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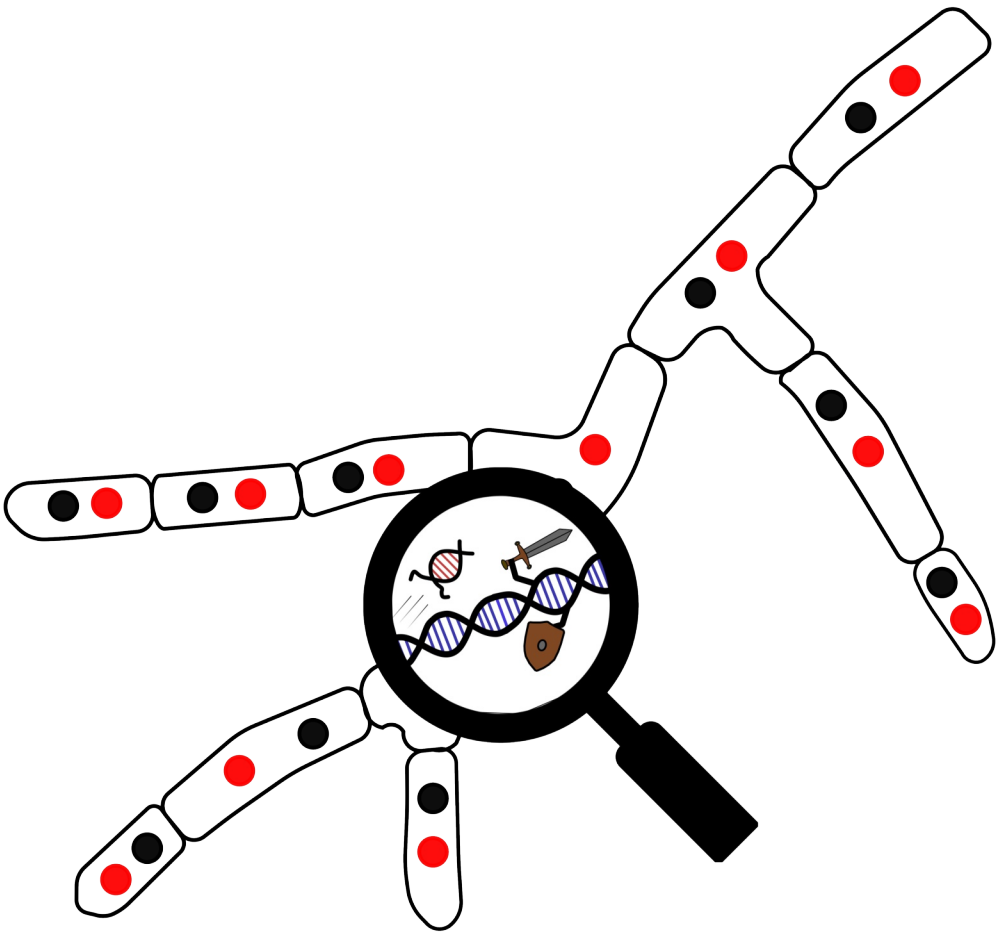
# The evolutionary interplay between transposable elements and genome defense in filamentous fungi

Ivar Westerberg



Doctoral Thesis in Ecology and Evolution at Stockholm University, Sweden 2025

# The evolutionary interplay between transposable elements and genome defense in filamentous fungi


## Ivar Westerberg


Academic dissertation for the Degree of Doctor of Philosophy in Ecology and Evolution at Stockholm University to be publicly defended on Friday 16 May 2025 at 13.00 in Vivi Täckholmsalen (Q-salen), NPQ-huset, Svante Arrhenius väg 20.


## Abstract

Transposable elements (TEs), so called "jumping-genes", are DNA sequences that are able to proliferate in the genomes of organisms. Their movement in the genome can be disruptive by inserting either into or near genes, but sometimes they provide beneficial mutational variation that natural selection, the ultimate force in evolution, can act upon. Despite their potential benefits for the organism, their overall movement is often thought to be at odds with the rest of the genome leading to them having been referred to as selfish. To counteract the negative effects of TE's movement, hosts have evolved defenses to control and prevent TE-related damage. In this thesis, I have studied TEs in fungi and their interaction with a fungal specific defense called repeat induced point mutation (RIP). RIP induces C-to-T mutations in any repeated region of the genome, including both TEs and duplicate genes. One of the key species where RIP has been described is the filamentous ascomycete *Podospora anserina*, which has been used as a model organism within genetics and evolution for over a century. In **chapter I** we were able to dive deeply into the interaction between a specific TE called *crapaud* and its evolutionary history, and discuss its potential interaction with RIP. In **chapter II** we discovered that RIP have been lost in a close relative to *P. anserina* called *Podospora pseudocomata* and that this loss may have been the cause of a total shift in both types and amount of TEs in its genome. This species also has smaller centromere regions than *P. anserina*. The centromere regions are the anchoring points when the chromosomes are pulled apart in every cell division. In many fungi the DNA of this region contains many TEs, and our result hints at a connection between the centromeres and RIP. *P.anserina* and *P. pseudocomata* are both part of an order known as Sordariales, which have species important to industry and contain model organisms such as *P. anserina* and *Neurospora crassa*. In **chapter III** we compared the genomes of nine families from this order using whole genomes and constructed a phylogeny of the order using phylogenomics. In **chapter IV** we developed in-depth methods to continue investigating RIP in the order Sordariales and discovered that *P. pseudocomata* is not the only species that has lost the RIP mutation pattern. We find 17 species lacking RIP, spread across the Sordariales phylogeny. In conclusion, this thesis presents a glimpse into the world of TEs and host genomes and their defense against TEs in filamentous ascomycetes, and the balance and conflict between them.



**Keywords:** *transposable elements, genome defense, filamentous fungi, centromeres, comparative genomics, evolution.*

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It doesn't stop being
magic just because you
know how it works.
- Terry Pratchett

# Abbreviations

TE - Transposable element
RNAi – RNA interference
RIP - Repeat Induced Point mutations
MSUD - Meiotic Silencing by unpaired DNA
MSA - Multiple Sequence Alignment
SSN - Sequence Similarity Network
LTR - Long Terminal Repeat
ChIP - Chromatin Immunoprecipitation
bp – base pair
kb – kilo-base pair

# List of Chapters

I. **Westerberg I,** Ament-Velásquez S.L, Vogan A.A, Johannesson H. (2024). Evolutionary dynamics of the LTR-retrotransposon *crapaud* in the *Podospora anserina* species complex and the interaction with repeat-induced point mutations. Mobile DNA 15, 1.

II. **Westerberg I,** Li M, Mercier E, Sandell L, Ament-Velásquez S.L,Vogan A.A, Grognet P, Malagnac F, Johannesson H, Reduced centromere size and shift in transposable element composition in the fungus *Podospora pseudocomata*: a link to the loss of genome defense? *Manuscript*

III. Hensen N, Bonometti L, **Westerberg I,** Onut-Brännström I, Guillou S, Cros-Aarteil S, Calhoun S, Haridas S, Kuo A, Mondo S, Pangilinan J, Riley R, LaButti K, Andreopoulos B, Lipzen A, Chen C, Yan M, Daum C, Ng V, Clum A, Steindorff A, Ohm R.A, Martin F, Silar P, Natvig D.O, Lalanne C, Gautier V, Ament-Velásquez S.L, Kruys Å, Hutchinson M.I, Powell A.J, Barry K, Miller A.N, Grigoriev I.V, Debuchy R, Gladieux P, Hiltunen Thorén M, Johannesson H. Genome-scale phylogeny and comparative genomics of the fungal order Sordariales. Molecular Phylogenetics and Evolution,Volume 189, 2023, 107938,ISSN 1055-7903.

IV. **Westerberg I**, Hensen N, Svedberg J, Vogan A.A, Slotte T, Johannesson H. Presence and absence of repeat-induced point mutations in the order Sordariales and its influence on genome evolution. *Manuscript*.

# Additional articles published during PhD

The following article was published during the time of my doctoral studies but is not included in the thesis.

Arnqvist G, **Westerberg I,** Galbraith J, Sayadi A, Scofield D.G, Olsen R-A, Immonen E, Bonath F, Ewels P, Suh A. A chromosome-level assembly of the seed beetle *Callosobruchus maculatus* genome with annotation of its repetitive elements (2024). G3: Genes, Genomes, Genetics. Volume 14, Issue 2

# Contributions

| | **I** | **II** | **III** | **IV** |
|---|---|---|---|---|
| Study design | LR | LR | No | LR |
| Sampling / data collection | No* | No* | No | No* |
| Wetlab experiments | n/a | Substantial | n/a | n/a |
| Bioinformatic analyses | LR | LR | Minor | LR |
| Interpretation of results | LR | LR | Minor | Substantial |
| First draft | LR | LR | No | LR |
| Manuscript review | LR | LR | Minor | Substantial |

*Data was available from previous studies

**Leading role (LR) =** Did a majority of the specified work

**Substantial =** Did a large part, but not a majority, of specified work

**Minor** = Did some of the specified work

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# 1 Introduction

There is a propensity to think of biological systems, whether they be organisms or individual cells, in the form of metaphors. When I was in middle school, the metaphor I clearly remember being taught is that DNA is being read like a blueprint to make proteins, which in turn are programmed to carry out precise tasks in the cell. Often these metaphors give a sense that biological systems are fine-tuned and hierarchical. Many metaphors exist in most biology subfields, metaphors like the blueprint metaphor of DNA, other metaphors of clocks, factories, machines, and scales, etc are abound in biological language. Not to say that the use of metaphors is bad (I use several widely used metaphors in this thesis), but some argue that it can come with problems (Taylor and Dewsbury, 2018). One potential risk, Taylor and Dewsbury argue, is that metaphors often miss the complexity of the concept they are trying to explain. In addition, I would further argue that metaphors, in particular, miss when biological systems do not function without fault or the patterns are not clearly hierarchical. In science, we often want to understand and explain natural phenomena in a structured way, and metaphors are a way of achieving this. However, biology is a "messy" science, where stochastic noise exists at every level (Tawfik, 2010). Anyone that has studied biology will be able to give examples where simple, structured explanations are difficult to make. To me, it is these complexities and the resulting quirkiness of biology that draw me in to pursue studying it. In this thesis I have studied the evolutionary genomics of filamentous ascomycete fungi. This is the largest phylum of fungi, and one that most people encounter as mold on their bread or cheese. However, many of the species that belong to this group are unseen to everyday human eyes, growing in a myriad of different ways and lifestyles. More specifically I study transposable elements, so called "jumping-genes", and their evolutionary interaction and conflict with the genomes they inhabit. Both fungal biology and transposable elements could in some ways be seen as "messy" biology. Fungi because they often do not follow the same life-cycles or life-histories as animals or plants, and thus, perhaps undeservedly, have been labeled as strange.

