thesis and I have studied a class of selfish genetic elements known as "Spore killers" and investigated their origins, function and evolutionary consequences. Below I will briefly discuss these two processes.

The evolution of linkage

In an asexual organism, all sites in a genome are linked and the only way to generate new variation is through mutations. In sexual organisms, new combinations of genetic variants can be created through meiosis and recombination. In this way, a huge amount of potential variation is distributed across all reproducing individuals in a population, and for this reason more diversity can be both maintained and realized.

In some cases, recombination can instead break apart advantageous combinations of genes, and if this happens at a significant frequency, selection for mutations that reduce recombination between genes is expected. Such linkage can have large consequences, as is perhaps most clearly illustrated by the evolution of highly divergent sex chromosomes, which are thought to have started from sexually antagonistic alleles being linked to the sex determining locus (Bergero and Charlesworth, 2009; Wright et al., 2016). While the theory behind the evolution of linkage (recombination rather than linkage) is well established (Ortiz-Barrientos et al., 2016), and while we can see variation in recombination rates across genomes, we still know relatively little about the different mechanisms that can reduce recombination and how common each of them are.

In most eukaryotes, recombination is concentrated to small regions called recombination hotspots (Choi and Henderson, 2015). What makes a region a hotspot is generally not well understood, though in some mammals, hotspots are regulated by a gene known as PDRM9, which recognizes certain DNA motifs where it methylates H3K4 histone residues and induces recombination (Baudat et al., 2013). Recombination rates can, in this case, be reduced either locally through mutations at each hotspot, or globally via mutations in PDRM9 itself. The ability of this mechanism to adaptively regulate local recombination rates is uncertain though, due to the general instability of PDRM9-mediated hotspots and the large epistatic effects mutations of PDRM9 are expected to have (Ortiz-Barrientos et al., 2016). Other epigenetic mechanisms, such as DNA methylation, have also been shown to affect recombination rates (Yelina et al., 2015), though the details are still elusive. Genes that reduce recombination over short distances have also been identified in for instance *Neurospora crassa* (Catcheside, 1981), but their mode of action is also poorly understood.

The type of genetic variants capable of affecting both large and small scale recombination patterns which have received most attention are structural rearrangements, and in particular chromosomal inversions. Inversions can, as single mutational events, affect recombination patterns throughout its length and into nearby regions (Kirkpatrick, 2010). Complex phenotypes caused by multiple genes have also been mapped to inversions, suggesting that inversions are important in linking beneficial gene complexes together (Hoffmann and Rieseberg, 2008; Kirkpatrick, 2016; Schwander et al., 2014).

Inversions are thought to reduce recombination through at least two mechanisms. One is by preventing synapsis at meiosis, and the other is through the production of unbalanced, inviable gametes, following an uneven number of within-inversion crossover events (Kirkpatrick, 2010), Recombinants will therefore be underrepresented in the offspring, and the recombination rate in the inverted region, measured on a population level, will be lower.

The production of inviable gametes comes at a fitness cost that increases with the size of the inversion, which is expected to lead to a general selection against them (Knief et al., 2016; Navarro and Ruiz, 1997). In some taxa, such as *Drosophila*, mechanisms that reduce these fitness costs have been identified (Sturtevant and Beadle, 1936), and consequently inversions appear to be common in these organisms (Krimbas and Powell, 1992), but in general, we know little about the conditions under which inversions are tolerated, and which mechanisms can mitigate their fitness effects.

There are two main explanations for how inversions occur (Casals and Navarro, 2007). Inverted repeats have sometimes been found near the breakpoints of inversions and ectopic recombination between these repeats has been suggested to lead to inversions (Finnegan, 1989). The second explana-

tion is that inversions are caused through faulty repair of a chromosome with two double-strand breaks (Ranz et al., 2007).

Genomic conflict

Different parts of a genome will sometimes be in conflict with each other. Some of these conflicts arise from accommodating different selective optima in a shared genome, such as sexual conflicts between males and females, but I will here focus on the effects of genetic elements that through their own activity will generate a reproductive advantage for themselves, at the expense of other parts of the genome. Through such activity these genetic elements end up in conflict with other parts of the genome, for instance the alternate allele at a specific locus, or – if the end result is a decreased fitness of the individual – the rest of the genome. Such genetic elements are often referred to as "selfish genetic elements", "selfish genes" or "cheaters" (Werren, 2011).

In their seminal overview of the field of genomic conflicts, Burt and Trivers (2006) identified three major strategies that selfish genetic elements can use to gain a reproductive advantage, or "drive". The first strategy is *gonotaxis*, or female meiotic drive, in which certain chromosomes can preferentially move towards the egg during asymmetric female meiosis, and in doing so, avoid becoming dead-end polar bodies (Pardo-Manuel de Villena and Sapienza, 2001). The second strategy is *interference*, where a genetic element disrupts the transmission of competing alleles, and by this process, results in more than the expected 50% of the offspring. A highly diverse set of selfish genetic elements uses this strategy, from single driving loci to whole chromosomes (which often are sex chromosomes) and even whole haploid genomes (Burt and Trivers, 2006; Lindholm et al., 2016; Lyttle, 1991). The third strategy is *over-replication*, which is employed by the most successful category of selfish genes: transposable elements, which will disperse by making copies of themselves throughout the genome (Feschotte and Pritham, 2007).

It has become increasingly clear that genomic conflicts can be major drivers of evolution (Rice, 2013; Werren, 2011), but different selfish genetic elements can affect an organism in very different ways. Some elements may quietly go to fixation without much notice, whereas others can have major effects. Selfish sex chromosomes will for example during male meiosis kill all sperm bearing the other sex chromosome, causing shifts in sex ratios. It is obvious that if such a sex chromosome were to spread in a population it could lead to the extinction of the other sex and accordingly, to the extinction of the population as a whole. However, it can also lead to the evolution of mechanisms that suppresses the ability to disable non-carrier sperm and

the selfish sex chromosome may end up in an arms race with the rest of the genome, with suppressors and counter-suppressors evolving at a highly elevated rate (McLaughlin and Malik, 2017; Meiklejohn and Tao, 2010). Such dynamics in male meiosis could potentially explain the high number and high evolutionary rate of testis-specific genes in, for instance, *Drosophila* (Meiklejohn, 2016).

Some selfish genetic elements are well known to affect large scale patterns of genome evolution. For instance, *transposable elements* that spread in a genome can have varied effects, most of which are considered deleterious (Charlesworth et al., 1994). Specifically, they can disrupt genes by inserting in the coding regions, they can cause rearrangements through illicit recombination between nonhomologous copies, or they can over time cause major changes in genome size and composition. Transposable elements have been extremely successful and almost all eukaryotic genomes bear traces of their activity, but the effects of uncontrolled proliferation of transposable elements is expected to be highly deleterious (Vinogradov, 2004) and for this reason, a diverse set of defence mechanisms have evolved to control them (Johnson, 2007).

Genome defence mechanisms are ubiquitous in eukaryote genomes. RNA interference (RNAi) has been shown to be widely involved in gene regulation, but it is thought to initially have evolved to control the spread of transposable elements (Agrawal et al., 2003), a function that it still retains. DNA methylation has evolved to perform a more general regulatory function, but is suggested to have an ancestral function for transposable element control (Chan et al., 2005), as is the case for heterochromatin (Henikoff, 2000). Two defence mechanisms specifically found in fungi are Repeat Induced Point mutations (RIP), which causes C-to-T mutations in any sequence found more than once in a genome (Galagan and Selker, 2004) and Meiotic Silencing of Unpaired DNA (MSUD), an RNAi based defence mechanism, that silences any part of the genome that is unable to synapse, and is therefore unpaired, during meiosis (Hammond, 2017).

Genome defence mechanisms can consequently have large effects on genome evolution. For instance, heterochromatin and methylation allow for regulated regional restructuring of the genome that can be activated or deactivated in a controlled fashion during the life cycle (Grewal and Jia, 2007). Similarly, RIP will prevent any gene duplications, and thus limit the evolvability of any organism with a functioning RIP system (Galagan and Selker, 2004).

