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Full circle

Rise and fate of genetic variation in Marasmius oreades fairy rings

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Abstract
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Genetic variation is a prerequisite for evolution. The degree of variability within a species is governed by forces including mutation, recombination and selection. In the kingdom of fungi, where periodic sexual reproduction may be interleaved with extended vegetative phases, generators of variability are not restricted to act only during sexual cycles. Such generators may be in the form of mutations to the genome, affecting single base pairs up to large-scale rearrangements, movement of transposable elements, or non-meiotic shuffling of genetic variants by mitotic recombination or parasexuality. Particularly in mushroom-forming fungi, where mycelia may become large and old, the evolutionary potential of variation acquired over vegetative growth is expected to be large. In this thesis, I have studied the rise and fate of variation gained during vegetative growth in the mushroom-forming fungus *Marasmius oreades*: a non-model species known for growing in 'fairy rings'. By taking advantage of state-of-the-art genome sequencing technology and developing new bioinformatics methods, the genome sequence of *M. oreades* was successfully reconstructed. This resource was combined with genome re-sequencing to identify different types of mutations in *M. oreades* fairy rings, and to investigate the transmission of such mutations into the next generation through sexual spores. The results presented in this thesis reveal that the *M. oreades* genome is extremely stable at all levels during vegetative growth in its natural environment. Furthermore, the few mutations that arise do not seem to be transferred to the sexual spores. A significant amount of transposon movement was however revealed in monokaryotic strains when separated from dikaryons and grown in the laboratory. The combination of these results suggests that fungi possess an unknown system to suppress the accumulation of mutations during growth in nature, and that the apparent lack of a segregated germline in fungi potentially has to be reconsidered. Thus, contrary to expectations, the vegetative life stage in long-lived mushroom-forming fungi does not contribute much genetic variation, making these organisms more similar to animals and plants than previously considered. Further studies are needed to reveal how fungi control mutation accumulation, and elucidate if transposon activity is high also in naturally derived monokaryons in the form of meiotic progeny. The findings in this thesis add to what is known about how genetic variation is introduced into natural populations, how fungi deal with mutations, and highlight the complexity of genetic systems in mushroom-forming fungi.

Keywords: mushroom, fairy ring, Marasmius, mutation, fungi, genomics

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”My most terrifying thoughts are of a finite universe. What, then, would we search for?”
Hexen

List of Papers

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

I Hiltunen, M., Grudzinska-Sterno, M., Wallerman, O., Ryberg, M., Johannesson, H. (2019) Maintenance of high genome integrity over vegetative growth in the fairy-ring mushroom *Marasmius oreades*. *Current Biology*, 29(16): 2758-2765.e6.

II Hiltunen, M., Ryberg, M., Johannesson, H. (2020) ARBitR: an overlap-aware genome assembly scaffolder for linked reads. *Bioinformatics*, btaa975.

III Hiltunen, M., Ament-Velásquez, S.L., Johannesson H. The assembled and annotated genome of the fairy-ring fungus *Marasmius oreades. Manuscript.*

IV Hiltunen, M., Ryberg, M. Johannesson, H. No inheritance of vegetative mutations into sexual spores of the fungus *Marasmius oreades*. *Manuscript.*

V Hiltunen, M., Ament-Velásquez, S.L., Ryberg, M., Johannesson, H. Dynamics of transposon activity and genomic rearrangements in natural and cultured isolates of the fairy-ring fungus *Marasmius oreades*. *Manuscript.*

Contents

Abbreviations

10XG – 10X Genomics
bp – base pairs
kb – kilobase (pairs)
LG – Linkage group
LTR – Long terminal repeat
MA – Mutation accumulation
ONT – Oxford Nanopore Technology
PacBio – Pacific Biosciences
PCR – Polymerase chain reaction
SNP – Single nucleotide polymorphism
SNV – Single nucleotide variant
SV – Structural variation/variant
TE – Transposable element
TIR – Terminal inverted repeat
TSD – Target site duplication
WGS – Whole genome sequencing

Introduction

The fungi constitute an extremely diverse and successful group of organisms. They range from tiny single-celled yeasts to thousand-year-old dynamic networks of cells, and can inhabit nearly every ecosystem on earth. In nature, they take the roles of nutrient-circulating saprobes, devastating parasites of a wide range of hosts, or essential symbionts that enable plant and animal life. The fungal kingdom is estimated to be the most species rich of all eukaryotic kingdoms (Larsen et al., 2017), and fungi impact our daily lives in e.g. food and medicine production. Many fungi consist of and grow as filaments called hyphae on or within a substrate. The collective network of hyphae is termed a mycelium and can be thought of as a fungal individual. The mycelium can occasionally give rise to sexual structures, either by themselves or by mating with another individual, and some of these structures are commonly recognized as mushrooms.

The success of the fungi has sparked the interest of ecologists, evolutionary biologists and geneticists, who have studied these organisms intensely over the last two hundred years. Owing to the relative ease by which many fungi can be manipulated and experimented upon while retaining similar cellular functions to animals, they have been used as models for studies in cell biology, genetics and evolution, and as a result, a significant body of fungal research has been produced. While many similarities exist between fungi and other eukaryotes, the gained knowledge has made it clear that there are also many differences. By directing the attention of our research towards fungal-specific characteristics and putting these into perspective regarding what we know of other organism groups, fungi can teach us alternative solutions to ubiquitous questions in evolutionary biology and genetics, providing a small but important piece for the puzzle of how life works.

During this thesis, my focus has been on the connections between life history traits, genetics and factors that drive evolution. I have used mushroom-forming fungi as a model to gain a deeper understanding of fungal biology, as well as general trends across eukaryotes as a whole. Where necessary, I have delved into more technical aspects to solve particular problems, but the overall goal has been to clarify how genetic variation is introduced into organisms at the most basic level.

What it means to be a mushroom

Mushroom-forming fungi (Agaricomycetes) belong to the phylum Basidiomycota, together with the rust and smut fungi. Basidiomycetes are defined by the special, club-like cell called the basidium, where meiosis happens and from which the sexual spores (basidiospores) are borne. Where exactly the basidium is located, and when it is differentiated, varies greatly between taxa. In the Agaricomycetes, the basidia are produced on fruiting bodies in the form of mushrooms or toadstools.

The general life cycle of the Agaricomycetes

Most members of the Agaricomycetes regularly mate by outcrossing (James, 2015; Nieuwenhuis et al., 2013). For mating to happen, cells of two monokaryotic fungal individuals (monokaryons; bearing haploid nuclei of a single genotype) must meet and fuse (called plasmogamy, or mating; Figure 1). The monokaryons need to be of different mating types for a compatible mating reaction. A dikaryon is formed during mating, bearing two genetically distinct nuclei in each cell. In most Agaricomycetes, the nuclei remain separated throughout the life span, only to fuse (i.e. go through karyogamy) briefly before entering meiosis and producing basidiospores. The spores are then dispersed, and can germinate to produce new monokaryotic mycelia. The new monokaryons are thus the recombined, sexual progeny of the two parent mycelia that initially mated. The alternation between haploidy and diploidy in Agaricomycetes is comparable to other eukaryotic life cycles where pairing of nuclei occurs, in e.g. animals and land plants. The biggest difference to these life cycles is that the nuclei stay separated as haploids for extended periods of time in Agaricomycetes, whereas they fuse immediately after mating in animals and plants.

The length of the dikaryotic stage differs in the basidiomycetes compared to the Ascomycota; the other fungal phylum where dikaryons are formed during the life cycle. In most ascomycetes, dikaryosis is confined to the ascogenous hyphae and lasts only a short time before karyogamy and sexual reproduction is initiated, whereas in basidiomycetes it can persist for almost the entire life span of the individual. Perhaps to enable this way of life without one of the nuclei outcompeting the other and taking over, many basidiomycetes have septate hyphae with a clamp connection at each septum, ensuring that there is exactly one of each nucleus in both daughter cells (Furtado, 1966). The clamp connection is useful in practice because it enables identification of dikaryotic mycelia, and can be used to determine the size of hyphal compartments.