Transposable elements because their role in eukaryotic evolution has a long history of being underestimated and misunderstood (see further down) due to their complex and "messy" interaction with their hosts.With this thesis, I do not aim to provide answers to all the complexity held within the topics studied. But my hope is that I will be able to convey the importance and impact of the exciting world of transposable elements inside of fungal genomes, and to add to the broader knowledge on the topic.

## 1.1 Different ways to view evolution

At the heart of biological research lies evolution. Since Charles Darwin published his On the Origin of Species (Darwin, 1859) (and even before then) many researchers have dedicated their careers and lives to figure out why there is such organismal diversity on earth and how species can adapt to their environment. Individuals carrying beneficial variation in a trait important for adaptation will have a higher fitness and thus have a better chance to pass the variation on, which will over generations increase the number of individuals carrying the variation in the population. At first this variation could only be studied on a morphological level, and measuring the heritability of traits was elusive. But major paradigm shifts such as the Modern Synthesis of evolution during the early 20th century (Julian Huxley, 1942), where several researchers reconciled Darwin's theory of evolution with Gregor Mendel's studies on genetics, made it possible to study selection on Mendelian traits using genetics. With the discovery of the structure of DNA in the 1950s, and the 75 years of research since then, the field of evolutionary biology today uses molecular techniques and genome sequencing to study evolutionary forces and patterns on individual genes, chromosomes and entire genomes.

Natural selection has classically been thought to act primarily on an individual level, this is both true in Darwin's thinking and in the Modern Synthesis (Darwin, 1859; Julian Huxley, 1942). But the prerequisites for natural selection, which comprises phenotypic variability, differential fitness and heritability, can occur at other levels in the biological hierarchy (such as genes, nuclei, cells, or populations) (Lewontin, 1970; reviewed in Okasha, 2006). Selection at different levels can be harmonious with each other, but can also lead to conflicting selection, not only

within, but also between the levels. Okasha (2006) gives the example of cancer, where at the cellular level the cancerous cells reproduce faster and are selected for, but at the organismal level it is maladaptive. The extent and relevance of multi-level selection and conflict has been hotly debated within the field of evolution. Tied closely to levels of selection, the "gene's-eye-view" or "the selfish gene" theory, takes a leap to consider that the focal point of selection is not on individuals, but instead on the genes themselves. The ideas behind the gene's-eye-view has been developed during a long time by many prominent scientists, even as far back as the modern synthesis, but the main concept of the gene's-eye-view has mainly been attributed to Williams (1966) and Dawkins (1976) (Williams, 1966; Dawkins, 1976). The concept has become one of the most well known in evolutionary genetics due to Dawkins' highly popular book "The Selfish Gene" that was aimed at the public. Dawkins argued that all genes are selfish, and that individuals are merely vehicles ensuring that the genes are passed on (Dawkins, 1976) (note that gene in his definition is any piece of DNA that has an effect on phenotype and not the definition of gene used in molecular biology). This universality of the gene's-eye-view is up for debate (Okasha, 2006; Ågren, 2021), but there are some types of genes that have clear selective conflict with the rest of the genome (Burt and Trivers, 2009). Dawkins called these genes "ultra-selfish" genes, but today they are most commonly (and slightly confusingly) referred to as selfish genetic elements. Selfish genetic elements have ways to ensure their own propagation at higher rates than by Mendelian inheritance. This means that they may (but not necessarily always) come into conflict with the rest of the genome, to the detriment of the organism. Some examples include meiotic drive elements that manipulate their propagation in meiosis (Burt and Trivers, 2009; Lindholm et al., 2016), sex-linked genes can behave selfishly and come in conflict between the sexes (Burt and Trivers, 2009; Mank, 2017), and transposable elements that are able to copy themselves and therefore increase their own transmission by horizontal transmission (within one generation) rather than only vertical (between generations). Of these examples, the work in this thesis only considers transposable elements, which requires a deeper introduction. Note that there are many more examples of other selfish genetic elements not mentioned or discussed in my work.

## 1.2 Transposable Elements

### Types of Transposable elements

Transposable elements (TEs) are mobile DNA elements capable of proliferating in the genome. TEs were discovered in the 1940s and 50s by Barbara McClintock (McClintock, 1947, 1950), who is seen as the founder of the field. For a long time, the majority of TEs were viewed as "junk-DNA" of no relevance to the host organism. With more attention given to TEs, we now know that they have an impact on several aspects of the life of organisms and their evolution (Finnegan, 1989; Biémont and Vieira, 2006; Oliver and Greene, 2009; Ayarpadikannan and Kim, 2014; Bourque et al., 2018; Mat Razali et al., 2019; Schrader and Schmitz, 2019). TEs are widespread and present in nearly all eukaryotes (Wells and Feschotte, 2020). The proportion of TEs in eukaryotic genomes is highly varied, ranging between no TEs, like in some apicomplexan protists (Kissinger and DeBarry, 2011), up to 85% in maize (Schnable et al., 2009). Many of the proteins of TEs also have deep evolutionary origins, with some having evolved prior to the split between eukaryotes and prokaryotes (Wells and Feschotte, 2020). TEs have evolved into a variety of different structures and transposition mechanisms, and they are mainly divided into two classes (Finnegan, 1989). Class 1, also called retrotransposons or commonly referred to as "copy-and-paste", transpose by transcribing themselves into an RNA-intermediate before reverse-transcribing into the genome again, resulting in a new copy in addition to the original copy. Class 2, also called DNA-transposons or commonly referred to as "cut-and-paste", instead transpose directly from DNA to DNA, which can lead to copies increasing in number if the transposition occurs during the replication cycle.

Apart from the highest classification level, further classification is done in a hierarchical structure with the levels of subclass, order, superfamily, family, and sometimes subfamily. This widely used scheme of classification was proposed by Wicker et al in 2007. The subclass, order, and superfamily levels of classification are governed by the mode of transposition, major differences in the insertion mechanism, and organization of the protein domains and structural features, while the two lowest levels are determined by sequence similarity between copies (Figure 1) (Wicker et al., 2007). Subclass is only used for Class 2 as there are some Class 2 TEs, such as helitrons (also called rolling circle

elements) and Mavericks, that were found to not move through cleavage of both DNA strands, i.e "cut-and-paste", but instead through a "copy-and-paste" different from Class 1 (Wicker et al., 2007). Order is based on differences in mechanism of transposition and structure of the element. Superfamily on protein conservation and ordering within the element. Family is based on DNA-sequence similarity, generally by the 80-80-80 rule, which states that copies above 80 bp in length that share more than 80% homology over more than 80% of coverage belong to the same family. Subfamily classification has been used to classify evolutionary patterns within families based on phylogenetic data, or in some cases to distinguish autonomous (intact copies able to transpose) from non-autonomous (copies lacking some or all components necessary for transposition (Wicker et al., 2007).

In filamentous fungi, TE proportions are, like in other eukaryotes, highly varied. Ranging from less than one percent of the genome (Ohm et al., 2012; Paun and Kempken, 2015) to up to 58% in the Périgord black truffle (Tuber melanosporum) (Martin et al., 2010; Paun and Kempken, 2015). Despite this variation, fungal genomes are on average much smaller (~40Mbp) and carry fewer TEs (around 1-4%) than those of animals and plants (Paun and Kempken, 2015).

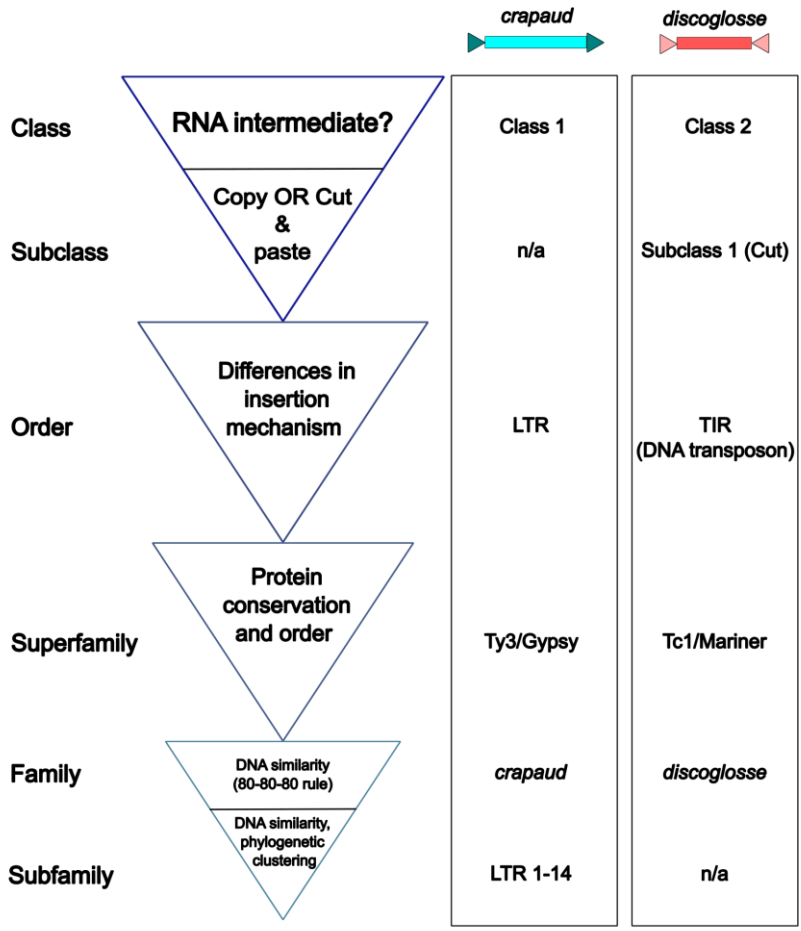


**Figure 1:** A visualization based on the hierarchical classification scheme from Wicker *et al* 2007 together with two example classifications of the *crapaud* and *discoglosse* TEs from the *Podospora anserina* species complex.