Another class of selfish genetic elements that can affect the evolutionary trajectory of a genome is *gamete killers*. These are a subgroup of meiotic drive elements that work at, or closely following, meiosis by disabling non-carrier meiotic products that are often, but not always, classified as gametes. Many well-studied examples of gamete killers are complex molecular systems, dependent on the interaction of multiple loci. These loci are often lo-

cated in large regions where recombination is low, which prevents the loci from being unlinked (Burt and Trivers, 2006; Lindholm et al., 2016; Lyttle, 1991). In several gamete killer systems, such as the Segregation Distorter (SD) complex in *Drosophila melanogaster* (Larracuente and Presgraves, 2012) and *t*-haplotype in mice (Lyon et al., 2000), suppression of recombination seems to be caused by chromosomal inversions in the vicinity of the active loci. If such inversions would go to fixation in a population or species, it would lead to a divergence in chromosomal structure. Furthermore, since the non-recombining region would stop exchanging genetic material with other chromosomes, its fixation would lead to strongly reduced levels of genetic diversity within the region (Charlesworth et al., 1993; Smith and Haigh, 1974).

The diversity of selfish genetic elements is vast, and my own research has primarily focused on meiotic drive in fungi. I will therefore review what is known of this field below.

Fungal spore killers

Spore killers are a class of meiotic drive elements found in fungi (Raju, 1994). Like male meiosis in animals and plants, fungal meiosis is symmetric and fungal spore killers will act like sperm killers in that meiotic products that carry a sperm killer genetic element (killers) will disable products that don't (sensitives). They do so by taking advantage of the fact that in many fungi meiotic products will initially share cytoplasm before the cell walls surrounding the spores are formed. This creates a window of opportunity where killer spores can attack their sensitive siblings.

Unlike most animal sperm killers, which are large multigene complexes located in regions of low recombination, such as chromosomal rearrangements or sex chromosomes (Burt and Trivers, 2006), many of the fungal spore killers are single genes. These typically kill through what can be described as a toxin-antitoxin mechanism, where a toxin is distributed among all spores and the antitoxin is limited only to the killer spores, and in doing so avoid self-killing (Grognet et al., 2014; Hammond et al., 2012; Hu et al., 2017; Nuckolls et al., 2017).

While spore killers can achieve high rates of drive, killing up to 100% of all sensitive spores, they are still thought to be weak drivers (Nauta and Hoekstra, 1993). Spores are progeny and not gametes, and will therefore only increase in frequency relatively, but not in absolute numbers, by killing their siblings and lowering the frequency of the non-killer genotype. When spore killers are rare in a population they will only have a very small effect

on population level frequencies and in order to efficiently invade a population, certain conditions must be met. These favourable conditions are high levels of sibling competition (mirroring the competition between sibling sperm to fertilize an egg), small (or structured) populations, or having some additional advantage, such as being linked to another beneficial locus, or by gaining resources from the killed spores. Despite these limitations, some spore killers are found at very high frequencies and it is not yet known whether any of these conditions are met or if our theoretical understanding of the phenomenon is incomplete.

Spore killers have so far only been identified in ascomycete fungi and they have primarily been studied in *Neurospora*, *Podospora* and *Schizosaccharomyces*. Spore killing has also been identified in *Fusarium* and *Cochliobolus*, but these spore killers have received less scientific attention. I will here summarize the current state of knowledge on all of these spore killer systems.

Neurospora

Neurospora is a genus of filamentous ascomycetes. It is sometimes referred to as "red bread mold", due to the distinct orange colour of the asexual spores and historical infestations in bakeries (Selker, 2011), but in nature it is most commonly found on burned substrates, for instance after forest fires. The most well-known species is *N. crassa*, which has been an important model organism for genetics and fungal biology for close to 80 years, and it was famously used for Beadle and Tatum's Nobel prize winning work on the one-gene-one-protein hypothesis (Beadle and Tatum, 1941). Several other species of *Neurospora* are morphologically very similar to *N. crassa*; among them *N. sitophila*, which harbours one spore killer element known as *Sk-1* and *N. intermedia* which harbours two others, known as *Sk-2* and *Sk-3*.

All *Neurospora* strains have both male and female sexual structures within one individual, and the species mentioned above are heterothallic, meaning that they are sexually self-incompatible and thus, can only mate with individuals of the opposite mating type as themselves, being either *mat a* or *mat A*.

Figure 1. A *Neurospora* mycelium will under the right conditions produce large amounts of conidia (asexul spores), making it completely orange. Photo: Raquel Pereira.

Sex in *Neurospora* can be induced by environmental cues such as nutrient starvation, and when in those cases an immature female sexual structure (protoperithecia) encounters male gametes (conidia, which can also function as an asexual spore), they can fuse and enter a dikaryotic stage where both nuclei are located in the same cytoplasm, causing the maturation of the protoperithecia into perithecia. The two nuclei will then go through karyogamy, forming a brief diploid stage in an oblong cell type known as an ascus or "spore sack", and quickly enter meiosis. The meiotic products will be distributed in an ordered fashion in the ascus (meaning that recombination events can be inferred from the order of the spores) and they then go through one round of post-meiotic mitosis, generating four pairs of identical twin nuclei, which all share the cytoplasm of the ascus. Finally, each nucleus will be encased in a cell wall, followed by yet another round of mitosis, and the previously transparent spores will mature and darken. The end result is eight black spores in a row (Figure 2) that can be shot out through an opening at the top of the perithecium (Raju, 1980).

When one of the mating strains carries a spore killer element and the other is sensitive to spore killing, the above described process will be identical up until the stage where the cell walls surrounding the spores are formed. Shortly after this stage, spores carrying the sensitive genotype will start to degrade, with the end result being four large, black, viable spores and four small, white, inviable spores in each ascus (Figure 2). If there has been no recombination between the centromere and the killer locus then all black

spores will be located at one end of the ascus and all the white spores at the other end. If recombination has occured, the pattern will instead be two black, two white, two black and two white. The pattern results from the ordered nature of *Neurospora* asci and allows for easy determination of linkage to the centromere. Except for a small number of sensitive spores which for some unknown reason can escape being killed, all surviving spores carry the killer element and will show killing in further crosses. This description is valid for all three *Neurospora* spore killers, with some minor differences that are elaborated upon below (Raju, 1979).

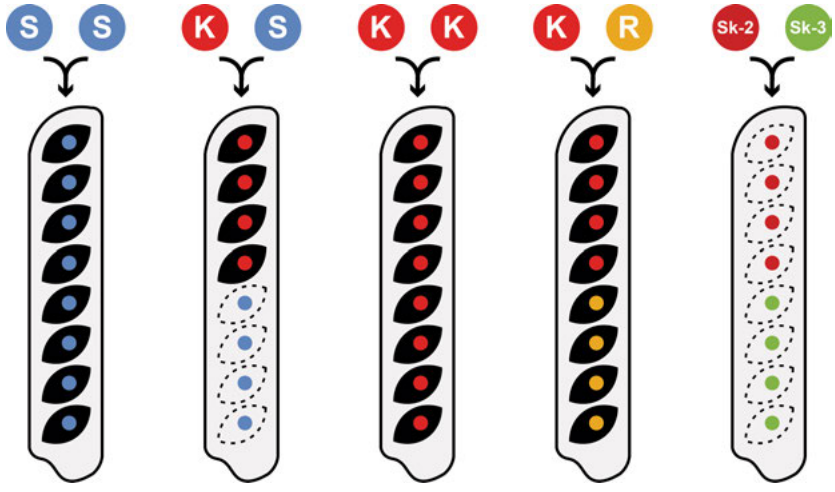

Figure 2. When two strains carrying a sensitive (S) genotype cross, all eight spores survive; as is the case when two strains carrying the same killer (K) genotype, and a killer and a resistant strain (R) cross. However, when a killer cross with a sensitive strain, only four spores mature into black spores and survive, and all are carrying the killer genotype. The other four remain white and die. When two strains carrying different killer genotypes (here *Sk-2* and *Sk-3*) mate, no spores will survive due to mutual killing, generating an empty ascus.

Sk-1 in *N. sitophila*

The first spore killer element to be identified in *Neurospora* were the *Sk-1* killers in *N. sitophila*. When crossing natural isolates from Nigeria to natural isolates from the US, a consistent pattern of four black, viable spores and four white, inviable spores was observed. When the surviving offspring was backcrossed to the parents, the pattern was only observed in crosses to Nigerian strains. Additionally, in crosses within each population dead spores could not be observed.