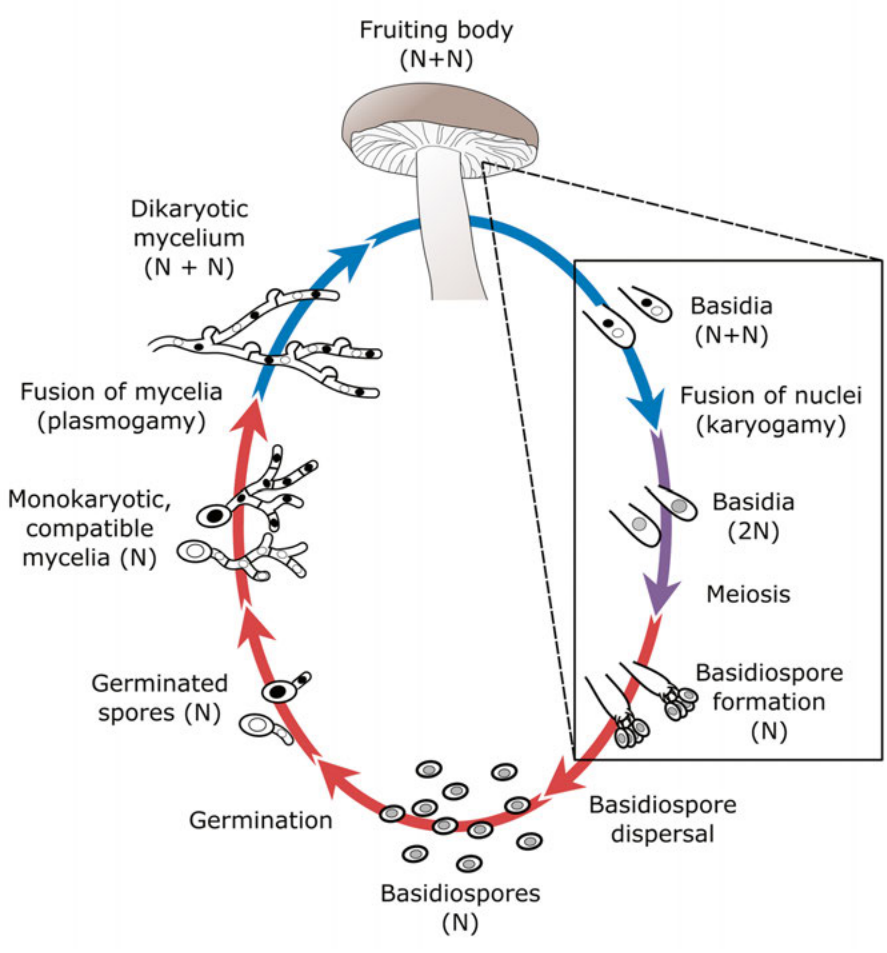

Figure 1. Life cycle of a typical mushroom-forming fungus. The fruiting body (mushroom) consists of dikaryotic tissue (with two separate, genetically distinct nuclei per cell), and specialized cells called basidia are found in the hymenium. Within these cells, haploid nuclei fuse (karyogamy) to form a diploid nucleus. Directly after, this nucleus enters meiosis to produce usually four haploid recombinant nuclei that are packaged into basidiospores. Spores are released, and if they land on a suitable substrate they will germinate to form monokaryotic mycelia. If two compatible monokaryotic mycelia meet, they may fuse (plasmogamy) and form a dikaryon, which is capable of producing new fruiting bodies. Figure modified from Hiltunen et al. (2019).

The curious case of the dikaryon

The fungal dikaryon (when nuclei are at a 1:1 ratio in each cell) is functionally similar to a diploid organism, in e.g. dominance and recessivity of alleles in different nuclei and hence sheltering of recessive lethal alleles (Day and Roberts, 1969). Given this kind of similarity, it is striking that the majority of

Agaricomycetes keep the nuclei separate up until moments before meiosis. This characteristic has puzzled mycologists over decades, and still no clear answer has been provided as to why. While diploid fungi do exist (Ullrich and Anderson, 1978) and dikaryons can at least in some species be manipulated into fusing their nuclei and forming a diploid (Anderson and Kohn, 2007; Casselton, 1965; Mills and Ellingboe, 1969), the difficulty in direct comparison of diploids and dikaryons has made it hard to draw conclusions on the effect of the nuclear organization on the life of a fungus. Nevertheless, keeping the nuclei separated can enable fertilization of adjacent monokaryons (Buller, 1930), or create novel heterokaryotic genotypes from vegetatively incompatible heterokaryons (Johannesson and Stenlid, 2004; Kay and Vilgalys, 1992; Nieuwenhuis et al., 2013). In a substrate that may be inhabited by a myriad of genotypes, it is possible that this genetic flexibility can have selective advantages, as opposed to the static nature of fused nuclei in diploids (James, 2015).

Humongous fungi

Agaricomycetes vary to great extent in their life history. While many species can be grown more or less indefinitely under laboratory settings, there is a turnover of genetically distinct individuals (genets) in nature. The turnover rate can vary significantly between species. Indeed, genets of *Gymnopus androsaceus, Amanita phalloides, Hebeloma cylindrosporum* and other species are small and short-lived in nature (Gherbi et al., 1999; Golan et al., 2019; Gryta et al., 1997; Holmer and Stenlid, 1991; Huai et al., 2003; Redecker et al., 2001). Others, such as *Suillus bovinus* and *Marasmius oreades,* are more persistent (Abesha et al., 2003; Burnett and Evans, 1966; Dahlberg and Stenlid, 1994, 1990). The extreme cases, however, belong to the genus *Armillaria*. After the discovery of an *A. gallica* genet in Oregon, estimated to be a minimum of 1,500 years of age, occupying an area of 15 hectares and weighing more than 10,000 kg, the term ‘humongous fungus’ was coined (Smith et al., 1992). More recent investigations have revealed even older genets in Michigan (Anderson et al., 2018).

A long lifespan can be advantageous because it allows for continual reproduction. In addition, longer time for growth may lead to an increased body size and more reproductive tissue being produced at each reproductive phase than in short-lived species. Longevity, defined as persisting and actively growing for many years (i.e. not in a resting state), is only found in diploid organisms (including, curiously, *Armillaria*), with the exception of dikaryotic fungi. While there are many components that determine the lifespan of organisms, somatic mutations are regarded as an important factor in aging (Kirkwood, 2005). The apparent lack of long-lived haploids can be hypothesized as being the result of having only one gene copy per cell, thus being more vulnerable to deleterious mutations than diploids and dikaryons (Orr, 1995). As a result,

selection might promote early reproduction in haploids, decreasing the likelihood of lethal mutations prematurely ending the cell lineage. This limitation could be less severe in diploids and dikaryons where lethal recessive mutations are sheltered, allowing long lifespans to evolve in these genetic systems.

Mutations as drivers of variability

All organisms are faced with the challenge to keep their heritable material, the DNA, to sufficient integrity to keep creating viable copies of themselves over time and generations. Meanwhile, as selection cannot act without standing genetic variation, some level of variation is desirable within a population, to allow for the ability to adapt to new circumstances.

Mutation rate evolution and the interaction with life history

The nearly neutral theory of molecular evolution states that most mutations are slightly deleterious, and that highly deleterious and advantageous mutations are relatively rare (Ohta, 1973; Ohta and Gillespie, 1996). More recent work largely supports this theory (reviewed in Eyre-Walker & Keightley 2007). If most mutations are deleterious, it should be in the interest of organisms to minimize the number of new mutations per unit time, i.e. the mutation rate, at least as long as the environment stays stable.

The idea of the mutation rate itself being an evolvable trait dates back to Sturtevant (1937). Since then, theoretical and experimental work has revealed that the mutation rate can indeed respond to selection, and in general evolves towards being lower (Baer et al., 2007; Lynch, 2010a, 2011). The lowest limit that the mutation rate can evolve to is thought to be determined by the effective population size, referred to as the drift-barrier hypothesis (Lynch et al., 2016; Sung et al., 2012a). The theory states that because large population sizes allow for more efficient selection (Charlesworth, 2009), organisms with large population sizes will have more streamlined genomes, less “junk DNA”, and a lower mutation rate than organisms with smaller population sizes.

Another recurring idea in mutation rate evolution theory is the interaction with life history traits (Cordero and Janzen, 2013). In particular, body size, generation time and longevity have been implicated as determinants of the mutation rate, and correlations have been observed with all of these traits (Promislow 1994, Nikolaev *et al.* 2007, Nabholz *et al.* 2008, Smith & Donoghue 2008, Galtier *et al.* 2009, Thomas *et al.* 2010, Hua *et al.* 2015). Large body size, or long generation time and lifespan, implies many replications of the genome, and as each replication carries the risk of introducing errors that lead to mutations, organisms that exhibit these traits are expected to evolve low mutation rates. Of course, these traits co-vary, and disentangling the causative factor from correlations is tricky, even more so when organisms with

large body sizes and generation times are not exactly associated with being attractive experimental systems. Probably, mutation rate evolution is governed by complex interactions between life history traits, population genetics and the molecular basis for evolution: the genes and the genome.

Mutations at the molecular level

While mutations in previous discussions in this thesis have been thought of in a phenotypic context, they are at the most basic level affecting the genotype of the organism. Mutations can be of many different types, affecting a single base pair (bp) up to large sections of chromosomes. I do not consider changes in the epigenetic landscape to be mutations in this thesis, as they are not changing the DNA, even though such changes can alter the expression of phenotypes (Felsenfeld, 2014). Mutations affecting a single nucleotide lead to single-nucleotide variants (SNVs). If such mutations are allowed to spread into a population by selection and drift, they give rise to new single-nucleotide polymorphisms (SNPs). Insertions or deletions of bases are referred to as indels, often affecting more than one base pair. Mutations that rearrange the large-scale structure of chromosomes, such as inversions, translocations, duplications etc., lead to structural variants (SVs). The distribution of mutation types that occur in an organism over a specified period of time is referred to as the mutation spectrum. In this thesis, mutations are investigated in different ways in Papers I, IV and V.