## Negative and positive impact of TEs on the host genome

By their nature as intragenomic mobile elements, TEs interact closely with the rest of the genome in a number of different ways. Most TE activity is thought to have negative fitness effects on the host, leading to intragenomic conflict. The most obviously negative effect TEs can have is that new insertions can disrupt or have regulatory effects on genes if they insert in or close to them, which

can cause disease. In fact, this was seen already in the maize studied by McClintock (McClintock, 1947), and has since been seen in many organisms (Bourque et al., 2018; Fueyo et al., 2022). In humans, TE insertions can cause a number of diseases, such as certain types of cancer (Ayarpadikannan and Kim, 2014). Another way in which TEs, including both active and remnants of TEs, can negatively impact genomes is by inducing genomic rearrangements through processes such as ectopic homologous recombination (Carvalho and Lupski, 2016).

All of the listed negative effects of TEs could in some circumstances have host-positive effects. Gene-regulatory effects or ectopic homologous recombination could have beneficial outcomes for the host, providing variation that is positively selected for (Schrader and Schmitz, 2019). TEs providing benefits can, over time, be co-opted or "domesticated" by the host, providing both important protein coding genes and non-coding RNAs (Jangam et al., 2017; Bourque et al., 2018; Fueyo et al., 2022). Genomic plasticity is a broader idea that has been proposed as a positive effect of TE activity. In this model, having some TEs within the genome could increase fitness by increased evolvability if the negative effects are tolerable (Oliver and Greene, 2009; Schrader and Schmitz, 2019) (Figure 2). The general presence and activity of TEs in a genome can increase an organism's evolvability and they can be key to adapting to new environments and lifestyles (Casacuberta and González, 2013). TEs can act as generators of variability through both gene regulation and TE-mediated rearrangements (Oliver and Greene, 2009; Chuong et al., 2017; Mat Razali et al., 2019; Schrader and Schmitz, 2019). Some even go as far as to say that "TEs are almost essential for significant continuing evolution to occur in most organisms" (Oliver and Greene, 2009). Another effect of TEs on genome evolution that is less clearly negative or positive is the relationship between TEs and genome size expansions in eukaryotes (Blommaert, 2020; Kidwell, 2002). Genome size evolution is itself a long researched conundrum, as it does not correlate with organismal complexity or effective population size (Gregory, 2001; Lynch and Conery, 2003; Gregory

and DeSalle, 2005; Blommaert, 2020; Marino et al., 2024). Furthermore, while some theory and experimental work has shown that increased genome size has negative fitness effects (Malerba et al., 2020), others have shown evidence that increased genome size can occasionally increase fitness and thus be selected for (Arnqvist et al., 2015; Boman and Arnqvist, 2023).

In filamentous fungi, many species have highly compartmentalized genome organization (i.e gene-dense parts and TE-dense parts). The TE-rich compartments can in some species be entire accessory chromosomes that can be transmitted between individuals of a population (Habig and Stukenbrock, 2020). These compartmentalized genomes have also been labeled as "two-speed" genomes where parts of the genome, carrying more TEs have a much higher rate of evolution than the core genome (Croll and McDonald, 2012; Raffaele and Kamoun, 2012; Dong et al., 2015). The "two-speed" genome model has been proposed as a way to increase evolvability, and has mainly been observed in plant pathogens that need to keep up with plant defenses. Another positive example of TEs in filamentous fungi is the recent findings and classifications of some of the largest TEs ever found in eukaryotes, called *Starships* (Gluck-Thaler et al., 2022; Gluck-Thaler and Vogan, 2024). *Starships* transpose through a tyrosine recombinase and have been found to be able to carry gene "cargo" as they transpose. Similar to accessory chromosomes (but at much greater evolutionary distances), *Starships* can horizontally transfer between genomes and carry with them genes important for adaptation (Gluck-Thaler et al., 2022; Urquhart et al., 2023; Gluck-Thaler and Vogan, 2024).

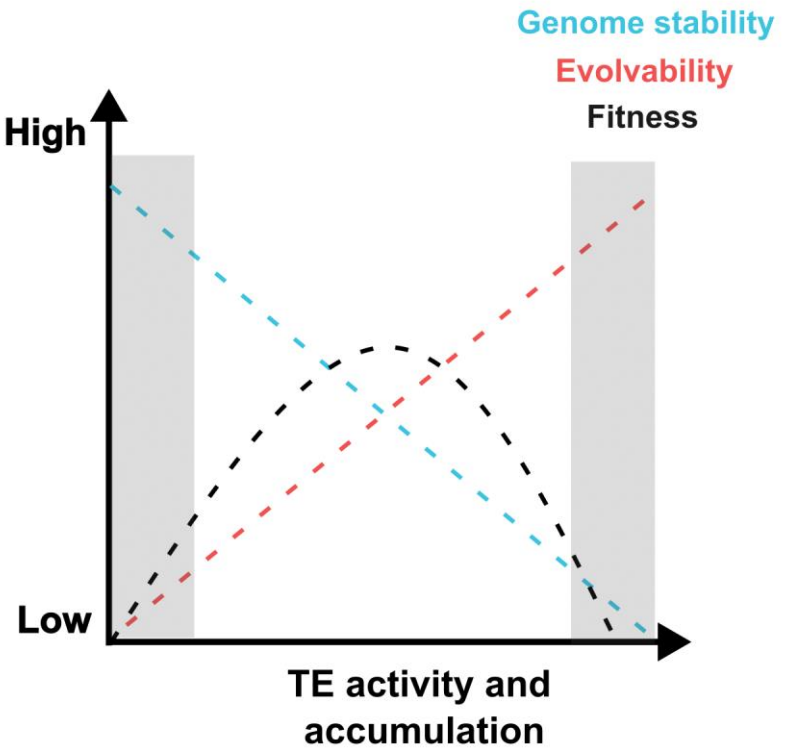


**Figure 2**: Hypothetical scenario based on Oliver and Greene (2009), where fitness (black) is a balance between maintaining genome integrity and potential for evolvability to adapt to environmental changes. Oliver and Greene (2009) argues that both extremes (grey areas) could lead to extinction of a lineage, and that natural selection should favour an intermediate level of TE activity/accumulation.

## Defense against Transposable Elements

As with other mutations, evolutionary processes such as natural selection and meiotic recombination during sexual reproduction can purge harmful mutations (which may include TEs) from the population. But to counteract negative impact, there have been several defense mechanisms that have evolved in eukaryotes to detect and stop TEs from proliferating. However, not all species carry specialized TE defense, so what governs whether TE suppression evolves in a population? Early population genetic modelling on TEs investigated under what conditions natural selection would favor the evolution of specialized defense. In large, free-recombining diploid populations, selection for specialized

defense would be low and unlikely to evolve (Charlesworth and Langley, 1986; Betancourt et al., 2024). But under certain conditions, the negative effects of TE insertion can be high enough for specialized defense to be favored by natural selection. These include scenarios where there is high linkage between the TE and a suppressor locus, for example when recombination is reduced, such as during asexual lifestyle or selfing (Charlesworth and Langley, 1986; Blumenstiel, 2011; Betancourt et al., 2024). But genome defense can also evolve when the genome size is small (as TE insertion has increased risk of inducing lethal or negative effects) or if the organism is haploid (as all mutations, not only dominant mutations,would be exposed and have effects on the host) (Charlesworth and Langley, 1986; Blumenstiel, 2011; Betancourt et al., 2024).

Some genome defenses are common to all eukaryotes and likely evolved early in eukaryotic evolution, others are more specific to certain lineages. Many repetitive regions, like TEs, are often epigenetically silenced, in the form of DNA methylation or histone heterochromatin modifications (Ikeda and Nishimura, 2015; Kabi and Filion, 2021; Di Stefano, 2022). In eukaryotic genomes different regions of the DNA are differentially packed, with tighter heterochromatic regions preventing expression and looser euchromatic regions allowing expression. These epigenetic regulations prevent TEs from expressing the necessary proteins for transposition. Another defense mechanism common to many eukaryotes is a series of protein pathways involving small interfering RNAs, called RNA interference (RNAi). While RNAi is evolutionarily conserved among eukaryotes, there are a wide variety of different pathways of RNAi with similar components but diverse functions (Aravin et al., 2007; Obbard et al., 2008; Lisch and Slotkin, 2011; Borges and Martienssen, 2015; Gladyshev, 2017; Nicolás and Garre, 2017; Carotti et al., 2023). Common to all pathways is the use of small RNAs to recognize expressed genes or TEs and then alter their expression by either cleaving transcribed RNA or by inducing methylation and heterochromatin formation (Obbard et al., 2008). Some, but not all, RNAi pathways are involved in defense against TEs and viruses, which is thought to be the reason why RNAi evolved (Obbard et al., 2008). Lastly, another mechanism to defend against TEs is to induce mutagenesis within TEs, which destroys the proteins necessary for transposition (Gladyshev, 2017).