Based on these results it was determined that the US strains were carrying a spore abortion factor that only acts on strains that do not carry it. This phe-

nomenon was named “spore killing” and the killing factor *Sk-1*. It was also observed that this phenomenon was functionally very similar to sperm killing meiotic drive systems found in animals and plants. The *Sk-1* spore killer shows close to 100% killing efficiency and 5% recombination rate from the centromere, but a lack of markers made further genetic characterization, such as assignment to a linkage group, difficult (Turner and Perkins, 1979).

Over 700 natural isolates of *N. sitophila* have been tested for spore killing and roughly 15% of these natural isolates display the phenotype (Jacobson et al., 2006; Turner, 2001). A single resistant strain that can neither kill nor be killed has also been identified. Killer strains have been found all over the world, but differences in regional distribution are large. Some geographic regions appear to be fixed for *Sk-1*, whereas it is completely absent in others. In Italy and Tahiti, dense sampling has revealed an even ratio between killers and sensitives, but we don’t yet know how to explain these differences.

Sk-2 and *Sk-3* in *N. intermedia*

A second spore killer element was later found in a *N. intermedia* strain from Borneo. It could be shown that it segregated in a similar way to *Sk-1* when crossed to sensitive strains of *N. intermedia*. Further sampling resulted in a total of four *Sk-2* strains, which all produced four spores when crossed to sensitive strain and eight spores when crossed to each other. This is explained by the fact that spore killers must carry a resistance factor that prevents self-killing. When crossed to another spore killer of the same type, all spores will carry the resistance factor and thus survive. One tested strain did not show this pattern. While it killed when crossed to a sensitive strain it would instead produce empty asci when crossed to strains classified as *Sk-2*. This could be explained by the fact that the two strains carried different incompatible killer systems and would therefore kill each other, and for this reason the last strain was classified as a new spore killer, which was named *Sk-3*. It was also observed that many strains were resistant to either *Sk-2*, *Sk-3* or both (Turner and Perkins, 1979), and that these were found at much higher frequencies than either of the killers (Turner, 2001).

Both *Sk-2* and *Sk-3* were introgressed into *N. crassa* to facilitate genetic analysis. Spore killing was still possible when crossed to most natural *N. crassa* isolates, but it was discovered that resistance was also segregating in this species (Turner and Perkins, 1979). Using the rich set of genetic markers established in *N. crassa*, both *Sk-2* and *Sk-3* could be mapped to a 30 map-unit region surrounding the centromere of chromosome 3 (from now on the sk region), where recombination was suppressed between killer and sensitive strains (Campbell and Turner, 1987). A small number of markers were introduced into the sk region, which could be used to show that *Sk-2* strains recombined with a normal rate when crossed to other *Sk-2* strains (as was the

case for *Sk-3*), and that the suppression of recombination could not be explained by a single inversion, but that several smaller inversions could still be a possibility.

The recombination rate between killers and sensitives was measured to 10^{-5}-10^{-6} crossovers per meiosis, and this rate is difficult to explain through known mechanisms. While recombination suppression can be caused by inversions, the suppression is expected to be upheld by the production of unbalanced, inviable gametes, generated by within-inversion crossovers (Campbell and Turner, 1987). Neurospora crosses heterozygous for inversions are for this reason characterized by asci with variable ratios of dead and living spores (Perkins, 1997), and this pattern is not observed in spore killer crosses, which instead generate a stable 4:4 living:dead ratio. Furthermore, double crossovers are expected to occur within an inversion (generating viable offspring) at a rate of 10^{-3} over short distances (Stadler and Towe, 1968), and this is much higher than the measured recombination rate. Finally, so called *rec* genes have been identified in *N. crassa* (Catcheside, 1981), which can locally lower recombination rates. These will also allow for higher rates of recombination (0.1 to 0.01 of normal rates) and they have only been shown to be able to suppress recombination over much regions. Such genes are also expected to lower recombination for homozygous killer crosses, which does not appear to be the case for either *Sk-2* or *Sk-3*.

Resistant strains recombine freely with sensitives and it was shown that resistance segregates as a single locus. This was utilised to map the locus to the left end of the sk region (Campbell and Turner, 1987) and later to a specific gene, which was named *rsk* (Hammond et al., 2012). Knockouts of the gene in *Sk-2*, *Sk-3* and resistant strains showed that this locus is responsible for resistance in all three cases. Sensitive, resistants and killers all have different alleles at this locus with up to 20% sequence divergence. In a phylogenetic analysis of the locus, sensitives were grouping with sensitives, *Sk-2* resistants with *Sk-2* killers and *Sk-3* resistants with *Sk-3* killers, suggesting that different gene variants would confer resistance to each specific killer type, or lack of resistance (Hammond et al., 2012).

The killer phenotype was later mapped to a 45 kbp region on the right side of the sk region, but the specific causative factor is yet to be (Harvey et al., 2014). This study also revealed that the 45 kbp killer region was bordering an inversion at least 200 kbp long extending towards the centromere, but the full extent of this inversion is not known.

When through the use of developmental mutants, a sensitive nucleus is packaged into the same spore as a killer nucleus, both genotypes will survive and can be extracted after germination (Raju, 1979). When the resistance locus was deleted in a killer strain, an empty ascus was produced instead of eight small dead spores. This suggests that the *rsk* gene was protecting all nuclei during the syncytial stage and it is the loss of this protection after spore delimitation that kills them. From these facts, Hammond et al. (2012)

created a model for how the spore killer mechanism works. The killer spores produce both a toxin and an antitoxin, which neutralizes the toxin. These both diffuse freely in the ascus during the syncytial stage and the antitoxin protects all nuclei. After spore delimitation, the sensitive spores lose access to the antitoxin, either because it is less stable than the toxin, or because only the toxin can pass through the cell walls, and they therefore die. Killer and resistant spores can instead continuously produce new antitoxin, and by doing so survive.

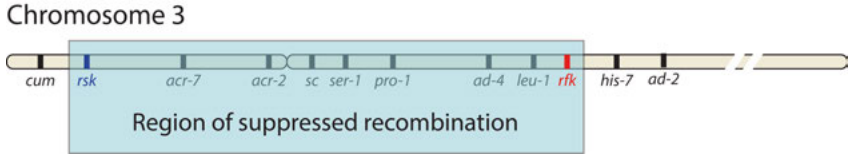

Figure 3. Map of the spore killer region on chromosome 3. The box marks the region where recombination is suppressed between spore killer and sensitive strains. *rsk* is the resistance locus and *rfk* the killer locus. The other loci are markers used for determining the size of the region (Campbell and Turner, 1987).

In contrast to the large number of killer strains found in *N. sitophila*, only a handful of killers have been found in *N. intermedia*. Instead resistant strains are common (Turner, 2001) and both phenotypes are only found in southeast Asia. Theory predicts that a high frequency of resistant strains can prevent the invasion of a killer in a population (Nauta and Hoekstra, 1993), and this could explain why *Sk-2* and *Sk-3* are so rare. But it could also be the case that since *Sk-2* and *Sk-3* are located within a region of suppressed recombination, they can accumulate deleterious mutations that lowers their ability to drive or the general fitness of any individual that carries them.

Podospora anserina

The Spok genes

Podospora anserina is a filamentous ascomycete which grows on herbivore dung. Its primarily selfing life cycle depends on the ability to pack two haploid nuclei of opposite mating type into a single ascospore, and for this reason it normally produces four binucleate spores per ascus instead of eight uninucleate spores as is the case for the *Neurospora* species described above. The proper packaging of both mating types into one spore is ensured by an obligate crossover between the centromere and the mating type locus, which means that any gene showing first division segregation (i.e. segregating with its parental centromere) is homozygous in the spore and any gene showing second division segregation (i.e. is unlinked by recombination from its parental centromere) is heterozygous.