The sources of mutations can be intrinsic and extrinsic (Chatterjee and Walker, 2017). Intrinsic ones in the form of replication errors are common, and while the DNA proof-reading and mismatch repair systems can remove many such errors, some are bound to remain and give rise to mutations in the genome at the next replication (Bębenek and Ziuzia-Graczyk, 2018; Schaaper, 1993). Extrinsic factors include ionizing radiation such as gamma rays and ultraviolet light, the latter of which primarily causes DNA damage through covalent bonding between adjacent pyrimidines, which are resolved by C:G to T:A transition mutations. (Chatterjee and Walker, 2017; Rastogi et al., 2010).

Transposable elements can cause genetic variation

An important source of variability in most genomes is transposable elements (transposons; TEs). Transposons are selfish, mobile genetic elements, capable of moving and increasing their copy number in a host genome. New insertions and deletions of TEs can be considered mutations, and they can greatly impact the evolution of organisms (Biémont, 2010; McDonald, 1993). The most obvious way that TEs can be problematic for the host is by inserting into genes, thus interrupting them and possibly causing a dysfunctional gene product.

They can also affect organismal fitness e.g. by altering the expression of adjacent genes, shuffling non-homologous regions of the genome through ectopic recombination and horizontally transferring genetic material (Daboussi and Capy, 2003; McDonald, 1993).

Transposons are common in fungal genomes, and in mushroom-forming fungi they often constitute around 20% of the genome size (Miyauchi et al., 2020). Several types of TEs have been shown to be actively moving in fungal genomes (Borgognone et al., 2017; Chen et al., 2015; Ikeda et al., 2001). The movement of TEs, despite their abundance in most genomes, is usually restricted to periodic bursts or being induced by biotic or abiotic stressors (Anaya and Roncero, 1996; Miousse et al., 2015; Wessler, 1996). Under normal conditions, TE activity is suppressed by the host genome, often by epigenetic silencing (Zemach et al., 2010). It has been speculated that under stressful conditions, however, the novel diversity that TEs provide can be of selective advantage, though a perhaps more parsimonious explanation for the increased TE activity under stress is a shift in focus of the cellular machinery from TE suppression towards stress handling (Capy et al., 2000). In this thesis, TEs play a significant role in Paper III, and are the main players in Paper V.

Somatic mutation – does it matter?

Genetic variation that is introduced by mutation will directly influence the daughter cells to the cell that originally mutated. The number of mutations that can accumulate over a generation and impact evolution is expected to be a function of the time spent growing and the number of mitoses between each meiosis (Orive, 2001). In purely clonal organisms, any changes in mitotic cell lineages can affect adaptive evolution (Fagerström et al., 1998). In sexual organisms however, only mutations in the cell line that ultimately goes into meiosis will have evolutionary significance, since mutations in other cells will not be transferred to the next generation.

The separation between germline and soma

Weismann introduced the concept of separation between germline and soma based on observations in animals (Weismann, 1893). Germline cells give rise to gametes, making the germline immortal, in a sense. Somatic cells, however, are only used during the lifespan of the organism and perish along with it, making them irrelevant for evolution, under a Weismannian doctrine. The concept of germline/soma separation can be reasonably applied across bilaterian animals. In chordates such as humans and mice, and ecdysozoans such as *Drosophila*, early segregation of germline cells appears to have evolved in convergence (Juliano and Wessel, 2010; Saffman and Lasko, 1999). The number of germline cell divisions during a generation is strictly controlled in these

species (Drost, 1993; Drost and Lee, 1995). With mutation rate evolution theory in mind, it can be hypothesized that the germline should mutate at a lower rate than the soma. Results from humans and mice show that the germline indeed mutates about ten times slower than somatic cells (Milholland et al., 2017), supporting the notion that germline segregation has a function in keeping up genome integrity across generations.

In a broader phylogenetic perspective, also including plants, multicellular algae and fungi, the distinction between the germline and somatic cells becomes muddier (Buss, 1983). In these organism groups, germline segregation appears to occur at a later stage, and in some of them, continually over many seasons. Recently however, this view has been challenged in plants, fueled by the finding that the number of cell divisions during a generation is controlled in tomatoes and *Arabidopsis* (Burian et al., 2016; Watson et al., 2016). Stem cells in the meristematic central zone of plants divide slower than sub-apical cells (Kwiatkowska, 2008). Lanfear (2018) argues that the central zone cells serve as a functional germline, capable of generating supporting organs such as leaves and bark, but with the primary function to go into meiosis and produce gametes. Regardless of whether or not the meristematic central zone is set aside for gamete production, it serves as a source of "fresh" genetic material, to a large extent unburdened by somatic mutations (Groot and Laux, 2016). The pattern of asymmetrical cell division in plant meristems, where central zone cells are carried forward by sub-apical cells, can explain the low rates of mutation accumulation in long-lived trees, and the generation time effect that is observed on rates of molecular evolution in plants (Smith & Donoghue 2008, Lanfear *et al.* 2013, Xie *et al.* 2016, Schmid-Siegert *et al.* 2017, Schoen & Schultz 2019, Hofmeister *et al.* 2020).

In fungi, much less is known about germline segregation. The general notion is that most, if not all, cells in fungal mycelia are capable of entering reproductive development, producing fruiting bodies or other sexual structures. The fact that fungal mycelia and even fruiting bodies can readily be reformed from small sections of the fungus, such as hyphal tips and protoplasts (Brown, 1924; Magae et al., 1985; Ochiai Yanagi et al., 1985; Peberdy, 1979), suggests that cell fate is flexible, making a Weismannian germline concept inapplicable to fungi. However, a functional germline, similarly as suggested for plants, may still be present. During Paper IV, we delve deeper into the question of when germline segregation occurs in fungi.

Mutation suppression in fungi

Something similar to a plant meristem has been proposed as a mechanism to explain a low rate of mutation accumulation in *Armillaria*; the humongous fungus (Aanen, 2014; Anderson and Catona, 2014). In these organisms, Anderson and colleagues found that the genomes of samples collected far from

each other contained much fewer new mutations than anticipated under expected mutation rates (Anderson et al., 2018; Anderson and Catona, 2014). Naturally, this finding drew parallels to the emergent link between life span and mutation accumulation in plants, as *Armillaria* are the oldest and largest fungi, and arguably even organisms, we know of (Arnaud-Haond et al., 2012). As an explanation for the few mutations found, it was suggested that the rhizomorphs produced by these fungi contain a number of slowly dividing cells, similar to the central zone in plant meristems (Aanen, 2014). Rhizomorphs are cord-like aggregates of hyphae, used by *Armillaria* to infect new trees (Sipos et al., 2018).

Aanen (2014) suggested, as an alternative to the rhizomorph meristem hypothesis in *Armillaria*, that the mutation suppressing mechanism in fungi is by "immortal strands" (Figure 2). First proposed by Cairns (1975), the immortal strand hypothesis states that chromatid segregation at cell division can be non-random, such that one daughter cell is more likely to obtain certain chromatids. If those chromosomes contain the oldest DNA strands, they would carry fewer replication errors than newly synthesized strands. Such template-strand co-segregation could act as a way to retain the relatively mutation-free DNA in stem cells, lowering the chance for mutations to spread in an individual. The immortal strand hypothesis has been met with some controversy (Lansdorp, 2007), but empirical data supports the occurrence of template-strand co-segregation in a wide range of organisms (Conboy et al., 2007; Gurevich et al., 2016; Lark, 1967; Rosenberger and Kessel, 1968; Snedeker et al., 2017). In Paper I, we found similarly low rates of mutation accumulation over vegetative growth as in *Armillaria* in another fungus, *Marasmius oreades*. This species, however, does not produce rhizomorphs. Thus, a meristem-like method of mutation suppression can be excluded in this case, leaving template-strand co-segregation as a potential explanation for the finding of low mutation accumulation (Hiltunen et al., 2019).