Within fungi, several systems have evolved to defend against TEs, utilizing the mechanisms mentioned above. Some of these systems are homologous or analogous to systems found in animals or plants (Gladyshev, 2017). Furthermore, some are active in vegetative tissue and others during sexual reproduction. In somatic tissue the most well studied system is quelling, which was first found in *Neurospora crassa* (Romano and Macino, 1992). Quelling is an RNAi based pathway and it is also known as cosuppression in animals and plants. During meiosis, there are two additional systems for defense against TEs (both also originally described in *N. crassa*) that are thought to be complementary to each other (Gladyshev, 2017). The first one, meiotic silencing by unpaired DNA (MSUD) (Shiu et al., 2001), detects unpaired DNA and then triggers an RNAi response to silence that region. The second meiotic process is repeat induced point mutation (RIP), that induces C→T mutations in repetitive regions (Cambareri et al., 1991; Selker and Stevens, 1985). Related to RIP is another pathway, called methylation induced premeiotically (MIP), which instead of inducing mutagenesis induces DNA methylation (Goyon and Faugeron, 1989).

RIP functions through a methyltransferase gene, RID, and detects duplicated regions in the genome and then stochastically induces mutagenesis, specifically from cytosine to thymine, which has the extended effect of introducing stop-codons in coding regions. RIP has a size requirement that recognizes repeated sequences larger than ~400bp if they are close to each other and larger if they are far apart (Watters et al., 1999). However, weaker signals of RIP mutations have also been detected for shorter homology lengths, suggesting that the homology length requirements of RIP are more of a range than a clean cut off (Gladyshev and Kleckner, 2014). In addition, RIP also generally detects sequences sharing sequence similarity above 80% (Cambareri et al., 1991), although this similarity requirement could be lower if there are smaller homologous regions that are interspersed (Gladyshev and Kleckner, 2014, 2017).

RIP is unique to fungi, and while it has only been studied more thoroughly in some species, the signatures it leaves on repetitive regions have been detected throughout many filamentous fungi, both in Ascomycota and even in Basidiomycota (Clutterbuck, 2011; Meerupati et al., 2013; Hane et al., 2015; Amselem et al., 2015; Clutterbuck, 2017). However, in Basidiomycota it is likely a similar but functionally differ-

ent process (Clutterbuck, 2017). Interestingly, while found in many species, RIP has also been lost in some lineages, like within the class Leotiomycetes (Badet and Croll, 2025) and in *Sordaria macrospora*, a close relative to *N. crassa* (Le Chevanton et al., 1989; Walz and Kück, 1995; Nowrousian et al., 2010). Loss of RIP suggests that it is not essential in filamentous fungi and there are genomic conditions where selection against losing RIP is relaxed.

## The TE life-cycle and co-evolution of TE and host

Across eukaryotic evolution, TEs are widespread and frequently transfer horizontally between species (Schaack et al., 2010; Walsh et al., 2013; Gilbert and Feschotte, 2018; Blumenstiel, 2019; Betancourt et al., 2024). At the same time, it is known that some TEs have long standing evolutionary histories in single host organisms, where they maintain in and co-evolve with their host (Smit et al., 1995; Ågren and Wright, 2011; Sookdeo et al., 2018; Luo et al., 2020). Some of these long term interactions between host genome and transposable elements have often been compared to an arms race. Biological arms races have been discussed using the concept of the "Red Queen Hypothesis", which is a hypothesis of coevolution in predator and prey (or host/parasite) interactions. The original hypothesis was proposed by Van Valen in 1973 and states that any relevant mutation in either predator or prey would lead to a reactionary mutation in the other (Van Valen, 1977, 1973). This interaction leads to a stalemate where neither of the two can get ahead of the other but always keep "running" but not getting anywhere, just like the Red Queen said to Alice in Lewis Carrol's novel "Through the Looking Glass". While the original idea has since been taken a lot further, it is an interesting framework for thinking about coevolution. The framework of the Red Queen hypothesis is often used to discuss the coevolution of TEs and host defense, and there are several examples of arms race interactions. From a TE perspective, the evolution of both L1 and Alu retroelements in mammals has been thought to be related to evading host defense (Price et al., 2004; Smit et al., 1995; Sookdeo et al., 2018). These elements have, over recent evolutionary time, radiated into multiple subfamilies in a ladder-like manner that reflect several points of TE evasion after suppression by host defense. This pattern has been proposed to be typical of retrotransposons (Storer et al., 2021). In addition, the RNAi defense components, specifically small RNAs called piRNAs, have been observed to coevolve with TEs in fruit flies

(Luo et al., 2020). As mentioned previously, maintaining some TE activity could be beneficial to keep up the evolvability from the genome's perspective (Oliver and Greene, 2009; Schrader and Schmitz, 2019) (Figure 2). However, simulations of TE dynamics have shown that over evolutionary time, TE families are highly unlikely to persist for long within a genome (Le Rouzic et al., 2007). Another way for a TE to be maintained within a genome is through acquiring some selective benefit for the host (Oliver and Greene, 2009; Schrader and Schmitz, 2019). If the chance for copies that carry adaptive potential is high enough, they can be maintained within the genome for a long time according to simulations (Le Rouzic et al., 2007).

To reconcile the evidence for long-term evolution of TE and host with the evidence that TEs frequently horizontally transfer between hosts, there have been so-called TE life-cycle models that have been developed (Blumenstiel, 2011, 2019; Betancourt et al., 2024). In the models the life-cycle of a TE could 1) be initiated in a genome either by a horizontal transfer from a different genome or reactivation of a silenced TE (Figure 3). 2) As the TE invades the new genome, a high initial transposition rate (also referred to as burst) is needed to avoid being immediately lost by genetic drift (Figure 3). Additionally, the new host may not have general TE defense or specialized defense against the TE. 3) Over time as defense activates or evolves the transposition rate of the element would be reduced (Figure 3). 4) As the TE is active in the host, it could acquire some adaptive function for the host (co-option) or have ways to overcome the host defenses (which could initiate an arms-race), which would allow it to prolong its life in the host. 5) If the TE can not overcome TE defense, it is likely to be silenced and possibly go extinct unless it can escape either by future reactivation or horizontal transfer. Different TE types may have different strategies, with some staying for extended times in arms-race and others in a "live fast, die young strategy" where they continually transfer to new hosts to ensure long-term survival (Schaack et al., 2010; Blumenstiel, 2011; Gilbert and Feschotte, 2018; Blumenstiel, 2019; Betancourt et al., 2024).

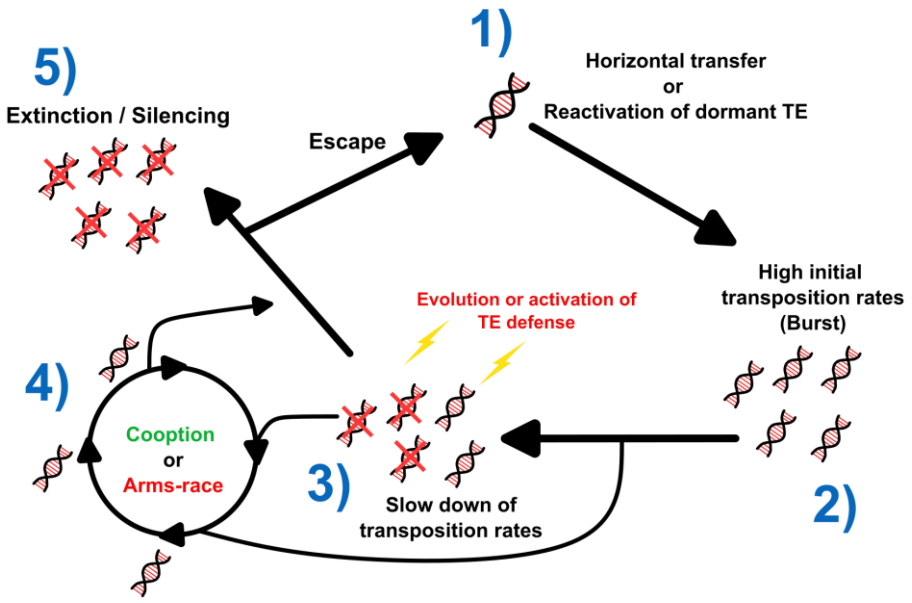


**Figure 3:** A model for the TE life-cycle in a host genome, inspired by Blumenstiel (2019) and Betancourt *et al* (2024).

Within fungi there has been evidence of both TE horizontal transfer (Rosewich and Kistler, 2000; Casacuberta and González, 2013; Gluck-Thaler et al., 2022; Bucknell and McDonald, 2023), co-option (Oggenfuss *et al*., 2024), and arms races in the form of TEs that evolve to escape RIP (Bhat and Kasbekar, 2001; Hood et al., 2005; Porquier et al., 2021). An example of potential combination of co-option and arms-race in fungi comes from Porquier et al. (2021) that described a family of LTR-retrotransposons in the plant pathogen Botrytis cinerea that express small RNAs involved in increasing pathogenicity by hijacking the RNAi based immune system of B. cinerea's host plants. Furthermore, these LTR-retrotansposons were classified in several subfamilies with different levels of RIP, and only the subfamilies with fewer RIP mutations produced the small RNAs involved in the increased pathogenicity. Porquier et al (2021) speculated that the subfamilies that had managed to escape RIP had done so due to positive selection related to the increased pathogenicity.

One can speculate how the model presented in Figure 3 relates to TEs in the genomes of filamentous ascomycetes. RIP as a defense universally recognizes any repeated sequences and therefore will target a TE burst quickly (given that sexual reproduction is frequent, which it may

not be in all lineages). A universal defense like RIP could be a way of preventing or mitigating the high initial activity of a horizontally transferred TE (step 1 and 2 of the model). Additionally, the mutagenic nature of RIP could increase the difficulty to reactivate after being silenced (step 5).