The first description of a fungal spore killer was done in *P. anserina*. When analysing two natural strains of *P. anserina* (T and T') a pattern of asci with either two or four living spores was observed and further analysis revealed that it was caused by two unlinked genes that independently could kill non-carrier spores (Padieu and Bernet, 1967; Turner and Perkins, 1991). Like in the experiments with spore mutants in *Neurospora*, the presence of a killer nucleus in a multinucleate spore will save all nuclei in *Podospora* as well, and the pattern of two and four-spored asci could therefore be explained by the fact that when the killer locus shows second division segregation, all spores are heterozygous for the killer element and survives, but when they show first division segregation, all spores are homozygous and only the spores that carry two copies of the killer locus will survive. This means that unlike the *Neurospora* spore killers, which all show close to 100% killing, the killing frequency of the *Podospora* spore killers depends on the genetic distance between the locus and the centromere (Padieu and Bernet, 1967).

Grognet et al. (2014) mapped a locus responsible for killing in the T strain by recurrent backcrosses to the standard lab strain S, always picking 2-spored asci. A gene close to the centromere of chromosome 5 that was unique to the T strain was identified and deleted. Instead of the expected loss of spore killing, the frequency of 2-spored asci went from 90% to 40%. A gene in the S strain showing 87% sequence identity to the T strain gene was identified and also deleted and by doing so all spore killing was eliminated. The two genes were named *spok1* and *spok2*. To summarize the results: *spok1* (the T strain gene) kills any strain that either carries *spok2* or no *spok* gene at all, while at the same time conferring resistance against killing from both *spok1* and *spok2*. *spok2* kills spores that do not carry any gene and confers resistance only from killing by *spok2*.

Tagging the C-terminus of the *spok1* protein with GFP produced only 4-spored asci, and tagging the N-terminus of *spok2* with mCherry lead to only empty asci. This suggests that the C-terminal end of the protein may be responsible for the killing function of the protein and the N-terminal end for the resistance, but truncating either end of spok1 led to complete loss of function, indicating that the functions may not be completely separable. Both in silico prediction and microscopy of GFP and mCherry tagged proteins indicate that the protein has a primarily nuclear localization.

A search for homologous genes in other species showed that such genes are commonly found among ascomycetes, often as many copies in a single genome. The gene tree does not follow known species trees, suggesting the possibility of widespread horizontal gene transfer. To test if *spok1* could show spore killing in a different species, it was introduced into *Sordaria macrospora*. A cross between a transformant *Sordaria* strain and a wildtype produced asci with four black and four white spores and all tested spores displayed the same phenotype in further crosses, in line with meiotic drive.

A *spok*-like gene from *Nectria haematococca* was also introduced into *P. anserina*. Crosses including this gene produced empty asci, suggesting the gene product can kill, but not confer resistance.

The presence of a spore killer gene in the standard strain S was highly surprising, since this strain has been thoroughly studied in the lab. A PCR screening for both *spok1* and *spok2* in 22 natural isolates of *P. anserina* revealed that *spok2* was found in most tested strains, whereas *spok1* could not be found. This result would explain why the spore killing activity of *spok2* has not previously been noticed, since most strains would be resistant.

In a screening of over a hundred natural isolates from the Netherlands and France, van der Gaag et al. (2000) identified seven new types of spore killers, defined by different killing percentage and genomic localization. Six of the seven types could be placed in a hierarchy where they showed either dominant epistasis (like T and S) or mutual resistance (though still being classified as different killers due to having different genomic localization). Two aspects of these results are especially interesting. One, that the hierarchy of killing interactions suggests that the different killer types are caused by *spok* genes, or genes that interact with *spok* genes in the same way, and two, that ~75% of all tested strains showed no killing with S, and were therefore classified as sensitive. This suggests that there may both be a large diversity of *spok* genes within *P. anserina* and that *spok2* is very common.

All of this points to the *spok* genes as being part of a highly successful family of meiotic drive elements, that can go to fixation within a species and continuously diversify to generate new, dominant driving alleles. Its peculiar mating system makes *P. anserina* an unusually difficult environment for genes like the *spoks* to invade, both because a high degree of selfing makes spreading in a population slower and because the heterokaryotic nature of *P. anserina* spores generates a certain protection that only first division segregation can overcome. This may be the reason why we find such high degree of polymorphisms within *P. anserine*; in an obligate outcrosser with uninucleate spores the *spok* genes may go to fixation so quickly that it is unlikely that we would be able to observe killing.

Het-s

A second meiotic drive system was actually described even before the *spoks* were discovered (Bernet, 1965), but again it was not initially understood to be an example of meiotic drive. Het-s is a locus harbouring two different alleles (*het-S* and *het-s*) conferring heterokaryon incompatibility, which is a self-nonself recognition system that prevents hyphal fusion between genetically different mycelia. *P. anserina* contains at least 10 different het loci, and two mycelia must be identical at all of them to be able to fuse. If not, cellular apoptosis will be initiated in the fusion zone and the two mycelia will be separated by a band of dead cells forming a so called “barrage” (Paoletti, 2016).

The *het-s* allele produces a prion that can form amyloid aggregates, similar to those found in the brains of Alzheimer's patients (Coustou et al., 1997). These aggregates are necessary for the heterokaryon compatibility process to occur, but they will also induce spore killing under certain circumstances (Dalstra et al., 2003, 2005). If a strain which carries both the *het-s* locus and the amyloid aggregates is fertilized by microconidia (i.e. male gametes) from a *het-S* strain, spore killing will occur, generating ~25% 2-spored asci, and the *het-s* will be overrepresented in the surviving spores. Spore killing will only happen when *het-s* is in the maternal role, as the amyloid aggregates are located in the cytoplasm, and the transfer of cytoplasm from microconidia is very limited. It is an interaction between the amyloid aggregates and the het-S allele that induces the killing, as no killing happens in a *het-S* knockout (Dalstra et al., 2003). The heterokaryon incompatibility system is generally turned off during sporogenesis, but this interaction may indicate that spore killing is induced by a reactivation of this system (Dalstra et al., 2005).

The *het-s* system is unique among known spore killers in two ways. It is dependent on the cytoplasm and show differential killing depending on maternal and paternal roles. It is also temperature dependent, and will only kill at 18 degrees C, and not at higher temperatures. Unlike *Sk-2* and *Sk-3*, but like the *spoks* and the *wtf* genes described below, it is a single gene.

The prevalence of *het-s* was investigated in the same Netherlands population of *P. anserina* as mentioned above (Debets et al., 2012), and amyloid aggregates was found in 66 out of 112 tested strains (59%). Since *het-s* is a heterokaryon incompatibility gene, it is expected to be under balancing selection (Zhao et al., 2015) and it is unclear if that is the main cause of the measured frequency or if it is the result of the meiotic drive activity.

Schizosaccharomyces pombe

Like *Saccharomyces cerevisiae*, the ascomycete yeast *S. pombe* is an important model organism for molecular and cell biology. It display low levels of fertility between natural isolates, despite being highly similar on a sequence level, with natural strains exhibiting less than 0.05% average sequence divergence. Some of these reproductive barriers can be explained by chromosomal rearrangements, such as inversions and reciprocal translocations, which are common in the species, but these can not be the whole explanation. On top of the effects of rearrangements a complex pattern of segregation distortion across the whole genome has also been observed (Zanders et al., 2014).

Recently, two groups identified several genes which actively cause segregation distortion when studying different natural isolates of *S. pombe* (Hu et al., 2017; Nuckolls et al., 2017). These genes all belong to the large, *S. pombe*-specific *wtf* gene family (*wtf* = with *Tf*, a transposable element). The active *wtf* genes consist of six exons and contain several transmembrane do-

mains. They act through a toxin-antitoxin mechanism, with a spore-limited antitoxin and toxin that can diffuse throughout the ascus. The toxin and antitoxin are produced by two different, but overlapping transcripts from the same gene. Antitoxin transcripts include the first exon, but the toxin is transcribed from an alternate start codon between exon 1 and 2 (Nuckolls et al., 2017). A deletion of the C-terminus if the gene disrupts the toxin, but not the antitoxin (Hu et al., 2017).

Since *wtf* genes contain transmembrane domains, Nuckolls et al. suggests that a plausible killing mechanism would be through disruption cell membrane integrity. This mode of action is also seen in several bacterial toxin-antitoxin systems (Lee and Lee, 2016; Unterholzner et al., 2013).