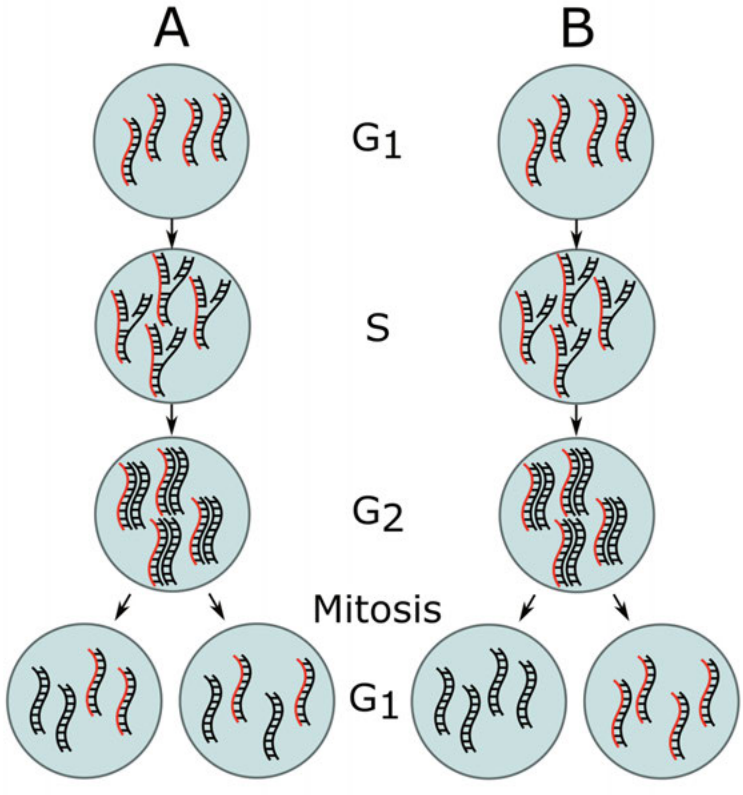

Figure 2. Strand segregation during mitosis. The oldest DNA strands are depicted in red and the cell cycle stage is indicated at the center. A. Strand segregation during standard mitosis, where chromosomes segregate at random and there is an equal chance for the daughter cells to inherit chromosomes with old or young DNA strands. B. Template-strand co-segregation, as suggested by the immortal strand hypothesis. Older strands of DNA may have a higher chance of segregating together during mitosis than at random. This way, the amount of replication errors that accumulate over time can be minimized in certain cells, since most such errors occur in newly synthesized strands.

How can we study mutations?

The spontaneous mutation rate is notoriously difficult to estimate. Historically, it has been studied using genetic constructs exhibiting specific phenotypes (Drake, 1991; Drake et al., 1998). Recently, however, technological

breakthroughs have improved our mutation detection ability by several orders of magnitude. Accordingly, the spontaneous mutation rates have since been measured in a multitude of model and non-model organisms (Farlow et al., 2015; Ho et al., 2020; Keightley et al., 2009; Kucukyildirim et al., 2020; Liu and Zhang, 2019; Lynch, 2010b; Ossowski et al., 2010; Saxer et al., 2012; Sung et al., 2012b). Different methods to infer mutation rates are outlined below.

Mutation accumulation experiments

In organisms prone to laboratory propagation and with short generation times, a mutation accumulation (MA) experiment is suitable for estimation of the mutation rate and spectrum (Halligan and Keightley, 2009). By having a genetically homogeneous starting population and propagating it over time, new mutations will start to accumulate and can be traced. A basic premise is to offset the selection-drift balance as far as possible towards drift as the major force, thereby minimizing the power that selection has as a determinant over the spread of new mutations in the experimental population. This effect can be achieved by minimizing the effective population size, in practice by random, sequential bottlenecks of cells or individuals. If drift maximization is achieved, the nearly neutral mutation rate can be estimated. Lethal mutations will however always be a source of underestimation for the number of mutations, as they will per definition not spread in the experimental population. Whole-genome sequencing (WGS) can be applied on the final population to find and quantify the number of new mutations. The main strength of MA experiments is the level of control that can be kept during propagation of the population, and it has been successfully applied to many organisms, primarily unicellular (Farlow et al., 2015; Liu and Zhang, 2019; Sung et al., 2012b), but also to filamentous fungi (Álvarez-Escribano et al., 2019; Bezmenova et al., 2020). However, for mutational studies in multicellular eukaryotes, other methods are more practical.

Trio sequencing

To investigate the mutations that arise across a generation in multicellular organisms, WGS of parents and offspring can be applied (Conrad et al., 2011). This method makes use of somatic tissue, e.g. blood, to sequence the whole genomes of two parents and their offspring, enabling the detection of mutations that have arisen in the germlines of either parent. Trio sequencing, or with the inclusion of more family members, pedigree sequencing, has revealed the generational mutation rates in a number of great apes (Besenbacher et al., 2019; Tatsumoto et al., 2017; Wu et al., 2020). When the generation time is clear and discreet, such as in bilaterian animals, trio sequencing provides a

robust estimate of the mutation rate per generation. In organisms with extended vegetative phases and presumably late germline segregation, such as sponges, corals, multicellular algae, some plants and fungi, reproductive age of an individual may span two or more orders of magnitude, making the generation time hard to robustly estimate. For example, some trees may start to produce pollen after just a few years after germinating from a seed, and continue to do so for hundreds of years. Trio sequencing may in those cases be less informative about the mutation rate per generation than in organisms with short time peaks in reproductive age. Nevertheless, an approach similar to trio sequencing has been applied in an Agaricomycete fungus, revealing a relatively high mutation rate in this species (Baranova et al., 2015).

Fungal fairy rings: natural experimental systems

The MA and trio sequencing experiments are direct in their way to track mutations over time. An alternative is to compare different cells that have a common origin at the same time point. This method has been applied in many different plant species, and is especially useful in long-lived trees where other methods fail (Hofmeister et al., 2020; Schmid-Siegert et al., 2017; L. Wang et al., 2019). The disadvantage is that selection cannot easily be ruled out, and has to be addressed in other ways. In plants, owing to their branching structure, it is generally clear to the eye which cells have a common origin. This kind of relatedness is not always as clear in fungi, where the mycelium is obscured in a substrate and other methods must be applied to determine if one or several genets are under study (Guillaumin et al., 1996). An exception, where the genet is distinguishable by eye, is fungal fairy rings.

A fairy ring is a circular mycelium, growing underground and leaving above-ground traces of itself (Shantz and Piemeisel, 1917). Such traces can be in the form of sparse-growing grass during dry periods, or lush, dark-green grass under humid conditions. Presumably, the thin fungal hyphae exceed the ability of grass roots to take up water, thus outcompeting the grass in dry conditions. Under humid conditions, the litter-degrading ability of the fungus may release nutrients into the soil that the grass can also utilize. During mushroom season, fruiting bodies appear along the edge of the ring, often as quickly as overnight, sparking tales of them being induced by dancing fairies, witches' cauldrons, demonic rituals, etc. While fairy rings are often associated with certain species, such as *Marasmius oreades, Lepista* spp*, Leucopaxillus giganteus* and *Agaricus* spp, there is no known genetic determinant that causes this growth pattern. Instead, the natural growth pattern for most filamentous fungi is outwards from a central point; evident for anyone who has cultivated a filamentous fungus on a petri dish with growth medium. If the growth substrate is reasonably homogeneous and undisturbed for some time, e.g. as in grasslands such as lawns and pastures, the fungal mycelium will continue to grow at the same rate in all directions. As nutrients deplete on the inner section

of the circle, the mycelium will degenerate and finally die in this section, giving rise to circular mycelia that are only active at the front line. Hence, the tendency of certain species to form fairy rings is likely caused by the natural growth substrates of these species. In a sense, fairy rings can be seen as giant petri dish cultures growing outside in nature.

Fairy rings are attractive as experimental systems for several reasons. First, the genet is clearly visible by eye, and genetic homogeneity of the whole ring has been shown experimentally (Burnett and Evans, 1966; Mallett and Harrison, 1988). Second, as the ring expands radially outwards over time, the age of fairy rings can be estimated from diameter and growth rate (Burnett and Evans, 1966). Third, the genotype of different sectors of a ring can be sampled from fruiting bodies arising in those sectors. By comparing the genotypes of different sectors from fruiting body samples, new mutations can be identified, similarly as has been done in trees and *Armillaria* (Anderson et al., 2018; Anderson and Catona, 2014; Hofmeister et al., 2020; Schmid-Siegert et al., 2017). The increased availability of WGS technology in the last two decades has made it possible to find mutations in virtually the whole genome.

In this thesis, the fairy-ring mushroom *M. oreades* has played an integral part, and is the study system in all papers that are included. Initially, this species was selected because of its abundance in Sweden and most of the Northern hemisphere. Most lawns around Uppsala contain some individuals of the species. In addition, previous investigations have shown that it can be cultured in the lab (Gordon et al., 1994; Mallett and Harrison, 1988). The downside was that before my work, no genomic data of the species was available.