## 1.3 Centromeres - arenas for conflict?

Centromeres are important structural areas that act as the primary constriction site of a chromosome where the kinetochore binds (Cheeseman, 2014; Darlington, 1936; Flemming, 1882)), and they allow for the proper segregation of chromosomes in both mitosis and meiosis. Centromeres have special nucleosomes containing a centromere-specific histone H3 variant, cenH3, also known as CENP-A. These nucleosomes are essential to build the kinetochore in most eukaryotes (Talbert and Henikoff, 2020). cenH3 nucleosomes act as an epigenetic mark that attracts the kinetochore proteins to initiate segregation (Cheeseman, 2014). The first centromeres investigated were the 125 bp long point centromeres in *Saccharomyces cerevisiae* (Clarke and Carbon, 1980; Carbon and Clarke, 1984). In *S. cerevisiae*, the DNA sequences determine centromeric positions along the chromosomes as transforming the centromeric DNA onto a plasmid creates a functional artificial chromosome (Clarke and Carbon, 1980). In most other organisms, despite having a conserved function, the position of the centromeres are determined independent from the DNA-sequence (Cheeseman, 2014; Talbert and Henikoff, 2020). In eukaryotes, the centromere DNA varies drastically, from the small point centromeres of *S. cerevisiae* to megabase-scale, repeat-rich regional centromeres in animals and plants. This phenomenon has been described as “the centromere paradox”, as it has eluded researchers as to why the underlying DNA sequence is rapidly evolving, when the centromere function is conserved (Henikoff et al., 2001). In non-point centromeres, centromere DNA is often repetitive, either made up of specific repeats, TEs, or tandemly repeated sequences. TEs may be specific to centromeres and play a role in centromere evolution and function (Wong and Choo, 2004; Gao et al., 2015; Chang et al., 2019; Seidl et al., 2020; Hemmer et al., 2023; Shimada et al., 2024). Some LTR retrotransposons specifically target centromeric heterochromatin as insertion sites (Gao et al., 2008; Sultana et al., 2017; Hemmer et al., 2023; Tsukahara et al., 2025),

which further suggest the evolutionary relationship between TEs and centromeres. However, while TEs inside centromeres may promote centromere function, centromeres can also act as safe havens for TEs to avoid getting purged by negative selection (Gao et al., 2008; Lisch and Slotkin, 2011).

Much of the early research on centromeres was based on studies from model systems of ascomycetous yeasts such as *S. cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans* (Talbert and Henikoff, 2020). In filamentous fungi, centromeres are regional centromeres, where the underlying DNA varies in length from small regional centromeres, which are only a few kb long, to larger regional centromeres, which can be several hundred kb in size (Guin et al., 2020). The composition of the regional centromeres is often repetitive and longer (>20kb) than the small regional centromeres found in yeasts. TEs can be found in centromeres of both Ascomycetes and Basidiomycetes and have been studied in several fungi including *N. crassa*, *Magnaporthe oryzae*, *Cryptococcus neoformans* and the *Verticillium* genus (Cambareri et al., 1998; Yadav et al., 2018, 2019; Seidl et al., 2020). These TEs are often fragmented by RIP and silenced by RNAi mechanisms (Cambareri et al., 1991; Seidl et al., 2020). Centromeres have been observed to be linked to RNAi in *Cryptococcus* where loss of RNAi led to shrinkage of the centromeric regions and fragmentation of the TEs inside (Yadav et al., 2018). While not true for all TE-rich centromeres, studies have reported enrichment for specific TEs at centromeres both in shorter (*Cryptococcus*) and longer (*Verticillium*) regional centromeres (Yadav et al., 2018; Seidl et al., 2020).

## 1.4 Fungal Genomics

### Fungi - Masters of adaptation and genome control

The fungal kingdom is diverse, ranging from single-celled yeasts to the largest organism on earth (Ferguson et al., 2003). Many fungi are masters at adapting to their environment and are able to grow under a variety of different lifestyles and conditions. Fungi have been found living in some of the most extreme conditions, such as hot springs and arctic conditions (Coleine et al., 2022), and even in disinfectants such as formaldehyde (Yu et al., 2014). Many fungi are also effective pathogens,

able to infect animals, plants, and other fungi (Sun et al., 2020). One reason for the large variety of lifestyles and environments is the production of enzymes used for breaking down complex compounds, as well as the production of secondary metabolites that can have vast ecological functions (Bills and Gloer, 2016; Nagy et al., 2017).

From an evolutionary perspective, fungi have several interesting features that need to be considered when studying them. Many fungi are able to reproduce both sexually and clonally by producing asexual spores (Nieuwenhuis and James, 2016). Fungi, as far as we know, do not have early germline sequestration. This means that any cell has the potential to be involved in sex, and in extension any mutations of that cell is carried over to the next generation. The lack of early sequestration increases the possibility of multi-level selection and conflict (Okasha, 2006).

## Sordariales and the *Podospora anserina* species complex

The filamentous Ascomycete order of Sordariales is one of the most diverse fungal orders and houses many species with different life-styles. Several fungal model organisms, such as *Neurospora crassa* and *Podospora anserina* belong to the order, and they have had a great impact in understanding eukaryotic biology. As previously mentioned, most of the current knowledge on the process of RIP comes from these two species. The order also contains species producing a diverse set of secondary metabolites (Charria-Girón et al., 2022). There have been several efforts to sort out the phylogenetic relationships within the order and the topic has been the source of both debate and controversy (Huhndorf et al., 2004; Miller and Huhndorf, 2005; Kruys et al., 2015; Wang et al., 2019; Ament-Velásquez et al., 2020; Huang et al., 2021). There are currently nine families belonging to Sordariales of which three; *Sordariaceae*, *Chaetomiaceae*, and *Podosporaceae*, have the most isolated species and are the most well studied.

The aforementioned model species *P. anserina* is part of a species complex together with six other closely related species (Silar, 2020). *P. anserina* has a coprophilous lifestyle, which means that for the spores to germinate, they need to first pass through the digestive tract of grazing animals. After that, mycelia of the germinated spore grows out on the dung and undergoes sexual reproduction (Silar, 2020). *P. anserina* has

been a model organism for over a 100 years and has been studied for several molecular processes such as senescence and cell differentiation (Silar, 2020). In terms of research on selfish genetic elements, *Podospora* has also been extensively studied for gene drive in the form of spore killers (van der Gaag et al., 2000; Grognet et al., 2014; Vogan et al., 2019, 2021). In addition, *P. anserina* has been one of the key species for understanding the mechanism of RIP (Graïa et al., 2001; Bouhouche et al., 2004; Grognet et al., 2019). The seven species in the species complex are reproductively isolated and have been found in different regions all across the globe (Silar, 2020). Their genomes are otherwise highly syntenous, which indicates a recent divergence time (Vogan et al., 2019, 2021; Ament-Velásquez et al., 2024). Recently, high quality, telomere-to-telomere genome assemblies of the seven species were made available (Vogan et al., 2019, 2021; Ament-Velásquez et al., 2024). This species complex is thus ideal for studying evolutionary genomics over a short evolutionary time.

I would like to end the introduction, and the *P. anserina* section, with a small note on the nomenclature of TEs in *Podospora*. Biologists have a tendency of naming things in playful ways and nowhere is that more true than in the world of TEs. When the *P. anserina* reference genome was published together with the first complete TE annotation of the genome, the TE families of *P. anserina* were named after different names for frogs and toads, most of them in french. Names like *crapaud* and *grenouille* literally mean toad and frog respectively, whereas others are named after the taxonomic names of frogs, such as *discoglosse* is named after the *Discoglossus* genus (painted frogs).

# 2 Aims of the thesis

The overarching aim of this thesis was to develop and use filamentous ascomycetes of the order Sordariales to investigate TEs and their interaction with the genome defense RIP. In extension it has aimed to investigate and discuss the balance between evolvability and stability of these genomes. In the next paragraphs I will describe the specific aims of each chapter.

In **chapter I** we aimed to describe the evolution of a variable TE family of the LTR-retrotransposon superfamily called *crapaud* in the *P. anserina* species complex. Specifically, we wanted to describe the subfamily divergences of this TE and investigate the shared and species specific expansions and dynamics in the species complex. In extension we aimed to provide a general contribution to an improved understanding of how LTR-retrotransposons evolve in eukaryotes.

In **chapter II** we aimed to describe RIP patterns in the *P. anserina* species complex. In addition we wanted to localize and investigate the centromeres in the *P. anserina* species complex. Specifically, we wanted to comparatively describe the size and content of the centromeric regions in the species complex and understand how evolution has shaped these genomic regions.

The main aim of **chapter III** was to construct a robust phylogeny of the order Sordariales that can be used in comparative genomics and contribute to and help solve taxonomic discordances in the order. Additionally, we aimed to broadly describe key genomic properties such as evolutionary rate, genome size, and GC-content across the order and explore evolutionary dynamics related to lifestyle.

**Chapter IV** aimed to study the evolutionary dynamics and consequences of RIP across Sordariales. Most studies have either considered narrow sampling (one or a few related species) or broad sampling (across Ascomycota or all of the fungi). The Sordariales, in contrast, represent an intermediary range of species, where only a few species have been well studied and RIP status is largely unknown in the rest. With the data and phylogeny generated from **chapter III** we wanted to investigate RIP evolution over these intermediate evolutionary distances, within the order where it was first described.