20-30 *wtf* genes were identified in each of the analyzed strains (Hu et al., 2017; Nuckolls et al., 2017), most, but not all, of which were shared between strains. Only a smaller subset of these (4-7 per strain) contained the second start codon, which generates the toxin and furthermore, several *wtf* genes appear to be pseudogenes. A phylogenetic analysis revealed that all *wtf* genes with the second start codon grouped into two clades each with a different start position.

wtf genes are often bounded by LTRs (long terminal repeats) and it was originally suggested that they could proliferate in the genome through LTR mediated transposition. That cannot be the case though, since transposition would remove all introns. Instead transposable elements may preferentially land near *wtf* genes, due to their high transcription rate at meiosis (Hu et al., 2017).

Copies of *wtf* genes that share the same genomic localization between strains often show highly elevated levels of sequence divergence compared to the rest of the genome, with 80-90% sequence similarity instead of the average >99.5%. Since *wtf* genes can reduce fertility, Nuckolls et al. suggests that there could be a selection on unlinked genes to evolve suppression mechanisms. This could then start a molecular arms race, where *wtf* genes and suppressors could coevolve and quickly generate high levels of sequence divergence. This mirrors the high evolutionary rates seen in genes that are involved in host-pathogen interactions (McLaughlin and Malik, 2017).

Nuckolls et al. also suggests a model for the evolution of *wtf* genes, based on the existence both of genes that produce a toxin and genes that do not. According to the model, the original function of a *wtf* gene is meiotic drive. If such a gene has gone to fixation, toxin activity should decay before antitoxin, and the gene would then only confer resistance to killing. Finally, when no active copies of the gene are left in the population, the antitoxin activity would also degenerate and only a pseudogene is left.

Fusarium

Spore killing has been observed in two closely related species of *Fusarium*, *F. verticillioides* and *F. subglutinans*, both of which are plant pathogens. When crossing natural isolates of *F. verticillioides* from southern Europe, the US and Central America to a sensitive tester strain, 80% showed a spore killing phenotype of asci with 4 living and 4 dead spores, 15% a sensitive phenotype of asci with 8 living spores and 5% a mixed phenotype, with both types of asci present (Kathariou and Spieth, 1982). The killer locus has been mapped to a 102 kbp region on chromosome 5, containing 44 genes, one of which is unique to the killer strain analyzed (Pyle et al., 2016). It is yet not known if the killing is dependent on one or several genes. As in the *Neurospora* spore killers, death of sensitive spores does not occur until after meiosis, following spore delimitation (Raju, 1994).

10 out of 15 *F. subglutinans* strains sampled from a single maize field showed spore killing in one experiment (Sidhu, 1984). The same three phenotypes (killer, sensitive and mixed) were observed, but it is not known if this killer system is the same as the one described in *F. verticillioides*.

Cochliobolus heterostrophus

C. heterostrophus is also a plant pathogen, and the highly virulent T strain is the cause of "southern corn leaf blight". When performing a genetic analysis of the T-toxin pathogenicity factor, segregation distortion of this gene was observed, together with non-random spore death (Bronson, 1988). This was shown to be caused both by a reciprocal translocation in strains carrying the T-toxin and by an unknown genetic element in the less virulent O race, which caused spore killing. 5 out of 11 analyzed O race strains were shown to carry the element and none of the T race strains. Spore killing happens after meiosis, and sensitive spores are not observed to degenerate until after a second round post-meiosis mitosis occurring after spore delimitation (Raju, 1994).

Studying genome evolution in fungi

It is important to investigate a broad diversity of taxa widely spread throughout the tree of life, to understand how genomes evolve, but when studying specific questions, choosing an organism that is tractable can be a good strategy. Fungi have a number of characteristics that make them attractive systems for studying genome biology and genome evolution. They have small, often haploid, genomes that makes it cost-effective to generate both high-quality genome assemblies – which are useful when studying genome architecture – and densely sampled population genomics datasets.

Certain species, such as *N. crassa* and *S. pombe*, have been developed into model systems, with rich sets of tools for lab manipulations and for genetic and molecular characterization. Fungi are also eukaryotes with complex cellular machineries, such as RNA interference, DNA methylation and histone modifications that show both similarities and differences to those of animals and plants. Finally, they are of course fascinating organisms in their own right, showing an immense diversity in lifestyle, habitat and metabolic capabilities.

The study of spore killers is an example of how the advantages of fungal biology can be utilized. In *Neurospora* and *Podospora*, spore killing can be easily phenotyped using dissecting microscopes. In these two species, as well as in *Schizosaccharomyces*, the function of genes involved in killing can be confirmed by deleting and inserting them in laboratory strains.

In this thesis I take advantage of the small genomes of fungi, their molecular tractability and of the extensive collections of natural isolates that are available. Among other things, I generate high-quality genome assemblies, which I use to study chromosomal rearrangements and patterns of repeat expansion; I generate population genomics data from a large number of strains, which I use to identify a gene responsible for killing; and I take advantage of earlier work on histone modifications (Jamieson et al., 2013; Lewis et al., 2009) to generate ChIP-seq data in order to study the distribution of heterochromatin. Some of these things may have been difficult to accomplish in other organisms. In particular I have, with the help of many colleagues, generated almost twenty complete, or near-complete, *de novo* genome assemblies of several different species of *Neurospora*, and such high quality assemblies may be unique for studying any multicellular organism.

Methods

I will here briefly describe a few key techniques and technologies that were used in the four papers of this thesis.

Whole genome sequencing has in recent years become the main source of scientific data for many evolutionary biologists. When sequencing an entire genome, the DNA is fragmented into smaller pieces known as "reads" (Ekblom and Wolf, 2014). This step is necessary because no current sequencing technology can reliably generate chromosome-length reads. In order to analyse these data, the sequenced fragments must be organised into larger structures, and that can generally be accomplished through one out of two different main strategies.

If no previous genomic data available from the sequenced organism, the first step is often to puzzle together all reads into larger chunks, representing parts of, or whole chromosomes. This strategy is referred to as generating a "de novo assembly" of a genome (Baker, 2012). Depending on the size and complexity of the genome and the length and number of sequenced reads, this can generate a near-complete genome assembly, with all chromosomes puzzled together into complete sequences, or it can result in a fragmented genome assembly, consisting of potentially thousands of pieces, known as "contigs". This method is well suited for studying small scale variation, for instance single nucleotide polymorphisms (SNPs), but larger scale differences, such as chromosomal rearrangements, can be more difficult to identify (Chaisson et al., 2015).

If a high-quality genome assembly of the organism already exists, and one would want to compare different individuals of that organism, all individual reads can instead be aligned (or "mapped") to the high-quality assembly and identify the sites in the genomes where the individuals differ from each other (Trapnell and Salzberg, 2009).

The dominating sequencing technology has, for several years, been the Illumina HiSeq platform (Bentley et al., 2008). It can cheaply generate large amounts of genomic data, but because of the short length of the reads that it produces (generally <150 bp) it can be difficult to generate high-quality *de novo* assemblies from only Illumina reads. Illumina sequencing is therefore often combined with other techniques, such as optical or genetic mapping, that can bridge shorter contigs together (Howe and Wood, 2015). Illumina data is well suited for mapping against a reference genome, and that has

allowed for many "population genomics" studies, where within-species genetic variation can be assessed (Luikart et al., 2003).

A newer method for generating genomic data is Pacific Bioscience's (PacBio) single molecule real-time (SMRT) sequencing technology (Eid et al., 2009). It is more expensive per base pair than Illumina, and it generates less data per sequencing run. However, it is significantly faster and can generate much longer reads (5000-50000 bp). Such long reads make it easier to assemble genomes into large contigs *de novo*, and for smaller genomes, it is actually possible to produce complete chromosomal assemblies from only PacBio data (Chin et al., 2013).

For this thesis, we used both the Illumina and PacBio sequencing platforms to generate genomic data. In order to study structural differences between genomes, we used PacBio sequencing to generate high-quality *de novo* assemblies of several *Neurospora* genomes. This technology was essential for identifying the inversions carried by spore killer strains in Paper I and for determining that chromosome 1 is collinear in most *N. tetrasperma* lineages in Paper IV.