Separation of nuclei in dikaryons

The fact that most Agaricomycetes spend the majority of their life cycle at a dikaryotic ploidy level presents a unique opportunity: the ability to study the monokaryotic, parental nuclear types that initially mated to form the dikaryon. In contrast to other eukaryotes, where karyogamy occurs directly after mating, the constituent nuclei of fungal dikaryons can be recovered through different methods. The most widely used technique is called protoplast isolation and regeneration (De Vries and Wessels, 1973, 1972; Peberdy, 1979). This method uses enzymatic digestion of the chitinous cell wall of fungal hyphae to release droplets of cytoplasm termed protoplasts, with random cellular content that may include nuclei (Figure 3). An osmotic stabilizer, which can be in the form of sugar, salt or sugar alcohol solution, e.g. glucose, $MgSO_4$ or sorbitol, is required for released protoplasts not to explode from differences in pressure. After filtration of residual cell debris and undigested hyphae, the protoplasts are spread onto regeneration medium, also containing osmotic stabilizer, where they may regenerate the cell wall and start to regrow into hyphae (Kitamoto et al., 1988; Ochiai Yanagi et al., 1985). Colonies can be transferred from the regeneration plate and allowed to grow into mycelia, at which point

the ploidy can be determined by inspection for clamp connections. This way, the constituent nuclei of the dikaryon can be recovered into live, monokaryotic strains called protoclones.

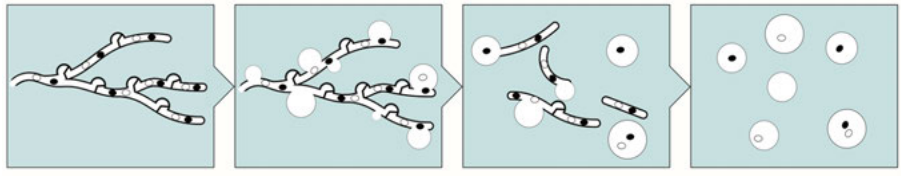

Figure 3. Protoplast isolation: a method to separate constituent nuclei of a dikaryon into monokaryotic strains. First, the dikaryon is subjected to a chitinase that digests the cell walls. As the hyphae disintegrate, nuclear components are released in the form of droplets of cytoplasm called protoplasts. After filtering away remaining cellular debris, a pure protoplast solution can be obtained. As some protoplasts may carry a single nucleus, they can reform the initial monokaryon upon regeneration. Nuclei of different genotypes are indicated in black and white.

Fungal genome sequencing: opportunities and challenges

As briefly touched upon previously, WGS is a wonderful tool for evolutionary studies. The early days of genome sequencing in Agaricomycetes made use of Sanger technology to sequence short fragments of the genome inserted into Bacterial Artificial Chromosomes (BACs). A single genome thus required extensive cost and effort from many researchers (Martin et al., 2008; Ohm et al., 2010; Stajich et al., 2010). Since then, different technologies have emerged that greatly facilitate the process, the main players in recent years being Illumina, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT). All three companies have several machines on the market, with differences in accuracy, read length, throughput and other factors (De Maio et al., 2019). Regardless of which technology is selected by the investigator, the genome sizes of most cellular organisms inevitably lead to a vast amount of data. A new challenge thus lies in data analysis.

The need for well-assembled genomes

Just because all DNA of an organism is sequenced, it does not mean that all information about its genome is available. To make sense of the enormous amount of data in a new sequencing project, it is necessary to reduce the complexity of the dataset by assembling sequencing reads into longer, more contiguous so-called contigs. The process is called genome assembly (Alkan et al., 2011; Green, 2002; Pop, 2009). The optimal assembly captures the full

sequence of each chromosome from start to end, and every letter in the sequence correctly represents the nucleotide of the biological "truth" (Koren and Phillippy, 2015). If sequencing reads are long and error-free, this process is straightforward. In reality however, this is never the case. Illumina reads can span between about 80-150 bp, depending on the sequencing platform. This length is short relative to PacBio, that can produce read lengths commonly in the kilobase pair (kb) range, and ONT, where reads can span up to the length of input DNA molecules. The major downside with the two latter technologies is the increased error rate, since they, contrary to Illumina, rely on single molecules for sequencing without any amplification of the target DNA. To make use of the advantages offered by either technology, new genome assembly projects regularly employ a combination of short, error-free Illumina reads with long, error-prone reads from PacBio or ONT (Bendixsen et al., 2020; De Maio et al., 2019; Jiao and Schneeberger, 2020; Rupp et al., 2018; Tan et al., 2018; Wallberg et al., 2019). The reason that error-free reads are desirable is self-explanatory, but why does read length matter for assembly? The answer lies in the complexity of most eukaryotic genomes, where certain sequences are often repeated many times, either in tandem, such as satellites, or interspersed across the chromosomes, as TEs often are (Britten and Kohne, 1968; Jurka et al., 2007). All types of repeats cause trouble during genome assembly, since the original position of a read cannot be unambiguously determined unless the read spans the entirety of the repetitive sequence including some unique flanking sequence. Longer reads can thus help resolve repeats during genome assembly.

But do we need to spend vast amounts of time, energy and resources on getting as much different sequencing data as possible? The answer depends on the research question being asked. Much fungal research today is centered around the evolution of gene family dynamics (Kohler et al., 2015; Miyauchi et al., 2020), or disentangling relationships between taxa through phylogenomics (Fitzpatrick et al., 2006; Spatafora et al., 2016; Zhang et al., 2017). For these types of questions, getting high-quality sequences of gene orthologs is necessary and should be the main focus, and can be achieved by using only Illumina sequencing. If the research question is instead based around synteny of genes, chromosome evolution, structural variation or the repetitive sequences that cause assembly issues themselves, genome assemblies with high contiguity, as can be produced by incorporating long reads, are of great help.

How are we doing in mushroom genome assembly?

While the genomes of many Agaricomycetes species have been sequenced by now (Zhang et al., 2017), the vast majority of them have relied only on Illumina sequencing, resulting in different degrees of fragmentation due to genomic repeats (Figure 4). Again, it is important to stress that this level of assembly has been enough to answer the questions posed for these sequencing

projects. Complete assemblies of Agaricomycotina, the subphylum where Agaricomycetes belongs, are however still rare, and most of them date back to the days of Sanger sequencing.

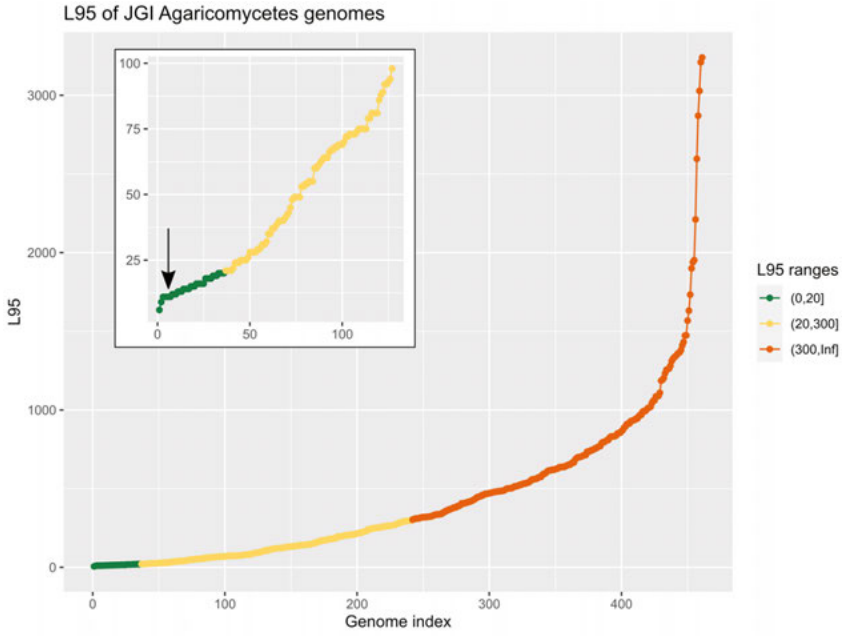

Figure 4. Contiguity of Agaricomycotina genomes available at JGI Mycocosm. L95 values of all 467 genome assemblies sorted from lowest to highest. Inset: assemblies with L95 of 100 or lower. The value of *Marasmius oreades* genome version Maror2 (Paper III) is indicated by an arrow (L95 = 11). Data obtained from Mycocosm, Joint Genome Institute, Jan 26, 2021.

Genome assemblies can be finished in different ways

In many cases, it is not possible to span whole repeat clusters even using long reads. To completely assemble this kind of genomes, additional genomic information is needed, which can be in the form of e.g. optical or linkage maps, chromosome conformation capture data (HiC), or linked-read sequences (Eisenstein, 2015; Foulongne-Oriol, 2012; Larraya et al., 2000; Lieberman-Aiden et al., 2009; Staňková et al., 2016).