# 3 Methods

## 3.1 Methods For Studying Evolutionary Patterns

One of the most powerful tools for understanding relationships of biological entities on different levels is the inference of phylogenetic trees. With an ever increasing amount of genome sequence data it has become one of the core methods for understanding evolution. At its core, a phylogeny is a tree of nodes that are connected by branches that are determined either by a set of characters or measurement of distances between the nodes (Yang and Rannala, 2012). For example, in a species tree, branching points represent speciation events. Phylogenies are inferred based on characters such as morphological characters or genomic sequences. The simplest phylogenetic method for inferring evolutionary relationships is maximum parsimony. This method assumes that the phylogeny with the fewest changes along its branches is the most likely to represent the true series of events during evolution. However, this assumption may not always be true and does not take any other information, like models of sequence evolution into account. As phylogenetic methods developed, several model-based approaches became more widely used than maximum parsimony. These methods, like maximum-likelihood and Bayesian inference, utilize information on sequence evolution stemming from models of population genetics to infer the phylogenetic relationships (Yang and Rannala, 2012). The maximum-likelihood method considers all the sequence characters in the tree to calculate a tree score based on the log-likelihood, where the score is the minimum number of changes for maximum parsimony (Yang and Rannala, 2012). Bayesian inference instead uses a prior distribution and then a posterior probability, which it infers by using a markov chain Monte Carlo algorithm. The models used by these two methods are substitution models of the DNA-sequences. The simplest substitution models assume equal mutation rates between all nucleotide base pairs. Whereas the most complex models are based on matrices with individual rates of mutations for all possible mutations. In modern tools for

inferring the phylogenetic relationships a model search algorithm is often implemented to find the best fitting model for the given data. Regardless of what method was used to generate the phylogeny, the resulting phylogeny is then considered as a hypothesis for how the true evolutionary events happened.

To test this hypothesis and gauge its accuracy, there are several methods for generating statistical branch support values. One of the most common ways to do this is to perform a bootstrap analysis. A bootstrap test is a statistical method to resample the data with replacement. In phylogenetics, it is used to evaluate how many of the resampled tree topologies resulting from the bootstrap contain a given node in the phylogeny. The bootstrap values at each node thus give a measure of confidence for each node (Efron, 1979; Felsenstein, 1985). The downside of this method has been that it is computationally demanding, a problem which has in the last ten years been solved through ultra-fast bootstrapping (Minh et al., 2013; Hoang et al., 2018).

In **chapters I and II**, we utilized maximum-likelihood phylogeny approaches to investigate the evolutionary relationships of TEs. We generated multiple sequence alignments (MSAs) of the TE copies using the tool MAFFT (Katoh and Frith, 2012), and then calculated the maximum-likelihood phylogeny using the software IQtree (Minh et al., 2020), which also has built in functions for evolutionary model testing and bootstrap support generation. In **chapter III** we also used a maximum-likelihood approach to estimate the species phylogeny of the Sordariales order. This was done using a phylogenomic approach, i.e an approach based on whole genomes (Patané et al., 2018), rather than a phylogeny based on fewer genes that has been done previously for the order. Within phylogenomics, there are a number of considerations to be made, but one typically identifies orthologs of highly conserved genes throughout the genomes of the taxa. Then either do a coalescent-based or concatenation-based phylogeny of those genes. In a coalescent-based phylogeny, each orthogroup is aligned to create single gene phylogenies. The many gene phylogenies can then be used to find the consensus phylogeny for which most single gene phylogenies agree. Contrastingly, in the concatenation-based phylogeny, which is what we used for the main phylogeny in **chapter III,** the genes are instead concatenated and analyzed as one super-matrix (Patané et al., 2018).

In some cases the evolutionary relationships are too complex to be described by a regular phylogeny (Huson and Bryant, 2006), for example if there are evolutionary events that would give rise to reticulated patterns, such as hybrid speciation, introgression, or horizontal gene transfer (Morrison, 2014). In these cases a reticulated network needs to be implemented to accurately determine the evolutionary relationships (Huson and Bryant, 2006; Morrison, 2014). The usage of networks to understand biological phenomena is not new as it has been widely used to infer gene regulatory pathways (Vijesh et al., 2013). One strength of using a network structure for inference is that one can utilize a long history of graph theory knowledge to analyze and find structures within the network (Clauset et al., 2004; Hagberg et al., 2008; Ronqui and Travieso, 2015). In **chapter I**, we implemented a network analysis based on sequence similarity of the variable LTR region of the *crapaud* element to disentangle its evolution.

## 3.2 Classification of Transposable Elements

Transposable element classification is based on hierarchical classification schemes (Figure 1), similar to how species are taxonomically classified. To arrive at such a classification for any particular set of TE copies there are several steps that are typically done to find and collect all representative sequences of all TEs in a species, a so-called repeat library. The first step is the usage of an automated repeat detection algorithm that identifies and classifies any repeated sequence in a genome based on known elements from databases and its structure. Using an automated identification method is usually enough for estimating overall repetitive content and higher classification in a genome through masking of the repeats (Smit et al., 1996; Goubert et al., 2022). However, the classification is usually not enough for detailed study of individual TE families and their evolutionary histories and relationships (Goubert et al., 2022). Because of this, repeat libraries generated by the automated softwares are usually manually curated to improve the accuracy of the classification (Goubert et al., 2022). Lately there have been several pipelines to incorporate curation into the automated library generation (Baril et al., 2024; Hu et al., 2024). These pipelines include iterative steps that incorporate MSAs of the TE copies to improve upon the initial classification. By applying these steps the pipeline is removing redundancy and generates improved consensus-sequences.

One of the most widely used softwares for TE identification and classification is RepeatModeler (Flynn et al., 2020). In addition, some curation pipelines implement both RepeatModeler and other tools for the repeat identification, such as the software EarlGrey (Baril et al., 2024). In **chapter III** we used RepeatModeler to classify the TEs of the Sordariales order, as we mainly sought to investigate overall TE patterns we deemed it to be enough with only RepeatModeler. In **chapter IV** we used the pipeline EarlGrey to reclassify the repeat libraries since in this study we wanted to investigate patterns of TE order and superfamily evolution.

## 3.3 Estimating the presence and effect of repeat induced point mutations (RIP)

A challenge when studying the interplay between TEs and genome defense is how to accurately measure the extent of RIP and if it is active in a given species. In both *N. crassa* and *P. anserina*, RIP primarily targets CpA dinucleotides (Clutterbuck, 2011). However, in other species this context may vary, for example in species belonging to *Chaetomiaceae*, also within the Sordariales, both CpA and CpG are favoured (Clutterbuck, 2011). To estimate the effect of RIP in a genome, methods to calculate "RIP-indicies" have been developed (Hane and Oliver, 2008; van Wyk et al., 2019). These are based on ratios of dinucleotide frequencies of nucleotides in the provided sequence, which can be single repeats or entire genomes. The three indicies are the Product, the Substrate and the Composite index. (See equations below, reproduced from van Wyk et al 2019):

Product index: $\frac{TpA}{ApT} : x > 1.1$

Substrate index: $\frac{CpA + TpG}{ApC + GpT} : 0.9 > x$

Composite index: $\frac{TpA}{ApT} - \frac{CpA + TpG}{ApC + GpT} : x > 0$

If all the calculated index values (x) are above or below the indicated values, the sequence is considered to be affected by RIP (Hane and

Oliver, 2008; van Wyk et al., 2019). To get the RIP affected level of an entire genome one can calculate RIP in sliding windows across the entire genome, and then divide the number of RIP affected windows by the total number of windows (van Wyk et al., 2019).

Another way to indirectly estimate if a given species is RIP-affected is to calculate the GC-content across the whole genome. A telltale sign that RIP mutations have happened is that the content of GC will be lower in repeated regions compared to the average GC-content of the genome (Testa et al., 2016). The overall GC-content of the genome will then, if RIP is strong enough, have a bimodal distribution. However, while this is true for some species the picture seems to be more complex in others (Amselem et al., 2015). Another difficulty with using this indirect estimation is that TEs may inherently have a different GC-content or there may be other factors affecting GC-content that differ between TEs and other parts of the genome.

In **chapter III** we investigated RIP across the order Sordariales by using both RIP-indicies calculated from the software TheRIPper (van Wyk et al., 2019), as well as investigating the relationship between GC-content and whole genome RIP proportions. In **chapter IV** we expanded upon this by analyzing RIP-indexes, GC-content, and dinucleotide frequencies across the whole genome, and in repetitive and non-repetitive regions.

## 3.4 Identifying Centromeres

Since most centromeres are not determined by any specific DNA motifs, the centromeric DNA sequences may be poorly conserved between the same centromeres of closely related species. The standard method to determine centromere locations is to identify the location of the cenH3 DNA binding sites. This can be done using a number of different methods but the most commonly used method is to perform chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-Seq) (Wiehle and Breiling, 2016). This widely used method can identify the DNA binding site of a protein, in our case the cenH3 protein. It does this through multiple steps: first the protein bound DNA is cross-linked, which captures a snap-shot of how the protein and DNA are bound. Secondly, the chromatin is extracted and sheared into

numerous pieces, to which an antibody is added that will bind specifically to the protein of interest. Then magnetic beads are added that bind to the antibodies with the protein and DNA of interest, and can be precipitated with a magnet. Finally the beads, the protein, and the DNA are separated from each other. The resulting DNA can be sequenced and compared with a negative control to find enrichment for the protein binding sites. Unlike regular H3 histones, cenH3 is less conserved between species. This means that ChIP-Seq experiments on cenH3 are often done using a transformed strain where a fluorescent tagged cenH3 has been incorporated. Instead of using an antibody targeting cenH3, an antibody to the fluorescent protein is used instead.

Once the ChIP product has been produced it can be sequenced with paired-end Illumina sequencing. Typically, sequencing is done for both the immunoprecipitated DNA sample (the sample where the specific tagged cenH3 is enriched), the input (DNA before the antibody was used to pull down the specifically bound DNA), and a mock sample. The sequences of each of the three are then trimmed and mapped to the reference genome assembly. Once mapped, enrichment peaks can be identified inside the immunoprecipitated sample by comparing it with the input and mock.

In **chapter II** we utilized this method to localize the centromeres of the *P. anserina* species complex. We specifically performed the ChIP experiment on two species, *P. anserina* and *P. pseudocomata*.