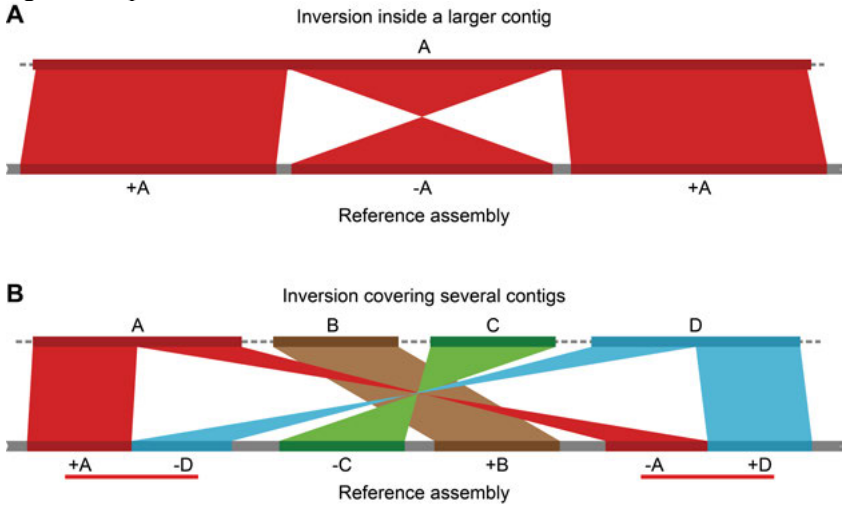

Figure 4. Schematic of method used for identifying inversions from fragmented *de novo* assemblies. Inversions can be identified by aligning two genome assemblies to each other, even if one of them is highly fragmented. **(A)** If an inversion is located entirety within a contig of one assembly, it is possible to identify it as a block that aligns in the opposite direction compared to the rest of the contig when aligned to the other assembly. **(B)** When inversions span several contigs, finding the exact size is more challenging, but if both inversion breakpoints are located within two different contigs, it will produce a characteristic alignment pattern at the breakpoints. In this figure, one assembly consists of several contigs (A-D) and the other is complete. When aligned to a complete assembly, contig A and D will be aligned next to each other at both inversion breakpoints (red underlined regions). Contig A will be aligned to the left of contig D at both breakpoints, and both contigs will have switched alignment direction (here shown with + or -) between the breakpoints.

We also generated Illumina HiSeq data from a large number of *Neurospora* strains. This data was, among other things, used to extract SNP data from over 50 *N. sitophila* strains, that could be used to identify the locus responsible for spore killing in *Sk-1* killer strains in Paper II. The identification was accomplished through a so-called genome-wide association study (GWAS), where the statistical association to the spore killer phenotype was calculated for each variable site. By looking for outliers I could identify a single locus that showed perfect association and that after molecular characterization could be shown to be the causative factor for spore killing.

While it is more difficult to study structural rearrangements from Illumina-only data, it is far from impossible. Methods where rearrangements are inferred from patterns of mapped reads are popular (Chen et al., 2009; Rausch et al., 2012), but in Paper IV I developed a method where fragmented *de novo* assemblies are screened from a type of rearrangements known as inversions. Identification of inversions is accomplished by aligning all contigs in the fragmented assembly to a reference assembly. If the genome of the fragmented assembly carries an inversion, a specific alignment pattern can appear at each inversion breakpoint (Figure 4), and this pattern can quite easily be identified.

Research Aims

The overall aim of my PhD was to study genome evolution in the ascomycete genus *Neurospora.* More specifically, I was interested in genomic conflict and recombination and how the interaction of these two phenomena affected population patterns and speciation in the ascomycete genus *Neurospora*. The main focus of my research was on the "spore killer" meiotic drive elements which can be found in two different species of this genus, and to use modern whole-genome sequencing technologies to study genome composition and structure, infer evolutionary histories, estimate population patterns and the mechanisms of speciation, and to identify the key genetic components the meiotic drive systems.

Specific aims of each paper

The aim of **Paper I** was to study, the evolutionary history, and genomic architecture of *Sk-2* and *Sk-3* spore killer strains in *N. intermedia*, by generating high-quality genomes of several *N. intermedia* strains which were either phenotyped as *Sk-2* or *Sk-3* spore killer strains, or sensitive to spore killing. The more specific purpose gathering this data was to characterize the differences of the nonrecombining region of the spore killer strains, compared to the sensitive strains and to each other, both in terms of genome structure, patterns of genetic diversity, repeat distribution and gene content. Furthermore, we wanted to determine the cause of the suppression of recombination in this region, and to investigate the evolutionary processes that underlie the potential differences of killer and sensitive strains.

With **Paper II** we wanted to **A)** identify the genes which are responsible for killing by *Sk-1* spore killers in *N. sitophila,* **B)** map population structure within *N. sitophila*, and **C)** assess how this population structure relates to the presence of spore killers. In order to do this, we used the extensive collections of *Sk-1* spore killer and sensitive strains of *N. sitophila* that was available in various strain collections by generating genomic data from several different populations, with different frequencies of *Sk-1* killers.

In **Paper III** we asked whether spore killing could affect reproductive isolation between different species of *Neurospora*. This hypothesis was based on

an observation made by Barbara Turner (Turner, 2001) that strains of the species *N. metzenbergii* showed extra high levels of reproductive isolation to strains of *N. intermedia* that carried spore killer elements, compared to *N. intermedia* strains that did not. Meiotic drive and other forms of genomic conflict have long been thought to increase reproductive isolation between diverging populations and, as a consequence, increase rates of speciation (Crespi and Nosil, 2013). We here set out to determine if the observation on reproductive isolation was correct and if we could elucidate the mechanisms behind this potential reproductive incompatibility.

Finally, the goal of **Paper IV** was to determine if the genomic rearrangements identified in the reference genome of *N. tetrasperma* (Ellison et al., 2011) were more widely shared within the species. If so, this would suggest that the rearrangements were central to the switch in mating system in *N. tetrasperma*. If not, this would instead indicate that other processes are important for maintaining both the differentiation seen on the mating type chromosome and for the switch in mating system.

Summaries of papers

Paper I: Spore killing and genomic structure in *N. intermedia*

Spore killers are a class of meiotic drive elements found in fungi, which causes sexual spores to kill their sibling spores, directly following meiosis. *Sk-2* and *Sk-3* are two different spore killer elements that were identified in *N. intermedia* over 40 years ago (Turner and Perkins, 1979). They have been mapped to a region on chromosome 3, where recombination between killer and sensitive strains is suppressed (from now on called the "sk region") (Campbell and Turner, 1987). A gene conferring resistance to spore killing was later identified on the left side of the sk region (Hammond et al., 2012) and a locus responsible for killing has been mapped to a 45 kbp region near the right edge (Harvey et al., 2014). The suppression of recombination therefore appears to be necessary to prevent these two loci from being separated by recombination, which would lead to self-killing. Little was known about the causes of the recombination suppression or how the sk region differs between both killers and sensitives, and between *Sk-2* and *Sk-3*.

In order to investigate this question, we generated high-quality genome assemblies of all known *Sk-2* and *Sk-3* strains, using PacBio SMRT long-read sequencing. A total of seven PacBio datasets was generated, most of which could be assembled into complete or near-complete chromosomes.

These genome assemblies revealed that all spore killer strains carried a dense cluster of non-overlapping tandem inversions, which covered the entire sk region. All four *Sk-2* strains shared gene order, but while the single *Sk-3* strain also carried inversions, it was a completely different set. These results indicate that *Sk-2* and *Sk-3* have established themselves as meiotic drive elements independently and may not be very closely related, and this is supported by a phylogenetic analysis based on SNPs from the sk region, which shows all *Sk-2* strains forming a monophyletic group and *Sk-3* being placed elsewhere within *N. intermedia*. Both *Sk-2* and *Sk-3* group within *N. intermedia*, which suggests that they have appeared after the species split, and also that they have not been introgressed from a different species.

The sk regions of both *Sk-2* and *Sk-3* were also enriched for transposable elements. We hypothesized that this could be due to less efficient genome defence mechanisms, such as RIP (Galagan and Selker, 2004), methylation and heterochromatin, but both bisulphite sequencing (to determine methyla-

tion patterns) and ChIPseq (to determine heterochromatin distribution) revealed normal patterns, consistent with previous results from *N. crassa* (Lewis et al., 2009; Selker et al., 2003).