Out of these options, one tractable method used to be linked-read data in the form of 10X Genomics (10XG) Chromium sequencing reads, but unfortunately, 10XG has recently discontinued linked reads. The technology partitions input DNA molecules into droplets where specific molecular barcode sequences are attached to the DNA fragments. The fragments are then sequenced using standard Illumina technology. The result is termed linked reads, where reads that share the same barcode sequence originated from the same

physical DNA molecule, and the high throughput of the Illumina system enables a large amount of linkage information to be generated. This technique was targeted towards larger genomes than what is generally found in fungi, thus being unsuitable for *de novo* assembly of small genomes. Nevertheless, the technology held great promise for scaffolding purposes; the method of stitching together assembled contigs into longer fragments, termed scaffolds, separated by sequence gaps represented by Ns. New methods for linking short reads together, called TELL-seq and stLFR, have been published recently, and it remains to be seen if they can fill the gap left by the death of 10XG linked reads (Chen et al., 2020; O. Wang et al., 2019). During Paper II, we developed a computational pipeline to produce scaffolds using linked reads, with 10XG Chromium and stLFR support.

Another alternative to infer which contigs belong together is by using a genetic linkage map (Fierst, 2015). A linkage map describes how genetic markers are distributed across the genome in terms of their genetic distance to each other, measured in centimorgan (cM). Such information is useful in many aspects of genetics. The basic principle is to measure the recombination rate between different markers; i.e. how often a specific marker pair carry the same combination of alleles in a population of recombinants. The analysis requires a recombined mapping population, from one or several parental crosses. Many species of fungi produce millions of sexual spores from a single fruiting body, and this characteristic makes them good systems for linkage mapping. Spores can be collected, grown in culture, and genotyped at marker loci or by WGS (Foulongne-Oriol, 2012). Owing to this characteristic, many linkage maps have been produced in mushroom-forming fungal species (Aimi et al., 2005; Foulongne-Oriol et al., 2016, 2011; Gao et al., 2018; Hoi-Shan and Hai-Lou, 2002; Labbé et al., 2008; Larraya et al., 2000; Lind et al., 2005). In Paper III, we present a linkage map of *M. oreades*, and use it to finish the genome assembly of the species.

Recent developments in long-read sequencing, HiFi reads of the PacBio Sequel II and the ultra-long reads of ONT PromethION systems, have made great improvements in throughput as well as read length and accuracy (Lang et al., 2020). It seems likely that scaffold gaps and fragmented assemblies are soon a thing of the past, just as BACs and Sanger-sequenced genomes, at least if the current trends of improvements and price reduction in DNA extraction and long-read technology continues (Mantere et al., 2019).

Research aims

As any PhD project, the start aims and end points of my PhD work diverged quite a bit. What started out as an investigation of parasexuality in basidiomycetes evolved into a project about variation generated in the form of mutations and recombination. Nevertheless, the overall aims have remained: to expand our knowledge of genetic diversification within basidiomycete fungi over vegetative growth. The ultimate goal is to understand basidiomycete fungi on a genetic and genomic level to build upon and expand what is known about the factors that govern evolutionary change in organisms in general. More specifically, my goals have been to map out the types of mutations that arise during vegetative growth in *M. oreades* fairy rings, from SNVs to transposons, using state-of-the-art genome sequencing techniques and bioinformatics methods. Furthermore, to take it full circle, my aims have been to trace the mutations through spores to the next generation.

Specific aims of each paper

For **Paper I,** the aim was to explore the mutations that arise during growth of *M. oreades* fairy rings and use this information to calculate the mutation rate, both by year and by generation. A requirement was first to create a *de novo* genome assembly of *M. oreades*. The purpose of this assembly was to use as a reference during variant calling in WGS samples from a number of fruiting bodies. In **Paper II**, sparked by our success in utilizing 10XG linked reads for genome assembly scaffolding, we aimed at developing a software tool that could be of wider use for this purpose. After the discontinuation of 10XG linked reads, we aimed to enable the use of other types of linked-read data in our pipeline. For **Paper III** the aim was to generate a comprehensive genome resource of *M. oreades*, fully assembled and annotated regarding genes and transposable elements. To achieve this purpose, we first aimed to construct a well-resolved genetic linkage map of *M. oreades*. During **Paper IV** the aim was to take the mutation investigation one step further by analysis of heritability of new mutations by the spores. Finally, during **Paper V**, we wanted to use the method known as protoplast isolation and regeneration in combination with the latest long-read sequencing techniques to get a detailed view of the individual nuclei that make up the dikaryon, from different sampling points in a *M. oreades* fairy ring. Here the purpose was to investigate mitotic recombination and transposition during growth of a fairy ring.

Summaries of papers

Paper I: *Marasmius oreades* is good at keeping its genome intact during growth

Most genetic mutations are deleterious, but organisms risk introducing mutations at each cell division. Selection should thus favor organisms with short generation times and few cell divisions between zygote and gamete. Contrary to this hypothesis, many long-lived species are found in nature, e.g. in vascular plants, corals, sponges, arthropods, vertebrates, and fungi. A way to minimize the number of mutations per generation is to separate a number of cells early on during development, which remain mitotically quiescent until sexual maturation when gametes start being produced. Such reduction in mitotic rate happens in bilaterian animal germlines and plant meristem tissue, but no similar system is known in fungi. The lack of a segregated germline begs the question of whether or not mutations accumulate to high levels or are being suppressed by some other mechanism in this organism group.

We used fairy rings of *M. oreades* to look into this question. By using a combination of whole genome sequencing technologies, we assembled the genome of *M. oreades* to chromosome level. We additionally re-sequenced 40 isolates, consisting of pre-meiotic, dikaryotic fruiting body stipe tissue, originating from six fairy rings of the species. We discovered surprisingly few mutations, reflecting findings in *Armillaria* (Anderson et al., 2018; Anderson and Catona, 2014). By measuring the ring radii and mean size of a hyphal compartment, we were able to estimate the number of cell divisions from establishment of the genets and up to each sampling point. Assuming symmetrical cell division and strand segregation, i.e. no germline/soma separation or template-strand co-segregation, we estimated a mitotic mutation rate of 3.8×10^{-12} mutations per genomic site and cell division. Such a rate would be lower than the previous extreme of 2×10^{-11} found in *Paramecium tetraurelia* (Sung et al., 2012b). We found no indication that purifying selection played a major role in removing mutations. Error sources were investigated by Sanger sequencing of a subset of the mutations, and by using our mutation calling pipeline for discovery of simulated variants, revealing that we were able to confidently call novel variants with our method.

Because fungi do not have exceedingly high rates of molecular evolution compared to plants and animals, it makes sense that vegetative mutation accumulation is limited in fungi. Indeed, by approximating the number of cell

divisions per generation in *M. oreades*, we show that the inferred low mutation rate per mitosis translates to a mutation rate per generation that is comparable to animals and plants. The mechanism that allows for this low level of mitotic mutation accumulation is yet unclear. Perhaps the mechanism is related to the DNA replication and repair machinery in fungi, but a pilot survey of the number of genes involved in DNA repair in a number of fungal genomes did not show much difference in species with different life history traits. Other potential explanations for the few mutations we found is that there is a hidden germline in fungi, protecting the cells that are destined for spore production from mutations, or template-strand co-segregation, where nuclei containing the oldest DNA strands are continually pushed to the growth front. Future studies should aim to investigate these different hypotheses.

Paper II: ARBitR is useful for genome assembly scaffolding

Contiguity in genome assemblies is important to study chromosome evolution, structural variation and TEs, among other things. A way to achieve higher contiguity is by 'scaffolding'; the method of using long-range information to link together previously assembled contigs into longer scaffolds. Such information can be found in linked reads: Illumina reads that are tagged with a molecular barcode during library preparation, where the barcode is common for fragments that originated from genomic regions in close physical vicinity to each other. A platform that can be used for producing linked reads is the 10X Genomics Chromium system, which makes use of microfluidic technology to partition input DNA molecules into droplets, where fragmentation, barcode attachment and fragment amplification takes place.

Current scaffolding procedures usually take no heed to overlapping ends of the contigs that go into the scaffold, instead merging them with a sequence gap in between ("N", repeated a number of times). The existence of such overlaps is dependent on characteristics of the genome under investigation, in addition to the sequencing technology and assembly algorithm that were used to create the initial assembly. Specifically, in small genomes where repeat clusters are short, that were assembled using overlap-layout-consensus of long reads, overlapping contig ends may be quite frequent. Merging contigs into scaffolds without paying attention to the sequence at the contig ends carries the risk of duplicating some sequence around the gap. A more refined way of scaffolding in such cases is by calculating an overlap between the contigs and collapsing this sequence into a consensus, resulting in a gap-free sequence.

Because of this limitation in previous software, we developed ARBitR: an overlap-aware assembly scaffolder for linked reads. We show the utility of ARBitR on different datasets, ranging in size from the small genome of *M.*

oreades to a human genome assembly. Particularly, the main advantage of using ARBitR compared to previous software is for assembling TE sequence at contig ends, where the Long Terminal Repeat (LTR) Assembly Index (LAI) is closer to reference values in ARBitR-scaffolded assemblies (Ou et al., 2018).