# 4 Summary of the chapters

## 4.1 TE activity in the *P. anserina* species complex

In **chapters I and II** we investigated the TEs inside the *P. anserina* species complex. **Chapter I** characterized an LTR retrotransposon family called *crapaud* in the *Podospora anserina* species complex.We showed that *crapaud* was the most abundant in almost all species and that it varied structurally among its copies, specifically in a structural feature known as the long terminal repeats (confusingly also referred to as LTRs) that sit on each end of the repeat. We thoroughly classified the *crapaud* family into 14 subfamilies based on this variation using a combination of a sequence similarity network (SSN) and maximum likelihood phylogenetics. We found that the *crapaud* subfamilies may have formed through recombination between the terminal repeats. This is similar to the TY-elements in *S. cerevisiae*, which are some of the earliest and best studied LTR-retrotransposons (Jordan and McDonald, 1998; I King Jordan and McDonald, 1999; I. King Jordan and McDonald, 1999), but which to our knowledge has not been described in other organisms. The part of the LTR that is variable is also where the element's promoter and enhancers are located, which led us to speculate on the role this has had in the evolution of the subfamilies. From a broader perspective we found that the *crapaud* subfamilies had evolved already in the ancestor of the species complex and had had continuous activity throughout the diversification of the seven species. This stood in contrast to other copy-rich TEs (*discoglosse* and *grenouille*) that had more explosive expansions at distinct points throughout the species complex evolution. Moreover, one can speculate whether these two transposition patterns are related to the presence of the defense RIP, as *discoglosse* and other TEs, but not *crapaud*, have expanded and remained in *P. pseudocomata* after the loss of RIP signature (**chapter I & II**). Other studies have suggested that TEs mainly survive through larger "burst" like expansions and have trouble persisting in a genome over longer time frames without horizontally transferring to other genomes (Le Rouzic et al.,

2007). The *crapaud* TE and its variable LTRs may be a strategy that have allowed it to persist, at least for as long as the *P. anserina* species complex has diversified, which admittedly is likely not that long based on their short (but unknown) divergence time (Ament-Velásquez et al., 2024). In **chapter II** we found that one species in the *P. anserina* species complex, *P. pseudocomata*, lacked the typical patterns expected by RIP mutations. We sought to investigate the impact of this assumed loss of RIP by investigating the repeat rich centromeric regions. By mapping the centromeric histone variant, cenH3, we successfully mapped the centromeres in this species and by extension were able to map this over to the rest of the species´ genomes. We found that the centromeres in *P. pseudocomata* were smaller and had a different TE composition. The *crapaud* family was the most abundant inside the centromeres of all species except *P. pseudocomata*, similar to the whole genome pattern found in **chapter I**. The DNA-transposon, *discoglosse* had instead had multiple expansions in the genome of *P. pseudocomata*, both inside and outside of the centromeric regions. Lack of RIP in this species and the drastic changes it has undergone, likely as an effect of loss of RIP mutation patterns, hint at the importance RIP has in shaping genome evolution.

## 4.2 Genomic properties across Sordariales

In **chapters III and IV** we studied the broader order of Sordariales. This order contains both families with important model species, such as *Podosporaceae* and *Sordariaceae*, of which *P. anserina* and *N. crassa* are part of respectively. But also the industrially important *Chaetomiaceae*, which contains the largest known number of thermophilic species of fungi.

In **chapter III**, led by fellow PhD-student Noah Hensen, we created an extensive phylogeny of the order Sordariales based on whole genome data. In the study we generated 59 new genomes, as a part of the JGI 1000 fungal genome project. These genomes were used together with already available genomes to generate a high support whole-genome phylogeny. The order of Sordariales has been controversial taxonomically, partly because previous order-wide phylogenies had markers with poor discriminatory power in Sordariales compared to

other orders. In our phylogeny we implemented a maximum-likelihood approach on high quality BUSCO genes. This was in contrast to previous phylogenies based on few marker genes. The result was a highly supported phylogeny, which resolved several of the previously ambiguous nodes. In addition we compared the genomes across the order to and identified large differences in genomic properties between the different families. We found large differences between the families of *Sordariaceae*, *Podosporaceae*, and *Chaetomiaceae*, with *Sordariaceae* having larger genomes, more repeats, and higher levels of RIP across their genomes. *Chaetomiaceae* instead had the smallest genome sizes, low repeat levels, and the highest GC-content. We speculated whether this may be because of the thermophilic lifestyle of several *Chaetomiaceae* species, which could lead to selection for genome reduction and codon-optimization. In **chapter IV** we continued investigating the genomic patterns of RIP across Sordariales and the relationship with TEs using a slightly updated dataset from **chapter III**, which included 81 genomes. We found that the differences in TE content across Sordariales was mainly contributed to by variations in retrotransposon content.

## 4.3 Loss of RIP signal across Sordariales

Perhaps one of the most important findings of the thesis is that our evidence points to several losses of the mutation pattern attributed to RIP in multiple lineages across Sordariales (**chapter II & chapter IV**). We conducted a thorough survey of RIP, GC-content and dinucleotide patterns across genomic, non-repetitive, and repetitive windows (**chapter IV**). Our survey showed that the signal of RIP has seemingly been lost in 17 of the 81 species investigated, spread across the phylogeny. It was previously known that RIP signal has been lost in *S. macrospora*, a close relative to *N. crassa*, despite having an intact RID gene (Le Chevanton et al., 1989; Walz and Kück, 1995; Nowrousian et al., 2010). These losses do not reach deep in the Sordariales phylogeny, and all are either unique in the taxa or shared between clades of two species. This may suggest that losing RIP infers fitness costs that impedes long term viability and diversification in Sordariales.

Loss of RIP signal can have multiple explanations: 1) The gene function of key RIP genes, such as *rid-1*, has been lost in these lineages, 2) the lineage may have rare or no sexual reproduction, which is where RIP is triggered, or 3) RIP may be active but the genome has few or no TEs and in extension no RIP mutations. Previous evidence from *N. crassa*, where recent gene duplications are absent, suggests that a major trade-off of RIP is loss of the potential for evolutionary innovation by generation of gene duplications (Galagan and Selker, 2004). We found that there indeed were an increased number of paralogous genes (resulting from duplication events) in RIP-signal absent species. However, we also found that *N. crassa* and its relatives were unique in their total lack of gene duplications. Other RIP proficient species had higher amounts of paralogous genes, suggesting that the relationship between RIP and generation of gene paralogs may be more complex than previously thought. We also found that TEs abundances were not larger in the species lacking RIP-signal, suggesting that loss of RIP signal may be explained by relaxed selection for maintaining RIP when TE load is low.

# 5 Concluding Remarks and Future Perspectives

The chapters presented in this thesis contribute to the knowledge of TEs and the study on genomic conflict between TE and host in a number of different ways:

One of the aims was to classify and describe evolution of the repeats in the *P. anserina* species complex. I think we have succeeded in doing this to a great level of detail in **chapters I and II**. In particular, we have described the subfamily evolution of the *crapaud* LTR family and we described the dynamics of different TEs in *P. pseudocomata*, where there has been loss of RIP signal. However, there are some avenues left to investigate. First, future studies could reveal more about how common the variable LTRs like in *crapaud* are (**chapter I**) and how this variation has been involved in the evolution of these elements. Second, I think elucidating the relationship between RIP and centromere size and contents in *P. pseudocomata* further would be important. In **chapter II** we ended up mainly describing the evolutionary patterns, but I think further molecular studies are needed to fully establish how RIP is linked to the centromeres in the species complex.

A key contribution of **chapter III** was to provide a resource of genomes as part of the 1000 fungal genomes project. This data has already been used in several published studies since its publication in 2023 and contributes to a large portion of the genomes in **chapter IV**. In addition, Sordariales has had taxonomic disputes, in which renaming several genera has been proposed. By generating a robust phylogeny of the order these discussions can have a stronger basis in the evolutionary relationships of the species. Today's technology and resources have allowed a much better sampling of the order than previous attempts to describe the relationships and comparative genomics of the order. However, there is still a lot of room to improve future sampling and analysis of this important order. In **chapter III** we

mainly focused on describing the three orders *Sordariaceae*, *Podosporaceae*, and *Chaetomiaceae* and saw large genomic differences between the three. In addition, we included genomes from six more families but only with a number of genomes ranging from 1-7 in each family (compared to 17, 20, and 41 in *Chaetomiaceae*, *Podosporaceae*, and *Sordariaceae* respectively). This means that there is a future opportunity to build on the result found in this chapter, especially in the under-sampled families.

The findings of several RIP signal losses in Sordariales (**chapter IV**) are intriguing from an evolutionary standpoint. Going back to the introduction, I think that Sordariales is a very good system for further investigation of the co-evolution between TE and host-defense. As RIP is costly for the genome (due to it also targeting gene duplications), there is likely a trade-off between its costs and benefits. Unfortunately, much of the information on lifestyle and ecology of the species is lacking, which would be needed for a thorough investigation of co-evolution. I think the study of RIP in Sordariales has at least three directions it can go in: Firstly, I think that the patterns observed in **chapter IV** can be investigated experimentally as they have been in *N. crassa* and *P. anserina*. Secondly, I think that mathematical modelling, simulating RIP, and its benefit and costs could unravel more about what specific conditions loss of RIP would occur in. Thirdly, further studying and incorporating ecology and genomic patterns of sexual reproduction could provide key clues that can be incorporated into studies of RIP.

There has been a trend within fungal genomics to study larger and larger datasets. Some examples that are relevant for this thesis include a recent study by Steindorff et al (2024), which studied thermophilic fungi across all fungi and a recent preprint by Badet & Croll (2025), which studied RIP in 1239 species across all fungi (Steindorff et al., 2024; Badet and Croll, 2025). While much can be learned from such broad sampling, there is a trade-off with how in depth the underlying questions can be studied. In the chapters presented in this thesis we have taken approaches that are slightly different. We have chosen to focus on the *P. anserina* species complex and the order Sordariales to study similar species and evolutionary topics. I think one of the key findings of this thesis is the prevalence of multiple losses of the pattern associated with RIP across Sordariales (**chapters II and IV**),

which has previously only been thoroughly described in *S. macrospora*. I think it shows that denser sampling can be just as important as broader sampling. Although, I think the future of the field will likely continue in the direction of bigger data, and more powerful tools, such as machine learning, to describe evolutionary patterns. Nevertheless, at least for now I think there are a lot of topics within evolutionary biology that likely could benefit from being investigated at different evolutionary scales.