Transcriptome sequencing was performed to identify novel genes within the sk region. A small number of unique genes were identified in both *Sk-2* and *Sk-3*, but no function that suggested involvement in spore killing could be inferred. These genes are prime candidates for further characterization using molecular techniques in future studies.

Patterns of genetic diversity in *N. intermedia* was investigated using SNPs generated from population level Illumina data. This revealed that the diversity of the sk region between all *Sk-2* strains was significantly less than in the rest of the genome and among sensitive strains that can recombine freely. The sk region also showed signs of molecular degeneration, in the shape of an elevated dn/ds ratio. This pattern is expected in regions of low recombination, where purifying selection is less efficient. This degeneration may also correspond to a reduced fitness of *Sk-2* strains, which could explain why they appear to be rare in nature.

In conclusion, this study thoroughly characterizes two different meiotic drive elements. The results which extreme effects the divergent selective forces of genomic conflicts can have on a genome.

Paper II: Population genomics of *N. sitophila* and the origin of the *Sk-1* killer element

Neurospora sitophila harbours *Sk-1*, the first spore killer element to be discovered in *Neurospora* (Turner and Perkins, 1979). Unlike *Sk-2* and *Sk-3* in *N. intermedia*, *Sk-1* is relatively common, with roughly 15% of all natural isolates showing the phenotype and certain geographical locations showing up to 100% frequency (Turner, 2001). Also unlike *Sk-2* and *Sk-3*, neither the genomic location nor the identity of any genetic components are known. While all three spore killer elements have a similar phenotype, it was not known whether *Sk-1* was at all related to the other killers.

We here gathered PacBio data from four strains, and Illumina data from over 50 strains of *N. sitophila*, sampling both *Sk-1* and sensitive strains. By comparing high-quality PacBio assemblies, we determined that *Sk-1* killer strains do not carry an inversion region on chromosome 3 or elsewhere in the genome. These results suggest that *Sk-1* is not related to either *Sk-2* and *Sk-3*, which is also confirmed by a phylogenetic analysis of the non-recombining.

The result also meant that we had no candidate region for the location of the killer locus, and we therefore used our population genomics dataset to perform a genome-wide association study for *Sk-1* spore killing. A single 5 kbp region on chromosome 6 showed perfect association with the phenotype

and a deletion mutant confirmed that this locus was responsible for killing and resistance. This makes *Sk-1* more similar to other spore killer systems found in *Podospora anserina* (Grognet et al., 2014) and *Schizosaccharomyces pombe* (Hu et al., 2017; Nuckolls et al., 2017) than to *Sk-2* and *Sk-3*.

The killer locus harbours a truncated copy of a gene that contains a methyltransferase domain, which is found at the same locus in sensitive *N. sitophila* strains and in *N. crassa* (where it is named NCU09865). We cannot yet determine if this fragment is necessary for killing, or if death is caused by a nearby sequence. A phylogenetic analysis of the killer locus and of two neighbouring genes suggests that they may have been introgressed from *N. hispaniola*, but further analysis is needed to confirm this.

An analysis of whole genome SNP data reveals signs of population structure within *N. sitophila*, with three major clades. One of these clades is fixed for *Sk-1*, and in another *Sk-1* is completely absent, this despite strains from both clades being sampled at the same site. In contrast, the third clade is polymorphic for spore killing and we do not yet have an explanation for these differences.

Paper III: Can spore killing drive speciation in *Neurospora*?

Selfish genetic elements like spore killers are expected to be able to increase reproductive barriers between population, which in turn can increase rates of speciation (Crespi and Nosil, 2013). While this phenomenon is predicted by theory, it can be difficult to study in nature there are only a small number of cases where genomic conflict appears to be strengthening reproductive isolation (Fishman and Saunders, 2008; Hu et al., 2017; McDermott and Noor, 2010; Nuckolls et al., 2017).

In a study on the geographic distribution of spore killers within *Neurospora*, Turner (2001) reported that *N. intermedia* strains from Mexico and New Zealand showed near-complete sterility when crossed to *N. intermedia* strains carrying either *Sk-2* or *Sk-3* spore killer elements. This pattern was in contrast with more normal levels of fertility to sensitive *N. intermedia* strains and to the 50% loss spore production that is expected from spore killer-sensitive crosses. The strains from Mexico were later reclassified as a new species, named *N. metzenbergii*, which was placed as a sister species to *N. intermedia* (Villalta et al., 2009).

We decided to more closely investigate the interactions between spore killers and *N. metzenbergii*. None of the *N. intermedia* strains from New Zealand had been included in the study that described *N. metzenbergii*, and we therefore wanted to determine if these strains were also *N. metzenbergii* or a different species. Four nuclear markers (Dettman et al., 2003) were se-

quenced from eleven New Zealand strains and a phylogenetic analysis based on these showed that all New Zealand strains grouped with *N. metzenbergii*, rather than *N. intermedia*. When New Zealand strains were crossed to both *N. metzenbergii* and *N. intermedia strains*, fertility was higher with other *N. metzenbergii*.

In order to determine incompatibility patterns spore killer specific incompatibility patterns, we crossed five *N. metzenbergii* strains from different localities to three sensitive *N. intermedia* strains, two *Sk-2* strains (one natural isolate and one that had been backcrossed with the normal Taiwan tester background). When crossed to sensitive *N. intermedia* strains, the crosses to the subclade NiA showed lower fertility than to other testers, indicating that variants causing reduced fertility is segregating within the species. When crossed to the two *Sk-2* strains, the wildtype *Sk-2* showed lower fertility than the *Sk-2* strain backcrossed to the Taiwan background, but in general both *Sk-2* strains showed severely reduced spore production compared to sensitive *N. intermedia* strains.

Different *N. metzenbergii* strains also displayed variation in reproductive output in different crosses, with *N. metzenbergii* strains from Madagascar showing zero spore production with any spore killer strain. The fertility of crosses was affected by incompatibilities manifesting themselves at different points during development. While crosses produced plenty of perithecia, consistent with low premating barriers, fertility was affected by the number of spores that matured, that germinated and that established viable mycelia, indicating complex patterns of molecular interactions behind the observed reproductive incompatibilities.

While direct interactions between the genes responsible for spore killing and specific loci in *N. metzenbergii* could be responsible for the observed reproductive barriers, a potentially more likely explanation is that the nonrecombining region of the spore killer strains has captured another genetic variant that causes inviability in a *N. metzenbergii* background. And since spore killing will force the inclusion of this variant in surviving spores, the number of surviving spores will be very low.

While there is no evidence for spore killing being involved in generating reproductive barriers that were important for the split between *N. intermedia* and *N. metzenbergii*, these results are a proof of concept that reproductive isolation can be driven by genomic conflicts.

Paper IV: Suppression of recombination in *N. tetrasperma*

Meiotic recombination is important for both maintaining and creating variation within sexual organisms, but it can sometimes also break up interacting combinations of genetic variants, which in turn could lower the fitness of a recombinant offspring. In such cases modifiers that locally reduce recombination rates, for instance chromosomal inversions, are expected to evolve. When measuring recombination rates in various species, regions of low recombination are often observed. One example of this is the selfing fungus *N. tetrasperma*, which ensures that self-compatible spores are produced by suppressing recombination between the centromere of chromosome 1 and the mating type locus (Raju and Perkins, 1994).

A study sequencing the two haploid genomes of one heterokaryotic strain of *N. tetrasperma* (Ellison et al., 2011) indicated that this suppression of recombination was maintained by a set of inversions, and we set out to determine if these inversions were a shared feature of the species. We went about this by generating eight high-quality genomes, using either optical mapping in combination with Illumina data, or PacBio long-read sequencing.

Surprisingly the genomes revealed that none of the sequenced strains carried the previously identified inversions. We also developed a method to screen fragmented de novo assemblies for inversions, which we used on a dataset of 92 *N. tetrasperma* genome assemblies (Corcoran et al., 2016). This revealed that only two lineages carried the previously identified inversions, and that while large inversions are enriched in the region of suppressed recombination, they are all derived.

From these results we concluded that another, non-structural, mechanism must maintain suppression of recombination in a region covering up to 86% of chromosome 1 in *N. tetrasperma*. To our knowledge, this is the first time suppression of recombination on such large scales have been observed in the absence of structural rearrangements in any species. We explain the observed enrichment of derived inversions in the region of suppressed recombination with the fact that inversions have fitness costs, since within-inversion crossovers can lead to the production of unbalanced, inviable gametes. If recombination was suppressed in a region by some other mechanism, these fitness costs would be reduced and inversions may accumulate.