Paper III: Completion of the *M. oreades* genome assembly by linkage mapping

Genetic mapping is a useful method for determination of long- and short-range linkage of genetic loci. Because of how recombination works, there is usually a clear correlation between physical and genetic distance between loci, meaning that linkage maps can be utilized to determine the higher-order structure of contigs in a genome assembly. Furthermore, to be able to investigate the potentially disruptive effect of mutations, high-quality gene annotations are required. For the identification of complex genetic variants, a library of repetitive sequences is a great advantage.

During this project, we isolated 95 single-spore cultures from four fruiting bodies of one *M. oreades* fairy ring. The single-spore strains are the sexual progeny of this fairy ring. We sequenced their whole genomes with Illumina technology, and used this information to construct a linkage map with the LepMAP software (Rastas, 2017). The analysis revealed 11 high-confidence linkage groups (LGs), putatively corresponding to the chromosomes of the species.

Re-assembly of the sequencing data we collected for Paper I, including scaffolding by ARBitR and now also utilizing the newly constructed linkage map, resulted in a near-complete genome assembly of *M. oreades*. Genes were identified using Funannotate (Palmer and Stajich, n.d.), resulting in ca. 14,000 gene annotations. The genome was mined for repetitive sequence, and the results were manually curated into a repeat library consisting of 217 sequences. By applying this library for repeat identification in the genome, we found that it consisted of about 22% repetitive sequence, mostly LTR TEs but also a significant fraction of DNA elements of the *Kyakuja-Dileera-Zisupton* (KDZ) and *Plavaka* groups (Iyer et al., 2014). Taken together, the results of this paper add detailed information about the genome characteristics of a mushroom-forming fungus, to a level that is previously only available for model species.

Paper IV: Vegetative mutations are not inherited to the spores

All changes to the genome that happen in the cell line between zygote and gamete can impact evolution. In fungi, as far as we know, each cell has the capability to enter reproductive development and produce gametes down the line, meaning that variants that have arisen during the vegetative phase can be transmitted to the spores. By investigating the transition between parent and offspring, the transmission rate of such variants can be quantified.

The aim of this study was to quantify the heritability of vegetatively arisen variants, and possibly investigate the selective potential of these variants. The hypothesis was that because the sheltering effect during dikaryosis is lifted once nuclei enter the haploid spores and form monokaryotic mycelia, recessive deleterious mutations would be expressed. As a result, we hypothesized that non-synonymous mutations in coding frames, along with their linked variants, would be found to a lesser extent than synonymous or non-coding mutations, in spore isolates derived from fruiting bodies.

To address this question, we made use of the same single-spore dataset as for Paper III: WGS of 95 single-spore isolates from four fruiting bodies of one *M. oreades* fairy ring. The fruiting bodies were collected from the cardinal directions of ring 43, located in Berthåga, Uppsala. In addition to this dataset, we sequenced the genomes of the stipes from the same fruiting bodies that we collected the spores from, and four fruiting bodies collected the year before, from nearby positions in the ring. By using our mutation-calling pipeline established in Paper I, we found that as expected, the fruiting bodies from both years had accumulated new mutations, and a higher number of mutations was found the second year. However, to our surprise, only two of the mutations were shared between the years. Furthermore, when we looked for the second-year fruiting-body mutations in the spore isolates, we found that they were never present. To answer our initial question, if new mutations were simply too deleterious to be tolerated in a haploid condition, we investigated linkage of the regions surrounding the mutations. Reasoning that if mutations were purged at the spore stage, there should be a signal of background selection on linked variants. We could not find such a signal, suggesting that the mutations we found in the fruiting bodies never made it into the spores in the first place. We did however find that some spore isolates carried variants that were not present in other spore isolates, or the fruiting bodies from which they originated, suggesting that some mutations may arise either at meiosis or afterwards when we propagated the mycelium prior to DNA extraction.

To try to explain our findings, we suggest three different scenarios in the paper: (1) mutations arise only in the fruiting body, after separation of hymenium from surrounding tissue, (2) cell fate is controlled even in the mycelium, such that specific cell lines are destined for spore production and kept free of mutations, or (3) mutations arise in the mycelium and fruiting body, and the

fruiting body is made up of hyphae of mosaic genotypes that distribute randomly in fruiting body tissues, many ending up in the hymenium and a few form supporting tissues such as stipe, cap, etc. All scenarios have observations that speak for and against them, and we discuss the scenarios in detail in the paper.

Paper V: Transposition and rearrangements in protoplast-derived monokaryons

Transposons are mobile genetic elements that are common in eukaryotic genomes, including fungal. Despite the observation that large genomic proportions often consist of transposons, active transposition appears to be restricted to short intervals during limited parts of the life cycle. During this study, we investigated if transposition occurs during vegetative growth of *M. oreades* fairy rings. We made use of protoplast isolation and regeneration to separate the constituent nuclei from four fruiting bodies of a fairy ring into monokaryotic isolates, and sequenced their genomes with the ONT PromethION system. With this method we were able to fully reconstruct the genomes of the two nuclei, allowing for detailed analysis of events across every part of their genomes. Furthermore, by comparing genome assemblies of the protoclones from the four fruiting bodies, we found large-scale structural variation, including new insertions of transposable elements belonging to *hAT* and LINE superfamilies. We found a new family of autonomous *hAT* type elements, encoding protein domains for transposition, and several other families without these domains that appear to parasitize the autonomous element for their transposition. Our experimental design allowed us to pinpoint the moment of transposition to either during protoplast isolation or during culturing thereafter, and no large-scale mutational events were inferred to have happened during growth in the natural environment. As it appears, the genome is highly stable during dikaryosis, and transposon activity is successfully suppressed at this stage. However, during monokaryosis in regenerated protoclones, transposon suppression is alleviated. It is likely that the stress imposed by protoplast isolation allows some transposon activity to slip through genomic defenses. Even so, the finding that some transposon insertion events have happened after regeneration of the protoplast suggests that either some lingering effects of protoplast isolation are at play, or that the monokaryotic condition in itself allows some transposition to occur. Future studies should clarify which of these explanations is the more likely one.

Future perspectives

As per the nature of the scientific process, new answers spark new questions, and my work is no exception. The mechanism by which long-lived fungi suppress the proliferation of mitotic mutations is highly intriguing and demands further inquiry. Template-strand co-segregation, as the immortal strand hypothesis predicts, could be investigated by radioactive tagging of nucleotides during initial cell culture followed by transfer to standard media and tracing of radioactive DNA during growth. If template-strand DNA is co-segregating, tagged DNA would show up at the growth front of the colony at a non-random frequency.

A potential link between longevity and mutation rate could be investigated in fungi. More specifically, mutation accumulation lines of short-lived species, such as *Coprinopsis cinerea*, or closer relatives to *M. oreades* e.g. *Gymnopus androsaceus*, could be grown in parallel with longer-lived species such as *M. oreades*. Isogenic lines are easily obtained in fungi by separation from a starting culture, but maximization of drift is harder to achieve, since most mushroom-forming fungi do not have a single-cell stage before basidiospore production. Nevertheless, small fragments of hyphae can be obtained by blending a culture at high speed. To rule out a phylogenetic signal, many pairs of long- and short-lived species spread across the fungal tree of life could be used.

The finding that mutations introduced during vegetative growth are not present in meiotic progeny needs to be confirmed by independent observations, also including other species to investigate how widespread this phenomenon may be. If this result is robust across clades of Agaricomycetes, the nature of the germline needs to be investigated in detail, e.g. in the model system *C. cinerea* where much is already known about fruiting body development.

Finally, the result that transposons stay inactive during the vegetative, dikaryotic phase of *M. oreades* in nature needs to be generalized and reconciled with the high proportion of fungal genomes that is often inhabited by TEs. It is likely that TE proliferation happens in short, periodic bursts instead of gradually over growth, and our results indicate that such bursts may happen during the monokaryotic stage of the life cycle. This hypothesis could be investigated in detail by quantifying copy numbers of different TE families in spore isolates and protoclones by quantitative PCR or whole-genome sequencing.

Concluding remarks

By the time of conclusion of this thesis, including a year of master level studies, I have spent six years with *Marasmius* as my main study system. During this time, we have sequenced no fewer than 159 genomes of the species, not counting the same genome with different technologies. When we started, we had some plans, but had no idea about how much this project would evolve. A few surprises arose during my work. The first was that, as my research group had previously almost only worked on Ascomycete fungi, the initial genome project turned out much more complicated than anticipated. When sequencing e.g. *Neurospora*, single PacBio libraries of 10 kb insert sizes generated almost finished assemblies of 10-30 contigs. After we did the same for *Marasmius*, we ended up with 160-something contigs, and thought something was wrong. Of course, genome complexity is highly variable, and over time, I came to realize that we had been naïve in assuming that the genome of a Basidiomycete mushroom would be similar to an Ascomycete mold, and 160 contigs was actually not a very fragmented assembly in the grand scheme of things. Of course, we still wanted to get the assembly to a similar level as we were used to from the Ascomycetes, so we continued by adding Nanopore data, 10X Chromium data, and finally a linkage map before the 11 *Marasmius* chromosomes started to fully materialize.