Throughout this thesis I have focused on viewing evolution from two perspectives, the host genome and the TEs inhabiting it. To investigate the complexities of the TE evolution one must consider their interaction with the rest of the genome and vice-versa. TEs can have a number of detrimental effects on the host, and the host has a number of ways to get rid of or silence the TEs while still preserving potential benefits TE proliferation may have. RIP as a defense system is quite dramatic, in that it induces mutagenesis to completely destroy TE copies. However, as we have shown in all four chapters (and many others have shown), TEs have different ways of continuing their proliferation. The field of studying TE and host co-evolution in fungi is already in a golden age. I think that the combination of better and more data, better methods for annotating and studying TEs, and increased interest of host/TE co-evolution means that we'll likely see more and more studies come out in the future. We have already seen some exciting results, for example the finding of giant *Starship* TEs, able to horizontally transfer large amounts of genes between species (Gluck-Thaler et al., 2022; Urquhart et al., 2023; Gluck-Thaler and Vogan, 2024).

For myself, the completion of this thesis has been a great personal achievement, and a great opportunity to be able to dedicate myself to such a complex and intriguing topic for almost five years. I started the PhD studies in November 2020 at Uppsala University, right in the middle of the covid-19 pandemic. But even after the pandemic there have been other challenges and uncertainties to overcome, like the move of our research group to Stockholm University. In the end I hope that the findings presented in this thesis, will spark many more projects to come to elucidate the role of RIP and TEs in evolutionary genomics of fungi.

# 6 English Summary

Evolutionary biology studies how variation can be created within a population and how it is passed on to the next generation. For a long time, researchers mainly considered the individual level to be where the forces of evolution primarily act. But, evolution can actually act at multiple levels. For example within populations, individuals, cells, genomes, and all the way down to single gene level. Not only that, there can be conflicting interests at different levels. One example of this is cancer cells, where the cancerous cells have their own agenda, distinct from the individual's. By dividing faster than other cells, they outcompete them on the cellular level. But at the individual level, this faster cell division results in disease. In this thesis, I have studied the so-called transposable elements. They are DNA-sequences that have the ability to copy themselves and "jump" to new locations within the genome. In this way they are similar to viruses, but instead of spreading between individuals they can increase their numbers and spread within a genome. Their spread can in many cases be damaging for the organism as they can both disrupt important genes but cause general mayhem. Transposons have been around for a long time and share deep evolutionary history with the organisms they inhabit. Many organisms are filled with transposable elements, like in the human genome that consists of up to 50% transposable elements. For a long time, transposable elements were thought to only be negative or non-functional and were referred to as "junk-DNA". But as research into them has progressed, scientists have come to the understanding that they are not only negative. Transposons can sometimes be "domesticated" by the genome, which means that they evolve to be helpful to the host. To limit the mayhem and to make the most out of the positive side of transposons, several defenses have evolved within organisms to control their movement. The interplay between transposons and the organism defenses can be seen as a balancing act between the negative effects and positive potential transposons have for the genome.

In this thesis, I present the work of four studies where we have studied the interaction between transposable elements and a defense system called RIP in filamentous fungi. RIP functions by inducing mutations into the transposable elements, which has the effect of stopping them from jumping around. In **chapter I**, we studied interaction between the transposable element *crapaud* and RIP in seven species of a genus called *Podospora*. *Podospora* is best known from *Podospora anserina*, which is a mold that grows on the dung of herbivores as an important part of its life cycle. The *crapaud* transposon was especially difficult to classify as parts of it had evolved into several variations. We could show that *crapaud* has had a unique evolutionary history compared to other transposons in the species complex and has been active since the formation of the species complex. In **chapter II**, we continued to explore the *Podospora* species and their transposable elements. We specifically studied their centromeres, which are the parts of the chromosomes that act as anchoring points to pull apart the chromosomes during cell division. Centromeres have an important and conserved function in eukaryotic organisms, such as animals, plants, and fungi. Despite this, the DNA sequences that build the centromeres are highly diverse between different organisms. In fungi, many centromeres are made of transposons, which indicate a double-edged effect of transposable elements in the genome. In this study we mapped where the centromeres are located in the genomes of the *Podospora* species. We discovered that RIP was absent in one species, *Podospora pseudocomata*, and that the centromeres were smaller in this species. We found that *crapaud* (the same as in **chapter I**) was the most abundant transposable element within the centromeres of all species except this one, where a different transposable element, *discoglosse*, had taken over. In **chapter III**, we described the evolutionary relationships of the entire order Sordariales, of which *Podospora* is a part of. We did this by creating a phylogeny, an evolutionary tree, based on the genomes of 100 species. A phylogeny is in itself a description of the relationships between species, but it can also be used to explore other taxonomic or evolutionary questions. In this study, we investigated how different genomic properties have evolved in Sordariales and found several distinct differences between them. In **chapter IV**, we used the tree generated from the third chapter to more specifically study how RIP has evolved in Sordariales. We found that at least 17 of the species have lost the mutation signature from RIP. These multiple losses were scattered across the phylogeny and we further investigated what trade-offs come with losing RIP signature.

# 7 Svensk sammanfattning

Inom evolutionsbiologin studeras organismers utveckling över många generationer där man försöker kartlägga hur variation inom populationer kan uppstå och föras vidare till nästa generation genom det naturliga urvalet. Evolution kan studeras på olika nivåer, till exempel populationer, individer, celler, genom (dvs den totala arvsmassan hos en organism) och ända ner på individuell gennivå. Inte nog med det, det kan ske i olika riktningar mellan olika nivåer vilket kan leda till konflikt. Ett exempel är cancerceller, där cancercellerna har en egen agenda som är separat från individens. De delar sig med högre frekvens än andra celler och om man ser till populationen av celler utklassar de de andra cellerna, men på individnivå har cancerceller en negativ påverkan och orsakar sjukdom. I den här avhandlingen har jag studerat transposoner, som är DNA-sekvenser som kan agera självisk genom att de har förmågan att kopiera sig själva och hoppa in på nya ställen i genomet. På detta sätt kan de föröka sig och sprida sig likt ett virus inom en organisms genom vilket i många fall är negativt för organismen. Interaktionen mellan transposoner och värdorganismerna har en lång evolutionär historia och många organismers genom är fyllda med transposoner. Till exempel är människans genom upp till 50% bestående av transposoner. Länge kallade man den del av genomet som består av transposoner för “skräp-DNA” då det inte ansågs ha något annat än en negativ roll för organismen. Men allt eftersom forskningen grävt djupare har man insett att transposoner inte bara är dåliga för värden utan kan också komma med en rad fördelar. Dels så kan transposonerna utvecklas till att vara helt hjälpsamma och på så sätt “domesticeras” över evolutionär tid. Men de har också genom att hoppa runt möjligheten att skapa genetisk variation, vilket är en viktig komponent för det naturliga urvalet att agera på. Flera försvarssystem har utvecklats hos eukaryotiska organismer för att skydda från de negativa effekterna av transposonerna och ta tillvara på de positiva. Ur ett större perspektiv pågår alltså en evolutionär balansgång mellan både försvar och transposon.

I den här avhandlingen presenterar jag arbetet från fyra studier där vi har studerat interaktionen mellan transposoner och ett försvarssystem som kallas RIP i filamentösa sporsäckssvampar. RIP fungerar genom att inducera mutationer i transposonerna och på så sätt förstör dem vilket förhindrar deras aktivitet hos dessa svampar. I **kapitel I** studerade vi interaktionen mellan en transposon, *crapaud*, och RIP i sju sporsäcksvampar i ett släkte som heter *Podospora*. Denna transposon var särskilt komplicerad att klassificera då vissa delar av *crapaud* har utvecklats till flera olika varianter. Genom studien kunde vi påvisa hur crapaud haft en unik evolutionär historia jämfört med andra transposoner i artkomplexet. I **kapitel II** fortsatte vi utforska *Podospora* och dess transposoner genom att beskriva och undersöka deras centromerer. En centromer är den del av en kromosom som agerar som förankringspunkt när kromosomerna dras isär under celldelning. Centromerer har en väldigt viktig och konserverad funktion i eukaryota organismer såsom djur, växter och svampar. Men trots denna bevarade funktion är de DNA-sekvenser som bygger upp centromererna otroligt varierade mellan olika organismer. I svampar är många centromerer uppbyggda av transposoner vilket talar för ytterligare en dubbeleggad effekt av transposonernas närvaro i genomet. I denna studie kartlade vi var centromererna sitter hos de sju *Podospora*-arterna och vilka transposoner som bygger upp dessa sekvenser. Vi upptäckte att en av arterna *P. pseudocomata* saknar RIP, och har annorlunda centromersekvenser än de andra arterna. I de andra arterna är det *crapaud*, samma transposon som vi studerade i första studien, som dominerar medan i *P. pseudocomata* är det en helt annan typ av transposon som heter *discoglosse* som har tagit över. I **kapitel III** beskrev vi de evolutionära släktskapen för en hel Ordning, Sordariales, som artkomplexet *Podospora* är en del av. Detta gjorde vi genom att generera en fylogeni, ett evolutionärt träd, baserat på genomen hos hundra arter. En fylogeni är i sig en beskrivning av arternas relation, men kan också användas för att utforska andra taxonomiska och evolutionära frågor. I studien utforskade vi hur olika genomegenskaper har utvecklats i Sordariales och upptäckte flera tydliga skillnader mellan dem. I **kapitel IV** använde vi fylogenin från tredje studien för att mer specifikt undersöka hur RIP har utvecklats i Sordariales. Vi upptäckte att 17 av arterna saknar RIP (vilket innefattar *P. pseudocomata* från andra studien) utspritt över deras fylogeni. Vi undersökte även huruvida RIP innefattar en evolutionär avvägning mellan att försvara sig mot transposoner och att tillåta andra evolutionära innovationer.

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