We do not yet know the cause of the suppression of recombination, but we speculate that epigenetic mechanisms, such as chromatin modifications, may play a role.

Concluding remarks

About four years ago, just a few months after I started my PhD, I wrote a research application to the Royal Swedish Academy of Sciences, asking for money to sequence some spore killer genomes. I didn't get the money then, but in it I wrote what was basically the first research plan for the *Neurospora* spore killer project, and I listed a number of questions that we wanted to answer. These were:

- What are the key molecular actors in the spore killer process?
- How old is spore killing?
- What is the phylogenetic relationship between the different spore killer types?
- What role has the region of suppressed recombination played in the evolution of spore killer? Is it older or younger than the active genes?
- Are there remains of inactive spore killer genes, or spore killer genes that have gone to fixation within phenotypically non-killing species?
- Can spore killers have acted as drivers of speciation within the *Neurospora* genus?

Looking back at these questions, I am fairly pleased with what we have accomplished since then. We have identified a key molecular actor in *Sk-1*, and our American collaborators are on well on the way to identify the killer locus in *Sk-2*. When I wrote this list, I thought that we would find that *Sk-1*, *Sk-2* and *Sk-3* were all just different variants of the same system, but as it turns out, there is no one spore killer with a single, specific age and there may therefore not be a phylogenetic relationship between them. This possible independent origin of spore killers in *Neurospora* is actually an even more interesting finding though, pointing to the possibility of ubiquitous spore killing among fungi.

We have not addressed the question of the age of the spore killers, and the non-recombining regions in this thesis, but I have good hope that we will have something to say on this subject before these manuscripts are published in scientific journals.

While we have not found any spore killers fixed within a species, we have found them fixed within subclades, and the finding of a possibly ancestral copy of the *Sk-1* killer locus in a strain of *N. hispaniola* opens up the tantalizing possibility that *Sk-1* may have a long history outside of *N. sitophila*.

Finally, we may not be able to show that spore killing has driven speciation in *Neurospora*, but we have at least established that the presence of spore killers like *Sk-2* and *Sk-3* can strengthen reproductive isolation, and in the right conditions potentially lead to speciation.

All in all, we have made strong contributions to our understanding of meiotic drive in *Neurospora* and I hope that this can in the future lead to a greater understanding of the consequences of spore killing in particular and of meiotic drive in general.

It has also been very gratifying to show the existence of large-scale suppression of recombination in the absence of structural rearrangements in *N. tetrasperma*. This phenomenon is constantly mentioned as a possibility in the literature on recombination suppression, but it has never really been shown before. I am convinced that we soon will see other examples of this phenomenon, and that work on identifying the underlying molecular mechanisms will generate some very interesting results.

Svensk sammanfattning

Genetiska konflikter kan uppstå mellan olika delar av ett genom. Exempelvis kan spermier som bär en viss allel döda alla spermier in inte bär på allelen och på så sätt denna spridas i en population. Om allelen är en självisk könskromosom med en mutation som får den att döda den motsatta könskromosomen (exempelvis X dödar Y), kan detta leda till en skev könsfördelning och i slutänden utrotning av det ena könet och därmed hela arten. Här ligger den själviska genen i konflikt med resten av genomet, och eftersom en skev könsfördelning förväntas leda till ett selektionstryck för att producera avkomma av det underrepresenterade könet kan snabbt mekanismer som motverkar den självisk könskromosomen att uppstå.

Även konflikter med mindre på spel än artens överlevnad kan lämna spår i ett genom, och det är idag allmänt accepterat bland evolutionsbiologer att denna typ av konflikter är, precis som parasiter och patogener, ett reellt hot mot alla arter och att de har haft ett stort inflytande på kanske alla levande varelsers evolution.

Jag har i min forskning arbetat med en typ av självisk gener som kallas "spore killers", eller spordödare. Spordödare har hittills hittats i mindre antal arter bland askomyceterna (sporsäcksvampar) och de är en klass av gener som får svampsporer att förgifta sina syskonsporer. Genom att göra detta förväntas spordödare också att kunna öka i frekvens i en population, men vi vet fortfarande mycket lite om konsekvenserna av att sådana gener cirkulerar i en art.

Tre spordödare har identifierats i två olika arter av svampen *Neurospora*. Med hjälp av modern sekvenseringsteknik har vi samlat genomdata från över 100 individer som antingen bär på dessa gener, eller kan bli dödade av dem. Utifrån denna data har vi gjort ett flertal intressanta observationer.

I **Artikel I** har vi använt oss av en ny genomsekvenseringsteknik från Pacific Biosciences (PacBio) i syfte att generera genom av extra hög kvalitet, vilket är nödvändigt för att studera storskaliga genomstrukturer. I två olika sorters spordödare i svamparten *N. intermedia* har vi kunnat visa på stora förändringar i genomstruktur (så kallade inversioner), och vi har dessutom kunnat visa både på en stor expansion av repetitivt DNA och att den inverterade region har ackumulerat mutationer i kodande sekvenser, vilket tyder på en försämrad renande selektion.

Slutsatsen från dessa observationer är att den självviska aktiviteten hos dessa spordödare har lett till omfattande förändringar av genomet och att de tecken på genetisk degeneration som vi ser kan tyda på att just dessa spordödare har misslyckats med att spridas i arten och är på väg mot utrotning.

I **Artikel II** har vi fokuserat på en annan spordödare i arten *N. sitophila*. Här har vi identifierat en enskilt gen som är orsaken till spordödandet. Denna gen är inte släkt med de gener som orsakar spordödande i *N. intermedia*, men vi hittade en liknande gen i den närbesläktade arten *N. hispaniola* och tecken som tyder på att genen kan ha spridits över artgränsen och överförts från *N. hispaniola* till *N. sitophila*.

Vi kan också se att *N. sitophila* uppvisar populationsstruktur, det vill säga att alla individer inom arten inte verkar para sig fritt med varandra utan främst inom undergrupper. Denna populationsstruktur korrelerar svagt med geografisk struktur, men istället mer med närvaron av spordödare. Vi kan ännu inte dra några slutsatser om orsaken till detta mönster.

I **Artikel III** undersökte vi hur närvaron av spordödare kan öka reproduktiva barriärer mellan populationer och eventuellt leda till uppkomsten av nya arter. En tidigare studie hade observerat fullständig reproduktiv isolering mellan spordödare och två olika populationer av *N. intermedia*. En av dessa populationer omklassificerades senare till en ny art kallad *N. metzenbergii* och vi undersökte först om den andra populationen också tillhörde denna art, vilket visade sig vara fallet. Vi testade sedan observationen av reproduktiv isolering och vi kunde bekräfta också denna. Vi tolkar dessa resultat som att spordödare bär på någon eller några gener som orsakar sexuell inkompatibilitet med *N. metzenbergii*, och att även om vi inte kan visa spordödare i detta fall har lett till att *N. metzenbergii* och *N. intermedia* har delats upp i två arter, så skulle ett liknande system kunna göra detta under de rätta förhållandena.

Ett fenomen som observerats i en mängd olika organismer är stora regioner i genomet där meiotisk rekombination sällan eller aldrig förekommer. Vi vet inte mycket om orsakerna bakom dessa mönster, men en populär förklaring är strukturella förändringar inom genomet, till exempel inversioner.

I **Artikeln IV** studerar vi en sådan region i *N. tetrasperma*, vilken täcker större delen av den största kromosomen. Tidigare studier hittade flera inversioner i regionen, men genom att generera högkvalitativ genomdata från åtta olika isolat kan vi visa att de flesta isolat saknar inversioner och att det måste finnas orsaker till de låga rekombinationsnivåerna som inte beror på genomstrukturen. Vi kan också visa att det trots detta finns flera stora inversioner i denna region jämfört med resten av genomet, vilket tyder på att den okända mekanismen kan minska negativa effekter som inversioner ibland förväntas orsaka.

Detta är så vitt vi vet första gången man kan visa i någon organism att låga rekombinationsnivåer i stora genomregioner orsakas av något annat än strukturella förändringar.

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