The second surprise was the realization that I enjoyed programming so much that I ended up writing a Python application and publishing a paper about it. Before starting as a PhD student, I had barely any coding experience, but this was remedied over time. While I am by no means an expert software developer, I have learned to solve most bioinformatical problems with relative ease and am enjoying doing so.

The third surprise came from the spore sequencing project that ended up as Paper IV. After being inspired by discussions with Anne Pringle (University of Wisconsin–Madison), and reading a paper about reproductive aging in *Populus* trees (Ally et al., 2010), I came up with the idea of sequencing parent fruiting bodies and their offspring spores. At the same time, we could get linkage information to create a genetic map, so it was a win-win. Imagine our surprise when we found that no mutations were actually inherited, and we had to redesign the project into a discussion of the possibility that fungi have a hidden germline.

When I started as a master student in Hanna's group, we discussed using protoplast isolation to separate the nuclei in *Marasmius* before sequencing

them. Thankfully, we did not end up doing that during my final year as a Master student. Instead, I took on the task later on and have spent countless hours of my PhD work in the lab perfecting a protocol that worked for *Marasmius*. While "protoplasting" is a fairly established method in fungal genetics, it is surprisingly tricky to reconstruct the method from the literature. Finally, for Paper V, I managed to isolate both nuclei from all four fruiting bodies, and the results were that the nuclei had not actually changed much during growth in nature, but rather started to accumulate surprising amounts of variation in the lab.

It is an exciting time to study the rise of genetic variation. Much progress has been made in the last two decades, not just in methodology, but also in theoretical aspects of how variation is introduced. In the end, I think my PhD work has highlighted and added important knowledge about some aspects of mushroom biology that are usually not thought much about. While my work in the fairy rings has gone full circle, at least for the time being, there is plenty left to do before we can fully understand the genetics of the complex world of mushrooms.

Svensk sammanfattning

Mutationer i arvsmassan är en förutsättning för att organismer ska ha möjligheten att utvecklas genom att skapa ny genetisk mångfald. Samtidigt är den mesta nya variationen som uppstår genom mutation negativ för individens överlevnad. Detta faktum leder till att organismer generellt utvecklas mot att nå eller bibehålla en låg mutationsfrekvens. Mutationer av olika slag uppkommer över tid och tillväxt från felaktigheter under replikation av DNA samt från utomstående faktorer så som radioaktivitet eller ultraviolett ljus. Ett sätt att skydda arvsmassan från mutationer är genom att minimera antalet celldelningar över tid, och att se till att viktiga celler inte utsätts för externa mutagener.

I min avhandling har jag studerat nya mutationer i häxringar av svampen *Marasmius oreades*, känd som nejlikbroskingen på svenska. Häxringar är bra system för att studera mutationer eftersom det är lätt att urskilja enstaka individer, samtidigt som man kan uppskatta individens ålder utifrån ringens diameter vilket gör att man kan beräkna mutationsfrekvensen. Genom att sekvensera genomen från olika delar av ringen och jämföra dem med varandra kan man hitta skillnader som har uppkommit då ringen har vuxit. I **artikel 1** i den här avhandlingen utnyttjade vi häxringar för just det ändamålet. Eftersom vi var först med att studera *M. oreades* genom så började vi med att rekonstruera svampens kompletta genomsekvens genom att kombinera olika sekvenseringsteknologier. Vidare sekvenserade vi DNA från fruktkroppar som vi samlat från olika delar av sex ringar, och identifierade mutationer som uppkommit. Vi hittade förvånansvärt få mutationer utifrån avstånden som ringarna hade vuxit. Vi drog slutsatsen att förutsatt att *M. oreades* inte har någon gömd könscellinje eller sorterar sina cellkärnor på unika sätt så måste svampen ha en av de lägsta mutationsfrekvenserna man någonsin hittat i någon organism.

Mutationer studeras enklast med hjälp av genomsekvensering idag, och kräver referenssekvenser för den organism man studerar. Att rekonstruera en hel genomsekvens att använda som referens utifrån läsningar av olika längd är inte en enkel uppgift, eftersom de flesta genom består av miljontals baspar och ofta har regioner som upprepas på olika ställen i genomet. Nejlikbroskingen är inget undantag, vilket ledde till att vi lade mycket arbete på att få en så representativ genomsekvens som möjligt. Bland annat så utnyttjade vi teknologin 10X Genomics Chromium, vilken kan producera korta läsningar som är länkade med varandra om de har kommit från närliggande regioner i genomet. När vi först fick resultaten från den här teknologin så var den relativt

ny, och ingen passande programvara existerade för att utnyttja dess information på det sätt vi hade som mål. Därför skrev jag ny programvara för denna uppgift som vi kallar ARBitR. Vi publicerade resultaten från programmeringen och ett antal tester i **artikel 2**.

Även om de nya resultaten från ARBitR gjorde stor förtjänst i att förbättra vårt referensgenom för *M. oreades* så återstod några regioner som vi inte var säkra på hur de hängde ihop. Vidare så ville vi studera nedärvning av mutationer i en häxring. För att kombinera de ändamålen sekvenserade vi genomen hos 95 sporisolat från fyra fruktkroppar som vi samlat från en ring. Vi konstruerade en länkningskarta i **artikel 3** som vi använde för att göra klart referensgenomet. I samma artikel gjorde vi också en djupdykning i annotering av gener och repetitiva sekvenser i genomet. Resultaten visade att genantalet reflekterade upptäckter i andra basidiomycet-genom. Vidare hittade vi ett stort antal repetitiva sekvenser, och kunde uppvisa att en stor proportion av dem tillhörde grupperna *Kyakuja-Dileera-Zisupton* samt *Plavaka*, vilka är understuderade grupper av DNA-transposoner hos basidiomyceter.

Vi använde även sporisolaten för att studera nedärvning av mutationer i **artikel 4**. I teorin så borde mutationer som uppstår under tillväxt följa med till nästa generation, förutsatt att det inte finns en segregerad linje av könsceller i organismen. Vi använde samma metod för att sekvensera DNA från fruktkroppar och identifiera nya mutationer som i artikel 1, men använde nu också fruktkroppar från året innan, samt sporisolaten från de ursprungliga fruktkropparna. Resultaten visade att mutationer hade uppstått likt tidigare resultat, men bara två av dem kunde identifieras i fruktkropparna från båda åren. Vidare kunde vi inte hitta mutationer som fanns i fruktkropparna i sporisolaten. I artikeln föreslår och diskuterar vi olika scenarier som kan förklara de resultat vi hittade.

Artikel 5 fokuserar på transposabla element, med frågeställningen om de förökar sig under tillväxten av en häxring. I samma ring som i artikel 4 isolerade vi kulturer från fruktkroppar. Därefter använde vi metoden protoplastisolering för att separera cellkärnorna och erhålla monokaryotiska kulturer med en av dem vardera. Efter att ha odlat upp en tillräcklig mängd mycel extraherade vi DNA från kulturerna och sekvenserade det med Oxford Nanopore PromethION-teknik. Med hjälp av vårt referensgenom för *M. oreades* kunde vi sätta ihop nästintill kompletta konsensussekvenser för kromosomerna som tillhörde de två cellkärnorna, och använde dem till att hitta mutationer i enstaka prover. På så vis lyckades vi hitta ett antal nya insertioner av *hAT*- och *Inkcap*-typer av transposoner. Vidare fann vi en stor translokation i ett av proverna, där några hundratusen baser av kromosom 1 hade duplicerats och ersatt en del av kromosom 3. Alla dessa händelser kunde vi spåra till att ha skett efter vi tagit in proverna till laboratoriet, och så vitt våra resultat tyder på händer ingen storskalig förändring av arvsmassan då svampen växer i naturen.

Sammanfattningsvis har våra resultat bidragit till att öka vad vi vet om hur mutationer uppkommer i naturen. Svampar verkar besitta ett system för att

minimera antalet av såväl stora som små mutationer som ackumuleras över tillväxt, vars exakta natur vi ännu inte känner till. Dessutom verkar inte de mutationer som faktiskt uppkommer föras vidare till nästa generation via sporerna, vilket skulle kunna tyda på att svampar segregerar sina blivande könsceller tidigare i utvecklingen än vi tidigare trott. Framtida studier bör fokusera på att undersöka hur denna mutationskontroll går till i detalj, så att vetenskapen på allvar får upp sina ögon för svamparnas intrikata genetiska, utvecklingsmässiga och evolutionära anpassningar.

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